Review

The role of microhomology in genomic structural variation

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Genomic structural variation, which can be defined as differences in the copy number, orientation, or location of relatively large DNA segments, is not only crucial in evolution, but also gives rise to genomic disorders. Whereas the major mechanisms that generate structural variation have been well characterised, insights into additional mechanisms are emerging from the identification of short regions of DNA sequence homology, also known as microhomology, at chromosomal breakpoints. In addition, functional studies are elucidating the characteristics of microhomology-mediated pathways, which are mutagenic. Here, we describe the features and mechanistic models of microhomology-mediated events, discuss their physiological and pathological significance, and highlight recent advances in this rapidly evolving field of research.

Microhomology as a mutational signature

Large-scale population studies, such as the '1000 genomes project', indicate that genomic structural variation is a major source of genetic diversity among individuals and populations [1,2]. Structural variation typically involves genomic segments over 100 bp in length and includes tandem duplications, insertions, and inversions, which can generate DNA copy number variants (CNVs), as well as translocations and complex rearrangements [3]. These ambitious research efforts have revealed that structural variants are abundant, and should be considered as important as single nucleotide polymorphisms (SNPs) and single nucleotide variation [1,4].

Germline structural variation can be phenotypically neutral, having no effect on the organism. However, if it affects gene expression, structural variation can have a significant impact on the fitness of an individual [5] by conferring disease susceptibility, giving rise to human disorders [6] or leading to traits that can be selected for if beneficial [5,7]. For example, the copy number of the human salivary amylase gene, *AMY1*, is higher in populations with high starch diets, where the increased amylase protein levels are likely to improve the digestion of starchy foods [8]. In somatic cells, genomic structural variation is also significant because it is a key mediator of neoplastic transformation and progression of cancer [9]. Furthermore, somatic structural variation has a normal physiological role at immunoglobulin gene loci, where it is essential for generating antibody diversity [10].

Recent high-resolution sequencing studies of germline and somatic rearrangement breakpoints have revealed molecular signatures that enable reconstruction of mutational mechanisms [11–13]. For example, blunt joins, or small insertions or deletions at the breakpoint junction, are characteristic of DNA double-strand break (DSB) repair through direct ligation by nonhomologous end joining (NHEJ), whereas long stretches of sequence homology at or near the breakpoint can be attributed to homologous recombination (HR). HR repairs DSBs using template sequences, and relies on the presence of DNA segments sharing extremely high similarity or identity.

Glossary

Class switch recombination: recombination event in mature B lymphocytes that generates immunoglobulin isotypes with different effector functions, switching from IgM or IgD to IgG, IgE, or IgA following an immune response.
Complex genomic rearrangements: rearrangements with two or more breakpoint junctions.
Flanking microhomology: microhomology adjacent to the junction of a genomic rearrangement but not overlapping it.
Genomic disorder: pathological phenotype resulting from structural rearrangements in genomic loci where architectural features render the genome unstable.
Junctional microhomology: microhomology occurring directly at a breakpoint junction of a genomic rearrangement. Given that the sequence is identical in each of the genomic segments that contribute to the rearrangement, it is not possible to identify the exact breakpoint, because the microhomology cannot be assigned to either of the respective segments.
Low processivity polymerase: a polymerase that incorporates a relatively low number of nucleotides before it dissociates.
Microhomology: two short DNA sequences that are identical.
Nonrecurrent genomic rearrangements: rearrangements with variable breakpoints at sites lacking extensive sequence homology.
Recurrent genomic rearrangements: rearrangements of the same genomic interval occurring repeatedly in multiple unrelated individuals, found at sites of extensive sequence homology.
Replication fork collapse: breakage of the replication fork and detachment of one arm.
Replication fork stalling: an abnormality arising during DNA replication, where DNA synthesis at the replication fork pauses. This can arise from low levels of DNA polymerase or nucleotides, or from the fork encountering a barrier, such as complex DNA architecture.
Single-ended DSB: a DSB with only one end, which can arise from telomere erosion, separation of partner strands of a DSB, or when progression of a replication fork is interrupted.
Structural variation: genomic insertions, duplications, or deletions, which are collectively termed 'CNVs', or translocation or inversion of segments of the genome.

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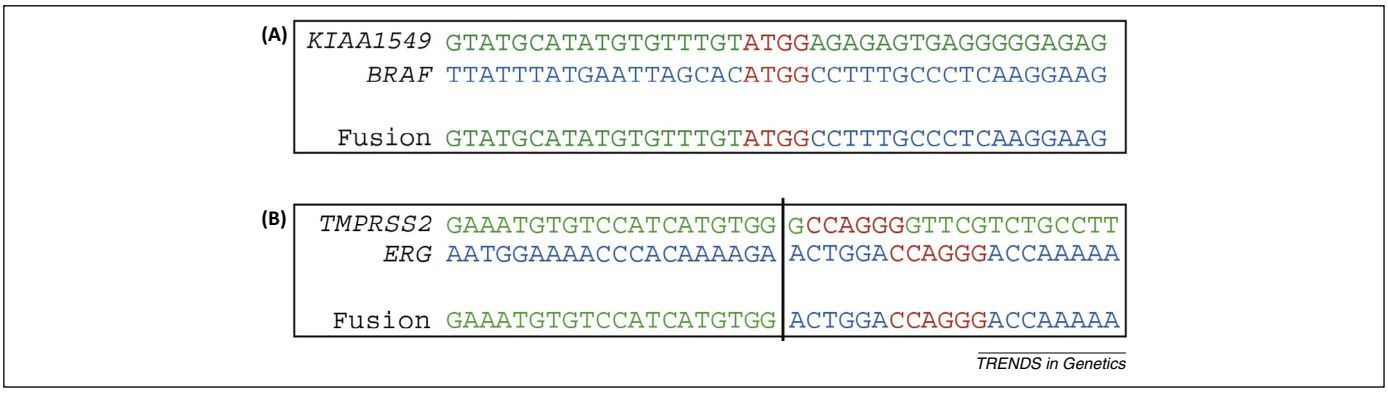

Figure 1. Microhomology at breakpoint junctions and flanking regions of simple gene fusions. **(A)** Junctional microhomology (red) at a *KIAA1549–BRAF* gene fusion in a paediatric low-grade astrocytoma. The exact breakpoint in each of the partner genes cannot be determined at a nucleotide level because the microhomology is present in both segments. **(B)** Flanking microhomology (red) at a *TMPRSS2–ERG* gene fusion in prostate cancer. The breakpoint is indicated by the black vertical line. Abbreviations: *BRAF,* v-raf murine sarcoma viral oncogene homolog B; *ERG,* serine 2- v-ets erythroblastosis virus E26 oncogene homolog; *TMPRSS2,* transmembrane protease. Adapted from [16] (A) and [18] (B).

These sequencing studies have also revealed short regions of DNA sequence homology, called 'microhomology' (see Glossary), at certain germline and somatic breakpoint junctions (e.g., [11,14,15]). Although definitions of breakpoint microhomology vary with respect to the length of the homologous region, it can be defined as a series of nucleotides (<70) that are identical at the junctions of the two genomic segments that contribute to the rearrangement (Figure 1A, Figure S1 in the supplementary material online). Microhomology has also been reported in DNA sequences that are adjacent to, but do not overlap, breakpoint junctions [16–18] (Figure 1B).

There is now evidence for additional repair mechanisms, besides the prevalent NHEJ and HR, that result in structural variation through the use of sequence microhomology. Whereas junctional microhomology of 1–4 bp can be a feature of NHEJ [19], as discussed below, one of these alternative mechanisms, termed 'microhomology-mediated end joining' (MMEJ), is independent of key proteins involved in NHEJ (Figure 2) [20,21]. MMEJ is error prone and frequently produces genomic rearrangements [22]. Further alternative mechanisms, termed 'fork stalling and template switching' (FoSTeS) and 'microhomology-mediated break-induced replication' (MMBIR), involve erroneous DNA replication, and template switching facilitated through annealing of microhomologous sequences [23,24]. These replicative mechanisms have been proposed to account for complex rearrangements that have multiple breakpoint junctions, insertions of DNA segments mapped to different genomic regions, as well as breakpoint microhomology, which together form a molecular signature inconsistent with NHEJ and HR. In this review, we examine the proposed molecular basis and regulation of these microhomology-mediated DNA repair mechanisms, and discuss their biological significance.

Microhomology-mediated end joining

DNA DSBs, which can be caused by a variety of agents, including reactive oxygen species, ionising radiation and UV light, are important mediators of structural variation [25]. The major repair mechanisms for DNA DSBs are NHEJ and HR [25]. NHEJ directly ligates broken DNA strands and is active throughout the cell cycle, although it predominates during the G0 and G1 phases. This repair pathway can lead to blunt joins, or small insertions or deletions at the breakpoint junction (reviewed in [19]). HR can lead to faithful DNA repair of DSBs during the S and G2 phases of the cell cycle, when a sister chromatid is available to serve as a template, but is often mutagenic during G1, when it relies on alternative homologous sequences, such as repetitive elements.

More recently, a further DSB repair pathway has been described that is thought to serve as a back-up repair process. MMEJ, which is sometimes referred to as an alternative NHEJ pathway, relies on the recombination of short stretches of microhomology for repair of DSBs [22]. Although understanding of MMEJ is still incomplete, it is emerging that this pathway can support DNA repair throughout the cell cycle [22], and shares elements with both HR and NHEJ.

MMEJ has been studied most extensively in yeast cells, where the mechanism was originally characterised, although mammalian functional orthologues have since been identified for most proteins (Table 1) [22]. As in HR, the essential initial step for MMEJ repair in mammals is digestion of the 5′ DNA strand by the Mre11–Rad50–Nbs1 (MRN) complex in association with retinoblastoma binding protein 8 (CtIP) to obtain a 3′ single-stranded DNA tail [26–28] (Figure 2). This occurs on each of the DNA strands beside the break. The exposed microhomologous sequences on the complementary 3′ ends then anneal to form a complex with gaps that need to be filled and ligated. The overhanging noncomplementary 3′ flaps are then trimmed by the endonuclease excision repair cross-complementing rodent repair deficiency, complementation group 4–excision repair cross-complementing rodent repair deficiency, complementation group 1 (Xpf–Ercc1) complex, whereas the gaps created on both strands through resection are filled in by a DNA polymerase, which has been proposed to be polymerase lambda [29,30]. DNA ligase I and ligase IIIa/X-ray complementing defective repair in Chinese hamster cells 1 (Xrcc1) are responsible for the subsequent joining of the DNA segments [31,32]. Given that MMEJ results in deletions of the DNA regions flanking the original break, it is an error-prone repair pathway. The mechanistic model of MMEJ, as it is

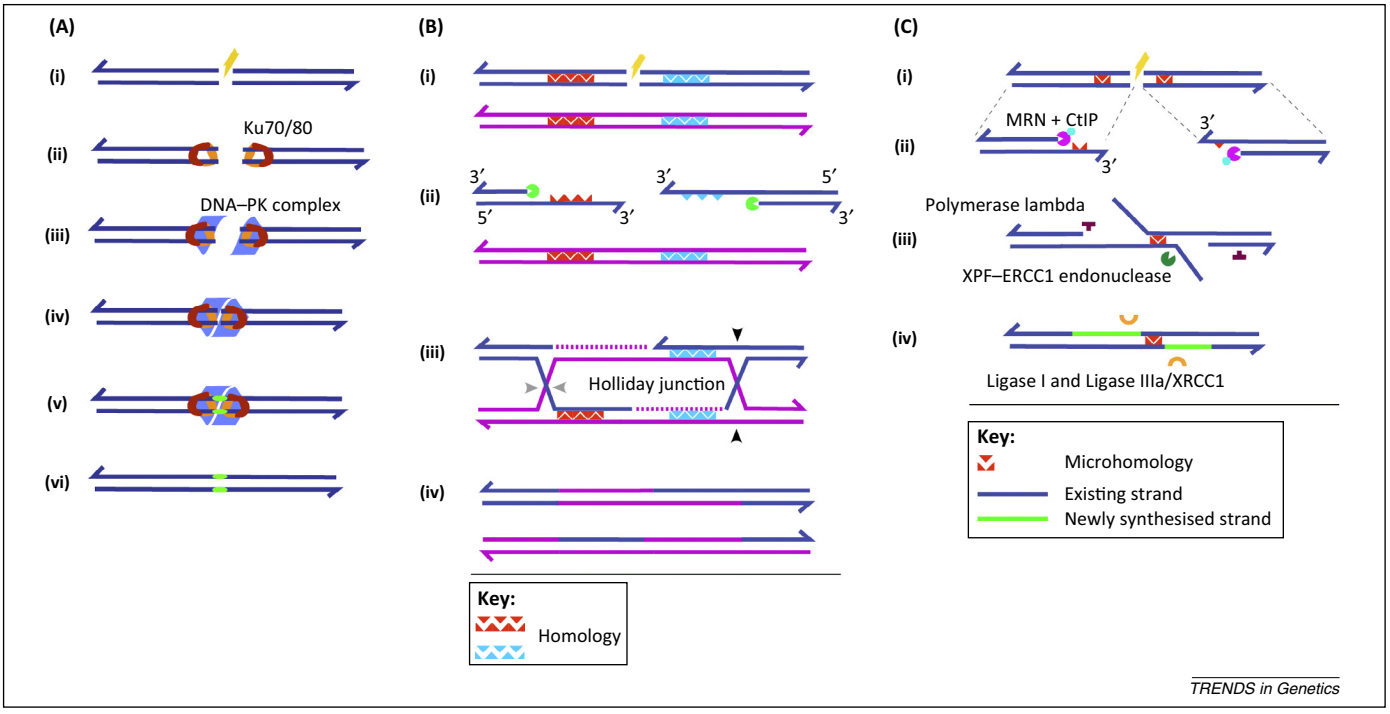

Figure 2. Nonhomologous end joining (NHEJ), homologous recombination (HR), and microhomology-mediated end joining (MMEJ). **(A)** NHEJ: (i) formation of a double-strand break (DSB); (ii) the Ku70/80 heterodimer binds to the break ends; (iii) the DNA-protein kinase (PK) catalytic subunit (DNA-PKcs) interacts with the Ku70/80 heterodimer to form the DNA–PK complex, which mediates end synapsis, as shown in (iv); (v) ligation of the break ends; and (vi) ligation can be preceded by insertion or deletion of a few nucleotides, leaving a characteristic molecular scar (green). **(B)** HR: (i) a DSB occurs in the presence of a sister chromatid, shown here in pink. (ii) 5′ to 3′ resection generates 3′ single-stranded overhangs exposing long stretches of nucleotides; (iii) the break ends invade the sister chromatid from both sides and anneal at homologous sequences, forming two Holliday junctions. Templated synthesis is initiated. The two Holliday junctions can be resolved in two ways by an endonuclease (grey and black arrows); (iv) Resolution. Alternatively, only one end of the break invades the sister chromatid. The invading strand disengages following templated synthesis and anneals to the homologous region on the single-strand overhang of its partner molecule (not shown). **(C)** Proposed mechanistic model of MMEJ: (i) formation of a DSB; (ii) 5′ to 3′ resection by the Mre11–Rad50–Nbs1 (MRN) complex and retinoblastoma binding protein 8 (CtIP) results in two 3′ single-stranded overhangs and exposure of short stretches of microhomology, shown in red; (iii) Following annealing of the microhomologous sequences, the noncomplementary 3′ flaps are trimmed by the endonuclease complex endonuclease excision repair cross-complementing rodent repair deficiency, complementation group 4–excision repair cross-complementing rodent repair deficiency, complementation group 1 (XPF–ERCC1) and gaps are filled in by DNA polymerase lambda; (iv) ligation of the phosphate backbones is carried out by Ligase I and IIIa/X-ray complementing defective repair in Chinese hamster cells 1 (XRCC1).

currently understood, has arisen from a series of functional studies. However, it is likely that knowledge of the pathway will be further refined and that alternative or additional proteins involved in MMEJ will be identified.

The selection of the MMEJ mechanism to rescue DSB repair is determined by three interdependent variables: (i) resection of the DNA ends flanking the breakpoint; (ii) phase of the cell cycle in which the DSB occurs; and (iii) the type and relative abundance of regulatory proteins in each phase of the cell cycle (Figure 3). In G1 phase of the cell cycle, HR and MMEJ are limited by the lower efficiency of MRN/CtIP-mediated resection of the DNA broken ends. This is due to lower levels of CtIP in G1 compared with other stages of the cell cycle, and to phosphorylated histone protein H2A histone family, member X (H2AX) recruiting the mediator of DNA damage checkpoint 1 (MDC1) protein to chromatin flanking DSBs to inhibit the activity of the small amount of CtIP that is present [33–35]. The resulting unresected DSB ends have high affinity for the Ku70/80 complex, which protects the broken DNA ends from nucleolytic degradation and commits the cell to NHEJ repair.

In G2, S, and M phases, DNA end resection is more efficient due to the higher abundance of CtIP and the reduced inhibitory effect of H2AX and MDC1 [33–35]. Thus, NHEJ repair of DSBs during these phases of the cell cycle is reduced. Following resection of the 5′ DNA strand, DSB repair can then either occur through the error-prone MMEJ, or through HR. The extent of DNA resection by MRN and CtIP is sufficient for MMEJ to proceed. However, HR requires further DNA resection by the helicase Bloom Syndrome protein (BLM) and exonuclease Exo1 [36].

Following the repair of a DSB, breakpoint microhomology involving a few base pairs cannot be unambiguously assigned to either NHEJ or MMEJ. However, whereas MMEJ relies on microhomology, this is not an essential requirement for NHEJ [19]. Furthermore, experimental studies have shown that MMEJ is independent of the key NHEJ factors, including Ku and DNA ligase IV/XRCC4 [20,21], and relies on factors that are not required by NHEJ, such as the MRN/CtIP complex, which is essential to obtain two single-stranded 3′ overhangs. Further studies also indicate that MMEJ operates in parallel when NHEJ is functional [27]. MMEJ is sometimes referred to as ‘alternative NHEJ’ or ‘back-up NHEJ’ and there is some debate about whether MMEJ should be regarded as an independent pathway, or should be classified as part of a more flexible NHEJ, which can function in the absence of key NHEJ factors, such as Ku or DNA ligase IV [19]. NHEJ is reported to use no microhomology most commonly, followed by 1-bp microhomology; in the absence of the DNA ligase IV complex, the peak length of microhomology used increases to 2–3 bp [19].

Table 1. Orthologous proteins reported to be involved in or inhibit MMEJ

Saccharomyces cerevisiae	*Homo sapiens*	Function in MMEJ	Involvement in other DNA repair mechanisms or replication	Refs
Involved in MMEJ				
Mre11, Rad50, and Xrs2	Mre11, Rad50, and Nbs1	5′ to 3′ resection to expose sequence homology	HR: as MMEJ	[26]
Sae2	CtIP	Interacts with Mre11 and Rad50 subunits of the MRN complex to promote resection	HR: as MMEJ	[27,28]
Srs2	No known orthologue	Promotes MMEJ	HR: inhibition of HR by interfering with protein complexes required for HR (Rad51 presynaptic filament)	[79]
Rad1 and Rad10	Xpf and Ercc1	Removal of overhanging noncomplementary 3′ flaps through endonuclease activity	HR: as MMEJ if recombination proceeds through single-strand annealing	[29]
No known orthologue	Parp-1	Facilitation of MMEJ; synapsis activity	NHEJ: competition with Ku for DNA ends with lower affinity binding than Ku; base excision repair	[80–82]
DNA polymerase IV	DNA polymerase lambda	Fill-in synthesis, able to promote the annealing of microhomology on single-stranded DNA 3′-overhangs	NHEJ: DNA synthesis	[30]
No known orthologue	DNA polymerase beta	Fill-in synthesis of DNA ends containing the (CAG)n triplet repeat sequence	NHEJ: DNA synthesis	[30]
Cdc9	DNA ligase I	Ligation	DNA replication: ligation of Okazaki fragments	[32]
No known orthologue	DNA Ligase IIIα and Xrcc1	Ligation	Base excision repair	[31,83]
Inhibits MMEJ				
Tel1	Atm	Inhibition of DNA end resection	Recruitment to DSBs; cell cycle delay	[24,84]
Ku70 and Ku80	Ku70 and Ku80	Protection of DNA ends from resection	NHEJ: DSB recognition, binding of broken DNA double-strand ends, recruitment of proteins involved in NHEJ; end synapsis	[85]
H2AX	H2AX	Recruitment of Mdc-1; inhibition of CtIP activity in G1	Cell cycle-dependent pathway choice	[34]
No known orthologue	Mdc-1	Inhibition of CtIP activity in G1	Cell cycle-dependent pathway choice	[34]
No known orthologue	53BP1	Protection of DNA ends from resection during CSR	NHEJ: promotion of NHEJ over MMEJ during CSR through protection of ends from nucleases	[86]
No known orthologue	Brca1	Cell cycle-dependent regulation of CtIP action	Cell cycle-dependent pathway choice	[87,88]

Whereas there is some overlap in proteins required for DSB repair by MMEJ and HR pathways (Table 1), few studies have directly addressed the distinction between these mechanisms. However, HR pathways are generally thought to rely on longer stretches of homology and, therefore, require more extensive resection for exposure of nucleotides [37]. Moreover, CtIP phosphorylation, which is essential for association of the protein with the MRN complex and breast cancer type 1 susceptibility protein 1 (BRCA1) is required for HR, but not MMEJ [38]. The roles of proteins involved in MMEJ in comparison to HR and NHEJ are described in Table 1.

Replicative microhomology-mediated mechanisms

Fork stalling and template switching

FoSTeS was the first of two replicative models proposed to explain the complex rearrangements leading to duplication of the dosage-sensitive proteolipid protein 1 gene, *PLP1*, in Pelizaeus–Merzbacher disease (PMD) [24]. In this model, the replication fork stalls at a DNA lesion, and replication is temporarily paused (Figure 4). The lagging strand disengages and invades an active adjacent replication fork through the annealing of microhomologous sequences. Synthesis of the lagging strand briefly continues at the invaded fork, before the lagging strand again disengages. The drifting strand sequentially invades other active replication forks, and synthesis is restarted at microhomology-primed sites before eventually resuming at the original template. Depending on whether the invaded replication fork is located downstream or upstream in relation to the original stalled replication fork, the resulting sequence can include deletions or duplications.

Microhomology-mediated break-induced replication

MMBIR was proposed as a more detailed and testable model for generating complex rearrangements compared with FoSTeS [23] (Figure 5). The model is based on the experimentally validated break-induced replication (BIR), which underlies chromosomal alterations in yeast, where BIR has been best characterised [39,40]. BIR is Rad51 recombinase dependent, therefore requiring the annealing of longer homologous sequences (70–100 bp), whereas MMBIR only needs short microhomologous regions [23,41,42].

BIR and MMBIR both require a single double-strand end during replication, which can arise following the collapse of a replication fork (Figure 5) or through the erosion of telomeres or the separation of two DSB ends [23]. Resection of the 5′ strand at the DSB generates a 3′

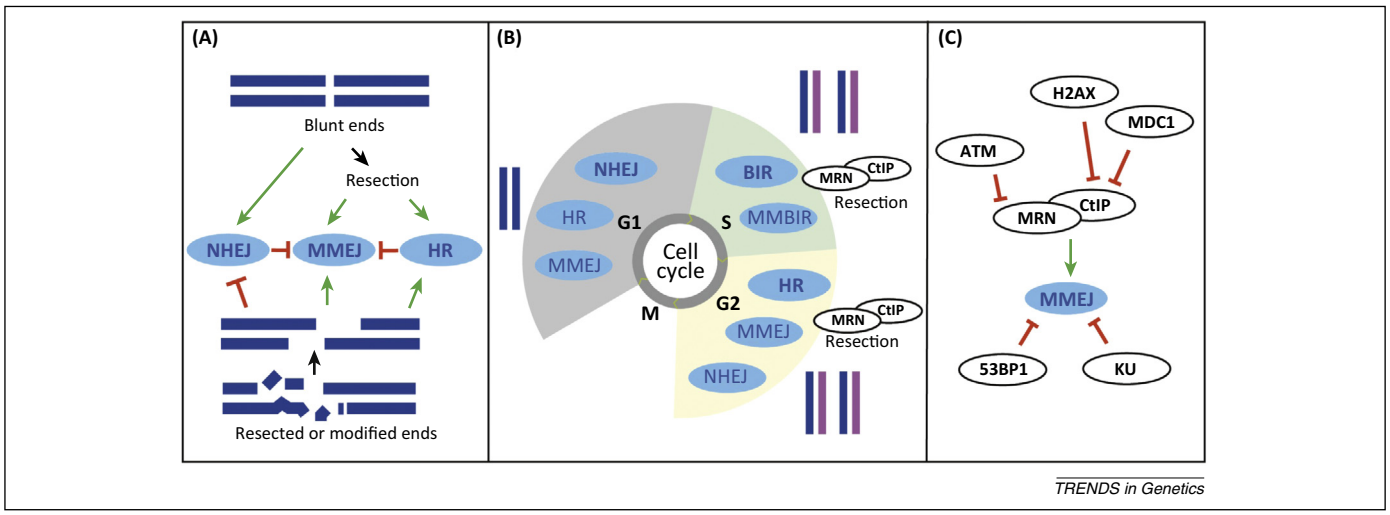

Figure 3. Double-strand break (DSB) repair pathway choice. **(A)** Status of breakpoint ends. Blunt ends following a DSB can be repaired by nonhomologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homologous recombination (HR). Resected or modified ends are repaired by MMEJ or HR, because NHEJ is inhibited. **(B)** Cell cycle phase. In S and G2 phases, end resection is mediated by the Mre11–Rad50–Nbs1 (MRN) complex in association with retinoblastoma binding protein 8 (CtIP), thus diverting DNA repair to HR. In S phase, DSBs formed following replication fork breakdown are primarily repaired by break-induced replication (BIR), whereas microhomology-mediated BIR (MMBIR) is used as a back-up mechanism. In G2 phase, DSBs are repaired by HR or, if HR is not available, by NHEJ or MMEJ. In G1, NHEJ is the preferred method of repair, with HR pathways and MMEJ as back-up mechanisms. **(C)** Molecular control of MMEJ. Resection by the MRN complex in association with CtIP is inhibited by ataxia telangiectasia mutated (ATM), histone protein H2A histone family, member X (H2AX), and mediator of DNA damage checkpoint 1 (MDC1). ATM inhibits the MRN complex, whereas H2AX and MDC1 inhibit CtIP. Ku and 53BP1 inhibit MMEJ by preventing the access of resecting nucleases to DNA break ends.

single-stranded overhang, which can invade a microhomologous region on a different DNA template to establish a new replication fork and restart synthesis. As in the FoSTeS model, multiple dissociations and invasions of new templates can lead to sequence complexity. It has been suggested that the proposed recurrent template switching of MMBIR is due to the low processivity of DNA polymerases at the beginning of the repair process, a finding previously reported for BIR in yeast [41].

Collapsed replication forks resulting in an unpaired DSB are usually repaired by BIR. However, use of error-prone MMBIR can be critical under specific circumstances. BIR is dependent on Rad51 to form the nucleoprotein filament, which mediates the homologous DNA pairing and strand exchange reaction [43]. Rad51 is generally in short supply, especially in conditions of cellular stress, such as hypoxia [44]. MMBIR is Rad51 independent, and Rad52 is suggested to catalyse the annealing reaction here [23]. *In vitro* evidence has demonstrated that Rad51 inhibits the activity of Rad52 and, therefore, a reduction in the amount of Rad51 could induce the use of MMBIR as a back-up repair mechanism [45].

Microhomology-mediated rearrangements in the germline

Germline rearrangements, including CNVs, arise from different mechanisms, including nonallelic homologous recombination (NAHR), the end-joining mechanisms NHEJ and MMEJ, the replicative FoSTeS/MMBIR, and insertions of mobile elements (Table S1 in the supplementary material online). Where CNVs affect dosage-sensitive genes, they can give rise to genomic disorders, including Mendelian diseases, birth defects, and complex traits [6,46].

CNVs can be divided into two classes: recurrent CNVs, which occur in a few genomic positions with breakpoints within low copy repeats (LCRs) that are clustered together in unrelated individuals, and nonrecurrent CNVs, which are distributed throughout the genome and have unique breakpoints [42]. The origin of recurrent CNVs has been attributed mainly to NAHR or defective crossing over during meiosis, when misalignment occurs due to the high degree of sequence similarity between LCRs [47]. NAHR has been shown to explain several recurrent rearrangements in both germline structural variation and cancer [47]. A large population study estimated the proportion of germline structural variation mediated by NAHR to be approximately 22%. [48]. The origin of nonrecurrent CNVs is less clear. Previous studies suggested NHEJ as the major mechanism creating nonrecurrent deletions [49–51]. More recently, sequence analysis has implicated microhomology-mediated mechanisms in the formation of numerous nonrecurrent CNVs [1,11,24,42,48,52–54] (Table S2 in the supplementary material online). Following the description of the novel replication-based microhomology-mediated mechanisms, a re-evaluation of the previous studies mentioned above [49–51] concluded that microhomology is present in more than half of the breakpoint junctions, a signature that is consistent with NHEJ, MMEJ, FoSTeS, or MMBIR [55].

Strikingly, the characterisation of CNVs in unrelated individuals showed that approximately 70% of deletions and 89% of insertions exhibited junctional microhomology [1]. However, the predominance of microhomology could not be reproduced for other types of chromosomal rearrangement. A recent study analysing the breakpoints of karyotypically balanced translocations and inversions revealed that microhomology was found in only 31% of the breakpoints analysed, whereas most rearrangements appear to occur through NHEJ [56]. These findings indicate that specific types of genomic rearrangement can differ in aetiology.

In addition to microhomology in genome-wide benign CNVs, microhomology-mediated replicative mechanisms have been implicated in specific disease-associated structural rearrangements. As discussed above, the FoSTeS

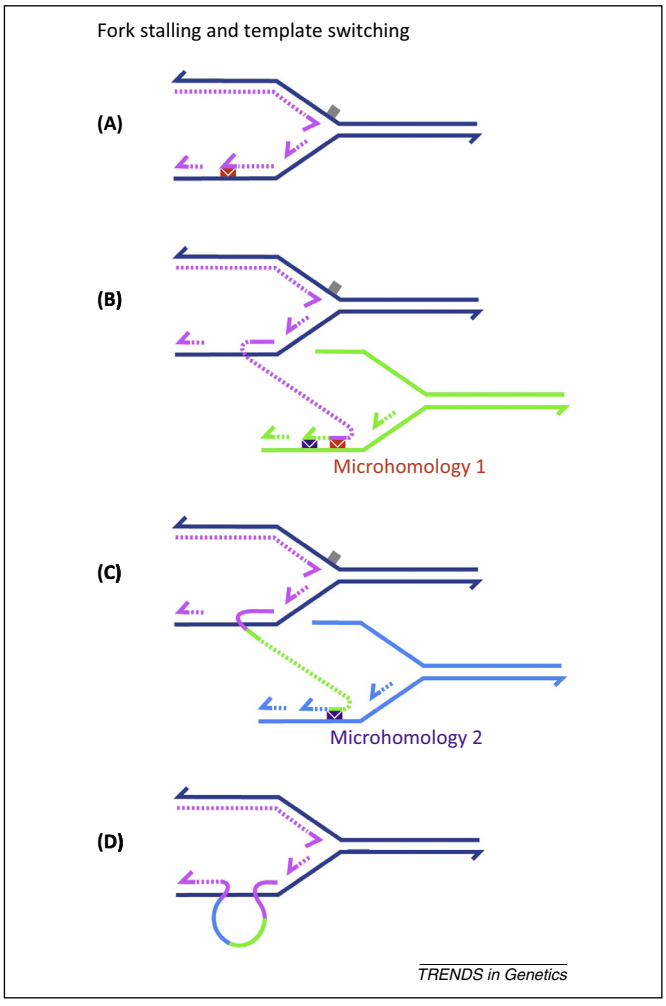

Figure 4. Mechanistic model of fork stalling and template switching. **(A)** The replication fork stalls at a DNA lesion (grey). **(B)** The lagging strand disengages, invades an adjacent active replication fork and anneals to a region with microhomology (red), which primes DNA synthesis. **(C)** The lagging strand disengages once more and invades a further adjacent active replication fork, where it anneals to another microhomologous region (purple) to restart synthesis. **(D)** Eventually, the lagging strand returns to the original replication fork to continue replication to the end of the chromosome.

replicative mechanism was first proposed to account for complex nonrecurrent rearrangements involving *PLP1* in PMD [24]. A FoSTeS/MMBIR mechanism has been proposed for numerous nonrecurrent CNVs associated with genomic disorders, such as haemophilia A and Cornelia de Lange Syndrome, as well as in rare pathogenic CNVs [53,57,58]. Strong support for replicative mechanisms also comes from experiments where *de novo* CNVs induced by low doses of the DNA polymerase inhibitors aphidicolin or hydroxyurea frequently exhibit microhomology at their breakpoint junctions [52,54], which occurs in the absence of Xrcc4-dependent NHEJ [20].

An interesting example of nonrecurrent CNVs where detailed sequence information provides mechanistic insights comes from characterisation of the large human neurexin gene, *NRXN1* [6]. Microdeletions within *NRXN1* confer susceptibility to a range of neurodevelopmental and neuropsychiatric disorders, such as autism spectrum disorders, schizophrenia, mental retardation, epilepsy, and Alzheimer's disease. Frequent microhomology has been found at the deletion junctions together with long terminal repeat (LTR) elements and non-B-DNA structures. These genomic features are likely to convey a complex chromatin architecture, thereby increasing the risk of DSBs, fork stalling, and microhomology-mediated template switching [6,55,59]. Similar architectural features identified at other rearrangement junctions provide further evidence for the widespread use of replicative mechanisms (Table S2 in the supplementary material online).

Increasing evidence suggests that extremely complex CNVs can arise from a single catastrophic phenomenon. This event, first described in cancer as chromothripsis, was thought to involve extensive chromosome shattering through multiple simultaneous DSBs and reorganisation of the DNA fragments [60]. Recent studies of *de novo* rearrangements in patients with genomic disorders have indicated that similarly complex patterns can also be observed in some constitutional rearrangements [56,61,62]. Although the molecular basis of chromothripsis is still uncertain, it is being recognised that more than one mechanism might create these complex rearrangements in the germline. The mechanisms proposed are ligation of multiple DNA DSBs by an end-joining mechanism, such as NHEJ [56,61], or erroneous replication, consistent with the microhomology-mediated replicative models of FoSTeS/MMBIR. The suggestion that chromothripsis has a replicative origin is based on observations that, alongside the complexity of rearrangements, some of the junctions have microhomology and templated insertions, which are inconsistent with an end-joining mechanism [62].

Gene duplications caused by retrotranspositions constitute an important distinct class of gene copy-number polymorphism. The actively mobilising long interspersed nuclear element 1 (LINE-1) retrotransposon has been shown to contribute to 19% of the structural variation identified in the germline [48]. Approximately 17% of the human genome comprises LINE-1 sequences, which replicate via RNA intermediates that are reverse transcribed and integrated into new genomic locations [63]. Microhomology identified at insertion breakpoint junctions suggests that MMEJ mediates LINE-1 insertions [64].

Somatic microhomology-mediated rearrangements

Microhomology-mediated ligation in immune cells

Microhomology-mediated ligation has been identified as a robust back-up mechanism for class switch recombination events in the immune system [65]. To generate a variety of antibodies, two highly specialised recombination events occur in lymphocytes. For the extensive repertoire of antigen receptors, developing B and T lymphocytes undergo V(D)J recombination, assembling different combinations of Variable (V), Diversity (D), and Joining (J) gene segments [66]. Once an antigen has been recognised, triggering an immune response, class switch recombination (CSR) enables mature B lymphocytes to generate immunoglobulin isotypes, switching from IgM or IgD to IgG, IgE, or IgA through recombination of the constant region of the antibody heavy chain [10]. This is a key immunological reaction because it generates immunoglobulin isotypes with different effector functions.

Each immunoglobulin heavy chain gene is preceded by repetitive DNA sequences termed 'switch regions'

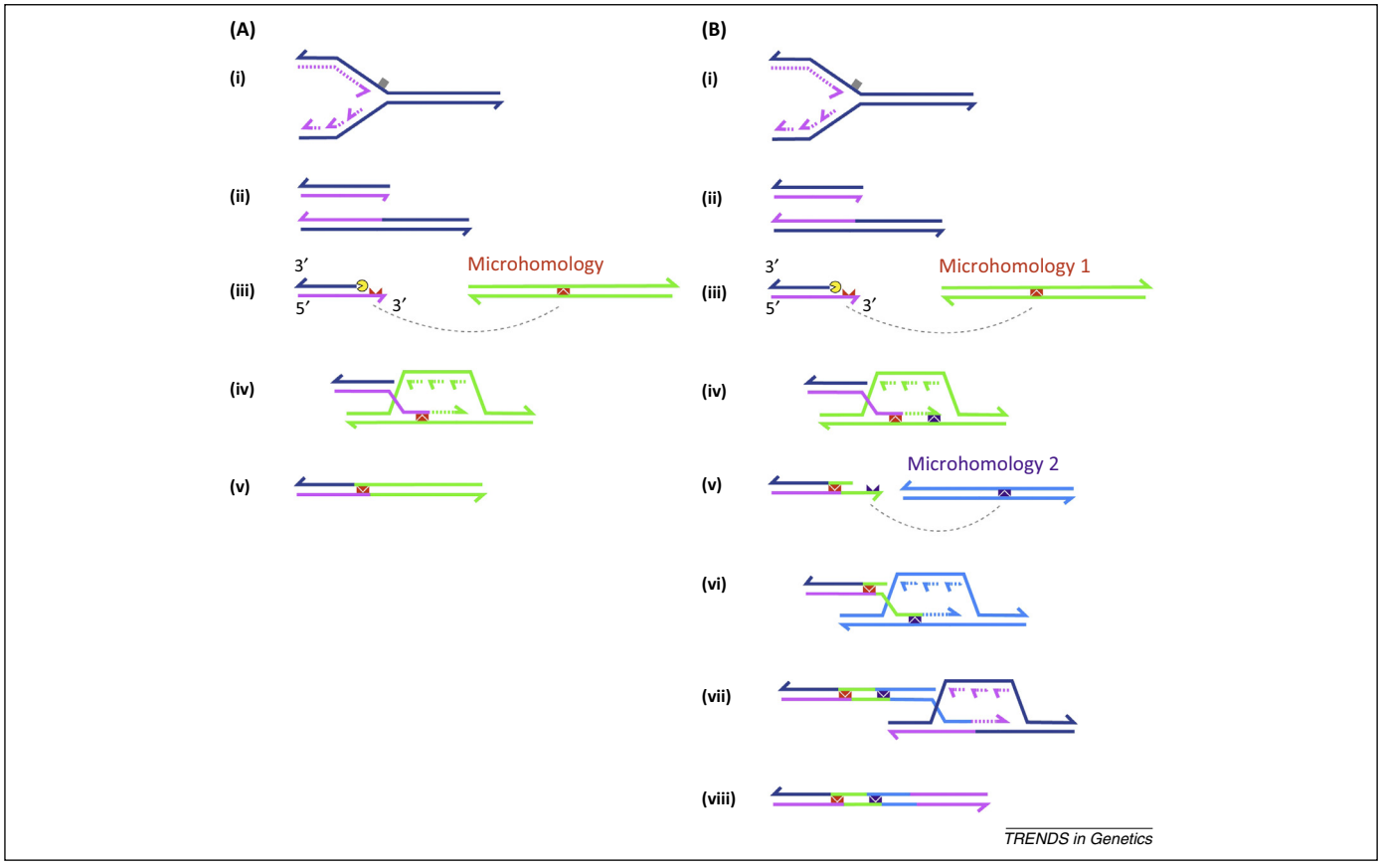

Figure 5. Mechanistic model of microhomology-mediated break-induced replication (MMBIR). **(A)** Simple genomic rearrangement: (i) the replication fork encounters a lesion (grey) and stalls; (ii) fork stalling leads to replication fork collapse and cleavage by an endonuclease creates a single-ended double-strand break (DSB), shown here as the shorter of the two strands; (iii) 5′ to 3′ resection exposes a region of microhomology (red) and generates a 3′ single-stranded overhang; (iv) the formation of a D-loop by the template strand is followed by the invasion of the 3′ overhang, which anneals to the microhomologous region to restart synthesis; and (v) synthesis is continued to the end of the chromosome. **(B)** Complex genomic rearrangement: (i–iv) As in simple MMBIR; (v) the invading strand disengages due to low processivity DNA polymerases; (vi) The 3′ overhang invades a different DNA segment and anneals to a microhomologous region. Further template switches occur until DNA polymerases with higher processivity enable continuous synthesis; (vii) eventually, synthesis restarts at the original strand and continues to the end of the chromosome; and (viii) following MMBIR with template switching, the replicated chromatid exhibits a region of complex rearrangements with junctional microhomology.

(S regions) [10]. Activation-induced deaminase (AID) deaminates cytidines to uridines within these S regions to generate staggered DSBs, which are predominantly repaired by NHEJ [67,68]. However, experiments in which NHEJ components, such as Ku70, XRCC4, and Lig4, are mutated have demonstrated that CSR still remains active [65,69], albeit at a substantially reduced level, with a reduction of approximately 80–90% in Lig4-deficient cells compared with wild type cells [70]. In cells with a functional NHEJ pathway, most junctions are either blunt or have 1–4 bp microhomology [65]. However, when NHEJ is abolished, no blunt joins are detected and the remaining junctions have longer microhomology [65,70]. Furthermore, NHEJ-deficient cells have increased CtIP binding to S regions and microhomologous breakpoint junctions, suggesting that microhomology-mediated repair acts as a back-up mechanism for CSR when NHEJ is not functional [27].

Whereas CSR can be rescued in the absence of key NHEJ components, V(D)J relies almost entirely on NHEJ, and junctions formed by other mechanisms are rare [71]. Notably, in the absence of NHEJ during both V(D)J recombination and CSR, the use of alternative pathways can lead to chromosomal translocations and the subsequent development of immune malignancies where a significant proportion of rearrangements exhibit breakpoint microhomology [65,72].

Importantly, V(D)J recombination offers a useful model to examine junctions formed by NHEJ in isolation. It has been reported that approximately 60% of the junctions generated by NHEJ have 1 or 2 bp junctional microhomology [73]. This finding strongly implicates junctional microhomology in canonical NHEJ and accentuates the difficulty in assigning junctions with short microhomology to either NHEJ or MMEJ based on molecular signature alone.

Microhomology-mediated structural variation in cancer cells

Genomic alterations in cancer cells arise from cumulative DNA damage and erroneous DNA repair processes. These alterations consist of ‘driver’ mutations, which are responsible for tumourigenesis and progression, and ‘passenger’ mutations, which have no phenotypic effect [9]. Importantly, microhomology has been identified at several rearrangement breakpoints in a variety of malignancies, including breast, colorectal, and prostate adenocarcinomas, as well as a high proportion of paediatric low-grade astrocytomas and adult glioblastomas [14–16,18,74].

The relative contribution of microhomology-mediated mechanisms to the formation of different genomic

rearrangements in cancer remains to be quantified. A large survey of breakpoint sequences across seven tumour types indicated that most complex genomic rearrangements are likely to be formed by NHEJ, and that up to 27% might be formed by microhomology-mediated mechanisms [74]. By contrast, a different study of somatic structural variation across ten tumour types indicated that microhomology-based and nonhomology-based mechanisms are equally important for generating deletions and translocations [13].

In breast and colorectal carcinomas, microhomology is particularly prevalent in tandem duplications [14,15]. In certain tumours, microhomology is present directly at breakpoint junctions, as well as in the adjacent flanking regions. For example, junctional and flanking microhomologies are present at the key *KIAA1549–BRAF* fusions, which are derived from tandem duplications in paediatric low-grade astrocytomas (Figure 1) [16]. The sequence profiles in these fusions suggest that they arise from MMBIR. The transmembrane protease–serine 2- v-ets erythroblastosis virus E26 oncogene homolog (*TMPRSS2–ERG*) fusions in prostate cancer also show junctional and flanking microhomology (Figure 1) [18]. In other malignancies, breakpoint microhomology has been linked to genomic stability and tumour behaviour. For example, invasive bladder tumours, which are characterised by genetic instability and loss of tumour protein p53 (TP53), show microhomology together with multiple errors at rearrangement breakpoints, indicating an active MMEJ pathway [75]. The invasive tumours simultaneously have reduced Ku-DNA binding activity. By contrast, non-invasive genetically stable bladder tumours do not show microhomology and display a faithful DNA repair profile.

Further insights into DSB repair processes that are active in cancer cells come from analysis of breakpoints in breast tumours associated with BRCA1/2 germline mutations [76]. A high incidence of microhomology was found at insertions and deletions, indicating that the cells are defective in HR-based DNA repair processes and that MMEJ or other microhomology-mediated mechanisms act in their place. A complementary study found an increase in microhomology-mediated repair processes in blood lymphocytes from women with hereditary breast and/or ovarian cancer risk and in younger women with sporadic breast cancer, compared with the levels in healthy controls [77]. Thus, identification of microhomology signatures at rearrangement breakpoints is indicative of defective DNA repair processes, and might in time be used to distinguish individuals at high risk of developing cancer.

Concluding remarks

The prevalence of microhomology at rearrangement junctions highlights the importance of microhomology in genomic plasticity, and the need for a better understanding of microhomology-mediated mechanisms. Knowledge of MMEJ and FoSTeS/MMBIR is still evolving and more functional studies are required to elucidate the molecular basis of these mechanisms. The rudimentary understanding of the causative mechanisms and the reliance on the molecular signature resulting from a mutational event as an end product, give rise to various controversies. First, NHEJ has been reported to use 1–4 bp microhomology in a proportion of junctions [19], which makes it impossible to assign junctions with short microhomology to either NHEJ or MMEJ. Furthermore, there is uncertainty about the length of microhomology that can be regarded as significant. Microhomology of one or two nucleotides, for example, can arise by chance, but might still be able to promote microhomology-mediated mechanisms. Moreover, it is likely that the length of homology required varies between different mechanisms. Whereas microhomology serves as an annealing point during MMEJ, it might act as a primer for DNA synthesis during FoSTeS/MMBIR [23,24].

The number of rearrangements with microhomology is likely to be higher than currently thought. Only recently have studies extended sequence analysis for microhomology to genomic regions flanking breakpoint junctions, and it might be necessary to reconsider previously identified genomic rearrangements that were restricted to microhomology overlying breakpoint junctions. In addition, high-resolution sequencing studies have identified an unexpected complexity of some genomic rearrangements that were previously thought to be non-complex, leading to the proposal that they result from FoSTeS/MMBIR [24]. As sequencing methodology progresses to enable rearrangements to be examined efficiently at base-pair resolution, more intrinsically complex rearrangements in seemingly simple mutational events may be uncovered.

Given that microhomologous sequences are widespread across genomes, DNA sequence alone is unlikely to account for the selective occurrence of microhomology-mediated events. To explain their frequency and distribution, it is critical to analyse them in the context of 3D genomic architecture. The way in which DNA is packaged could prevent or facilitate the formation of DSBs as well as alter the accessibility of microhomologous sequences to the enzymes required for microhomology-mediated events.

The prevalence of microhomology-mediated repair mechanisms during gametogenesis, development and throughout the lifespan of an organism remains to be established. Analysis of DSB repair in embryonic mouse cells has revealed a switch from predominantly NHEJ to MMEJ repair at a certain stage of development, which was associated with increased levels of CtIP, Mre11, Nbs1, and Ligase III [78].

Microhomology-mediated mechanisms have two outcomes with respect to genomic integrity. On the one hand, they can limit DNA damage, particularly when the predominant repair mechanisms NHEJ or HR are unavailable. On the other, these mechanisms can lead to genomic rearrangements due to their error-prone nature and, thus, generate structural heterogeneity that provides the framework for evolutionary processes and is critical in health and disease. Therefore, microhomology signatures open new avenues for deciphering fundamental pathogenic and evolutionary changes in the genome.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tig.2014.01.001.

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