Telomeres and Chromosomal Translocations

subtitled

(There's a Ligase at the End of the Translocation)

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Abbreviations Used:

A-NHEJ	Alternative nonhomologous end joining
APOBEC3	Apolipoprotein B editing complex 3
BFB	breakage-fusion-bridging
BRCA1	Breast cancer allele 1
BRCA2	Breast cancer allele 2
BLM	Bloom syndrome gene
C-NHEJ	Classic nonhomologous end joining
CtIP	C-terminal interacting protein
DNA2	DNA exonuclease 2
DNA-PK_{cs}	DNA dependent protein kinase catalytic subunit
DNA-PK	DNA dependent protein kinase complex
DN-hTERT	Dominant-negative version of human telomerase
DSBs	DNA double-strand breaks
EME1	Essential meiotic endonuclease 1
ERCC1	Excision repair cross-complementing 1
EXO1	Exonuclease 1
FANCN	Fanconi anemia protein N
GEN1	General endonuclease homolog 1
HDR	Homology-dependent repair
indels	Insertions and/or deletions
Ku	Ku70:Ku86 heterodimer
LIGI	DNA ligase I
LIGIII	DNA ligase III
LIGIV	DNA ligase IV
MRE11	Meiotic recombination defective 11
MRN	MRE11/RAD50/NBS1
MUS81	Mutagen sensitive 81
NBS1	Nijmegen breakage syndrome 1
NRT	Non-reciprocal translocation
nt	Nucleotide
PARP1	Poly(ADP-ribose) polymerase 1
PAXX	Paralog of XRCC4 and XLF

59	POLQ	DNA polymerase *theta*
60	RAD50	Radiation sensitive 50
61	RAD51	Radiation sensitive 51
62	RAD54	Radiation sensitive 54
63	RMI1	RecQ-mediated genome instability homolog 1
64	RPA	Replication protein A
65	SLX1	Synthetically lethal with unknown function (X) 1
66	SLX4	Synthetically lethal with unknown function (X) 4
67	ssDNA	Single-stranded DNA
68	STELA	Single telomere length analysis
69	TALEN	Transcription activator-like effector nuclease
70	TRF2	Telomere recognition factor 2
71	TOPO3α	Topoisomerase 3α
72	XLF	XRCC-4-like factor/Cernunnos
73	XPF	Xeroderma pigmentosum gene F
74	XRCC4	X-ray cross complementing group 4
75		

Abstract

Chromosomal translocations are now well understood to not only constitute signature molecular markers for certain human cancers but often also to be causative in the genesis of that tumor. Despite the obvious importance of such events, the molecular mechanism of chromosomal translocations in human cells remains poorly understood. Part of the explanation for this dearth of knowledge is due to the complexity of the reaction and the need to archaeologically work backwards from the final product (a translocation) to the original unrearranged chromosomes to infer mechanism. Although not definitive, these studies have indicated that the aberrant usage of endogenous DNA repair pathways likely lies at the heart of the problem. An equally obfuscating aspect of this field, however, has also originated from the unfortunate species-specific differences that appear to exist in the relevant model systems that have been utilized to investigate this process. Specifically, yeast and murine systems (which are often used by basic science investigators) rely on different DNA repair pathways to promote chromosomal translocations than human somatic cells. In this chapter, we will review some of the basic concepts of chromosomal translocations and the DNA repair systems thought to be responsible for their genesis with an emphasis on underscoring the differences between other species and human cells. In addition, we will focus on a specific subset of translocations that involve the very end of a chromosome (a telomere). A better understanding of the relationship between DNA repair pathways and chromosomal translocations is guaranteed to lead to improved therapeutic treatments for cancer.

Contents

5. Introduction

The concept of chromosomal translocations – in which a portion of one chromosome breaks off and fuses inappropriately to another chromosome – has been part of the scientific consciousness for the better part of eight decades. Chromosomal translocations were first described by Karl Sax in 1938 (Sax 1938) and then elaborated by Barbara McClintock in the construction of her seminal "breakage-fusion-bridging" [BFB] model during the 1940s (McClintock 1941). Chromosomal translocations gained significant clinical relevance a couple of decades later when it was demonstrated that a single recurring chromosomal translocation (the Philadelphia chromosome) was often found in patients suffering from leukemia (Nowell 1962; Rowley 1973). Chromosomal translocations are now well understood to not only constitute signature molecular markers of human cancers (solid tumors in addition to blood cancers) but to be causative in their genesis as well (Lieber 2016; Grade et al. 2015). As such, these translocations become extremely important for clinical diagnostics as well as treatment-related options, respectively. Moreover, with the advent of comprehensive cancer genome sequencing, it is now appreciated that translocations, causative or not, are a common feature of human tumors (Stratton et al. 2009; Bunting and Nussenzweig 2013). It is not surprisingly, therefore, that interest in identifying and quantitating chromosomal translocations has increased exponentially in the past decade. As a consequence of this interest – and the experimentation associated with it – tens of thousands of translocations in a veritable bevy of different human cancers have been catalogued and characterized (Stratton et al. 2009; Lieber 2016). While incredibly rich in molecular information, most of these studies suffer (biologically speaking) in being retrospective; that is, the mechanism for how the translocation occurred is often (correctly or incorrectly) simply inferred after the fact from the junctional sequences present at the site of a chromosomal translocation.

To try and address this dearth of mechanistic knowledge, this chapter will focus on the relationship between DNA repair (specifically DNA double-strand break [DSB] repair) and chromosomal translocations. An understanding of DNA DSB repair is paramount to our discussion because it seems obvious, if only intuitively, that a chromosomal translocation is the result of aberrant DNA DSB repair (Bohlander and Kakadia 2015; Iliakis et al. 2015; Roukos and Misteli 2014). However, DNA DSB repair is infrequently

– and probably only rarely – aberrant because it is responsible for the stability of the genome. Thus, it needs to be appreciated and emphasized that chromosomal translocations are by far the exception to the rule of the normally helpful processes (predominately DNA DSB repair) that keep the genome stable.

It is a tautology and a fact appreciated by all cancer researchers that a stable genome is highly desirable and is inherently anti-oncogenic. While this perspective is basically sensible, it is also important to remember that complete stability is antithetical with evolution/life. That is, perfect immutability is contrary to the process of evolution and thus nature must maintain a balance between accurate DNA repair and the formation of mutations (*i.e.*, the lack of – or mis-repair of – DNA) upon which selection can act. Thus, all DNA repair processes, DNA DSB repair included, have a bit of "sloppiness" inherent in their mechanism. It is likely that chromosomal translocations are the result of one of these rare sloppy DNA DSB repair events. Trying to identify how, why and when such events occur; and perhaps most importantly – whether they can be abrogated – is the clinically relevant goal for this field.

6. DNA Damage

As elaborated above, the human genome needs to be nearly (but not completely) immutable in order to ensure the survival of the species. This turns out to be an extremely tall order as the human genome is constantly being chemically assaulted by both endogenous and exogenous factors. The endogenous sources of damage likely vary from cell type to cell type, but can include: lesions associated with aberrant lymphoid gene recombination, DNA replication errors, transcriptional errors, the formation of reactive oxygen species during oxidative phosphorylation, as well as the spontaneous depurination or deamination of nucleotides [nts] due to the proximity of DNA to adjacent water molecules (Tubbs and Nussenzweig 2017) or the aberrant action of cellular deaminases (Swanton et al. 2015). Exogenous sources of DNA damage include, but are certainly not limited to, exposure to ultraviolet light, chemotherapeutic drugs, or ionizing radiation. Indeed, *in toto*, it is estimated that each human cell sustains an astronomical ~70,000 lesions per day (Tubbs and Nussenzweig 2017). Importantly, however approximately 69,975 of these lesions result in DNA damage on only one strand of the DNA duplex. Thus, although the type of damage can vary extensively and certain types of lesions require discrete DNA repair pathways (expanded upon in the next section) these processes are inherently high fidelity as a consequence of having an undamaged DNA strand upon which to template the repair events. Thus, in human cells there is a surprisingly high level of DNA damage occurring on a daily basis that is nearly completely neutralized by conservative DNA repair pathways that utilize undamaged DNA to restore genome integrity.

In contrast to all the other types of lesions combined, human cells suffer only about 25 DSB lesions per cell per day (Tubbs and Nussenzweig 2017). Again, the exact cause of the DSB can vary greatly depending upon the cell type. Some likely occur due to aberrant lymphoid recombination processes (Lieber 2016), whereas others may be due to reactive metabolic oxygen production, DNA replication errors (Barnes and Eckert 2017) or the inappropriate action of cytidine deaminases (Knisbacher et al. 2016). Whatever their exact origin, DNA DSBs are uniquely toxic to cells because when both strands of the chromosome are damaged most of the time the only way to restore the chromosome to its original state is if an undamaged homologous chromosome (or sister chromatid if the DSB should occur during S phase of the cell cycle) is available to

template the repair event. As a consequence, DSBs are inherently more mutagenic than most other types of lesions because of the difficultly in enacting their proper repair. A second parameter, which is relevant to this chapter, is that for all the other lesions, not only can the undamaged DNA strand help to enact error-free repair, but it also perforce holds the chromosome intact. In contrast, the formation of a DSB generates a window of opportunity, however small, for the two chromosomal fragments to move away from one another. If this happens, the chances of one of those fragments "repairing" itself onto another chromosome (*i.e.*, causing a translocation) rises astronomically.

In summary, the vast majority of the DNA lesions that a human cell experiences on a daily basis are generally rapidly and correctly repaired and are likely not relevant for the genesis of chromosomal translocations. Importantly, this is not to say that these types of lesions cannot cause chromosomal translocations. It is just likely that it is not a single single-stranded lesion *per se* that can trigger translocations, but the juxtaposition of two closely spaced single-strand lesions that give rise to a *de facto* DSB that are the culprit. Thus, DSBs and DNA DSB repair (or the lack thereof) have been firmly established as being mechanistically responsible for chromosomal translocations.

7. DNA Repair

7.1 DNA Repair Involving only a Single Strand

Due to the broad spectrum of lesions that can occur to DNA it is not surprising that discrete DNA repair pathways have evolved to correct these life-threatening alterations. Of all the lesions that damage only a single-strand of DNA most result in the formation of only a singly modified nt or an abasic site. These lesions are readily repaired by the base excision repair pathway (Figure 1A). This process involves the action of DNA gylcosylases, apurinic or apyrimidinic endonucleases and phosphodiesterases that ultimately convert the lesion into a single-stranded nick. This nick is then filled in by a DNA polymerase and sealed by a DNA ligase (Wallace 2014).

When the DNA lesion is bulkier than a single standard nt or when nts are fused together (*e.g.* via the formation of pyrimidine dimers) then a more complicated repair pathway, nucleotide excision repair (Figure 1B), is utilized that is capable of restoring stretches of nts (up to 24 nts in humans) in one event. In nucleotide excision repair, the bulkier lesion is recognized by a multi-subunit protein complex that introduces nicks 5' and 3' of the lesion. The offending lesion is then removed as an oligonucleotide by the action of a helicase and the resulting ~20 nt gap is filled in by a polymerase and then sealed by a DNA ligase (Spivak 2015).

A third common type of lesion is the mis-incorporation of nucleotides and/or generation of small insertions or deletions [indels] during DNA replication. These types of lesions are repaired by mismatch repair (Figure 1C). The mismatch repair machinery consists of large heterodimeric complexes that scan DNA and look for helical distortions due to the mispairing or indels. These complexes recruit additional factors including endonucleases that nick and exonucleases that degrade one of the strands resulting in the removal of the offending mispaired nucleotide and some flanking nucleotides. As before, the resulting gap is subsequently filled in by a DNA polymerase and sealed by a DNA ligase (Li et al. 2016).

All three of the above processes (base excision repair, nucleotide excision repair and mismatch repair) are critical for cellular and organismal well-being. Mutation of any of the factors associated with these pathways is generally either lethal or oncogenic, (although, pertinently, in the latter scenario not usually associated with chromosomal translocations). The importance of single-stranded DNA repair for genome stability is

further evidenced by the awarding of the 2015 Nobel Prize in Physiology or Medicine to the investigators responsible for the discovery and/or initial characterization of these DNA repair pathways (Kunkel 2015).

7.2 DNA Double-Strand Break Repair

7.2.1 C-NHEJ

Although DNA DSB lesions occur proportionately much less frequently than single-stranded lesions, they are so toxic that cells have evolved multiple pathways that utilize hundreds of genes to make sure the DSBs are quickly and (semi)-accurately repaired. The two major pathways are nonhomologous end joining [NHEJ] (Woodbine et al. 2014; Lieber 2010) and homology-dependent repair [HDR] (Jasin and Rothstein 2013).

In higher eukaryotes, DNA DSB repair proceeds most frequently by a process that does not require extended regions of homology. Specifically, mammalian cells – and humans in particular – have evolved a highly efficient ability to join nonhomologous DNA molecules together (Roth and Wilson 1985). This pathway is referred to as classic NHEJ [C-NHEJ] and it is generally error-prone. The evolution of a repair pathway that is error-prone may seem paradoxical but is likely due to 1) the increased percentage of non-coding DNA in higher eukaryotes, a feature that more readily tolerates imprecise rejoining (a luxury that bacteria and lower eukaryotes do not have) and 2) the requirement for productive error-prone repair during lymphoid recombination processes to generate a large immune repertoire.

Many of the details of C-NHEJ have been worked out, and the process is well (albeit certainly not completely) understood. Following the introduction of a DSB into a chromosome, DNA Ligase IV [LIGIV] will often (if possible) attempt to immediately and precisely rejoin the broken ends to generate a perfect repair event. Exactly how frequently such "error-free" C-NHEJ repair occurs is not known (Betermier et al. 2014), but it is now appreciated that it can occur much more frequently than had been believed (Oh et al. 2014; Waters et al. 2014). At least some fraction of the time, however, the ends cannot be properly rejoined (due, for example, to the loss of nts and/or to aberrant adducts at the break site). In these instances, the ends are bound by the Ku86:Ku70 heterodimer [Ku; reviewed by (Hendrickson et al. 2006)], a highly abundant protein complex that binds to the broken DNA ends to prevent unnecessary DNA degradation (Figure 2). The binding of Ku to the free DNA ends subsequently recruits and activates

the DNA-dependent protein kinase complex catalytic subunit [DNA-PK_{cs}, (Jette and Lees-Miller 2015; Blackford and Jackson 2017)]. DNA-PK_{cs}:DNA-PK_{cs} homotypic interactions (one molecule on each end of the DSB), in turn, are the critical feature required for synapsis, which retains the two broken ends near one another (Sibanda et al. 2017; Spagnolo et al. 2006). Once a Ku:DNA-PK_{cs} dimer [also referred to as the DNA dependent protein kinase complex; DNA-PK] is properly assembled at the broken ends it, in turn, activates a tightly-associated nuclease, Artemis (Moshous et al. 2003), to help trim any damaged DNA ends. The extent of deletion is usually only a few nts and generally does not extend much beyond 25 nts with few exceptions (Hendrickson et al. 1990; Gauss and Lieber 1996; Lieber 2010). Subsequently, the X family polymerases *mu* and *lambda* fill in missing nucleotides (Lieber 2010). The rejoining of the DNA DSB requires the recruitment (Critchlow et al. 1997b) of LIGIV and accessory factors: Paralog of XRCC4 and XLF [PAXX, (Ochi et al. 2015)], X-ray cross complementing group 4 [XRCC4, (Critchlow et al. 1997a; Li et al. 1995)] and/or XRCC4-like factor/Cernunnos [XLF, (Ahnesorg et al. 2006; Buck et al. 2006)] (Figure 2). Finally, it is relevant to note that most of the cells in a human being are either not cycling or in G_1 phase of the cell cycle. Because HDR (described below) is predominately restricted to cells in S phase (when a sister chromatid may be available for repair) C-NHEJ is perforce the preferred repair pathway in human cells and this accounts for its frequent usage. In summary, in humans the predominant pathway of DSB repair is C-NHEJ and it first utilizes LIGIV to try and simply re-ligate the ends of a DSB back together. Failing at that, C-NHEJ keeps the ends in proximity, polishes them up by limited resection and polymerization as needed and then uses LIGIV to religate the ends. Due to the nuclease and polymerase action on one or both of the ends, small indels are a classic and frequent hallmark of chromosomal junctions repaired by C-NHEJ.

7.2.2 A-NHEJ

It had long been appreciated that the kinetics of C-NHEJ were biphasic – most (~80%) of the ends were rejoined quickly (within 15' to 30' of the chromosome breaking), but some ends could take hours to finally be rejoined. For many years this was interpreted simply as some DSBs being "easier" to repair than others. It was the laboratory of George Iliakis that first suggested that the slow phase of DSB repair may in fact represent a completely separate repair pathway (Wang et al. 2003). This hypothesis

dovetailed nicely with earlier work done in yeast, which had genetically documented that in the absence of Ku, DSBs could be repaired by an alternative error-prone end-joining process that utilized microhomology (Boulton and Jackson 1996). Although still somewhat controversial (Pannunzio et al. 2014) significant evidence has accumulated over the past 15 years to substantiate the Iliakis hypothesis. The hallmarks of this pathway (generally referred to as alternative nonhomologous end joining [A-NHEJ] to distinguish it from C-NHEJ) are that it is Ku-independent and utilizes small (3 nt^{+}) regions of (Frit et al. 2014; Iliakis et al. 2015; Boulton and Jackson 1996) to facilitate end joining. The process of A-NHEJ is mechanistically simple and straightforward: both ends of the DSB are resected to generate 3' overhangs that are intermediate in length from those generated during C-NHEJ (which are a few nucleotides at most) and HDR (which are often hundreds or thousands of nt long). These resected ends can then base pair using now exposed stretches of "microhomology" (probably 3^{+} nt). Nucleases are recruited to trim the flaps that are often generated and the nicks/ends are then sealed by a ligase (Frit et al. 2014; Iliakis et al. 2015). Thus, A-NHEJ is inherently an error-prone repair process as it always generates deletions including one of the two regions of microhomology and all the DNA in between the two patches of microhomology.

Although the intellectual concept of how A-NHEJ occurs is clear, the genetics and biochemistry of the synapsis, processing and ligation of an A-NHEJ DSB repair event are still quite obscure leading to heavy debate by investigators in the field as to the precise mechanism. Several studies have suggested that, like Ku for C-NHEJ, the protein poly(ADP-ribose) polymerase 1 [PARP1] may bind to the DNA ends (Figure 3). Indeed, there is evidence that PARP1 may even compete with Ku for access to the ends thereby determining the choice of the NHEJ pathways used for the repair of specific DSBs (Cheng et al. 2011). Alternatively, the repair complex meiotic recombination 11/radiation sensitive 50/Nijmegen breakage syndrome 1 [MRE11/RAD50/NBS1; MRN] has also been implicated as the A-NHEJ end tethering activity (Dinkelmann et al. 2009; Xie et al. 2009). Regardless of whether recognition or tethering of the ends is carried out by either PARP1 or MRN, resection is required to reveal the microhomology that will subsequently be used to mediate the repair event. The initial resection is thought to be carried out by MRN and an associated nuclease, C-terminal interacting protein [CtIP]. The short resection mediated by MRN/CtIP is then elongated by DNA exonuclease 2 [DNA2] and/or exonuclease 1 [EXO1] (Mimitou and Symington 2008; Bernstein and

Rothstein 2009). Once sufficient 3'-single-stranded DNA [ssDNA] overhangs have been generated the strands can anneal through the exposed microhomology (Figure 3). Moreover, the presence of microhomology modulates further resection activity and stabilizes the junction to facilitate ligation (Paull and Gellert 2000). Finally, there are likely an additional number of enzymatic activities required for A-NHEJ including DNA polymerases and helicases, but most of these have only been inferred and not rigorously identified. Perhaps the only two enzymatic activities which seem clearly required are DNA polymerase *theta* [POLQ] and a flap endonuclease activity needed to clip off mispaired strands. In the case of POLQ, the evidence is strong that much of the microhomology introduced at DSB repair junctions is dependent upon this inherently error-prone enzyme (Ceccaldi et al. 2015; Mateos-Gomez et al. 2015). The flap endonuclease activity is very likely supplied by the structure-specific nuclease complex excision repair cross-complementing 1/xeroderma pigmentosum gene F [ERCC1/XPF] (Ahmad et al. 2008). Ultimately, the repaired DSB junction needs to be religated and DNA ligase III [LIGIII] appears to be the principal ligase used (Audebert et al. 2004; Della-Maria et al. 2011; Wang et al. 2005) although it is now clear that DNA ligase I [LIGI] can functionally substitute for LIGIII as well (Arakawa et al. 2012; Oh et al. 2014; Lu et al. 2016) (Figure 3).

In summary, in order for A-NHEJ to occur, the broken DNA ends must somehow bypass being repaired by C-NHEJ (how this occurs is poorly understood). If the ends are then subjected to significant, but nonetheless limited resection, they can utilize exposed microhomology to facilitate the repair event in a fashion that always generates deletions. Although most [albeit certainly not all, (Pannunzio et al. 2014)] investigators now accept that A-NHEJ is a discrete DNA repair pathway, it is confounded by two serious shortcomings. First, the most prominent feature of this pathway is the residual microhomology left at a repaired DNA DSB junction. However, the definition of microhomology is often investigator-arbitrary and may include microhomologies as short as 1 or 2 nts. Thus, there are a myriad of published studies where A-NHEJ is the inferred DNA repair mechanism because short microhomologies were observed at the repair junctions. This is unfortunate because C-NHEJ can also use and generate microhomologies of 1 or 2 nts during repair (Pannunzio et al. 2014). Thus, to be rigorous, at least 3 nt of homology is probably required before an assignment of A-NHEJ can confidently be given. When this criterion is utilized, the vast majority of DNA repair

events suggested to be caused by A-NHEJ is dramatically reduced [see, for example (Chiarle et al. 2011)]. The second failing of A-NHEJ is that there is no specific factor required for the repair event. LIGIII was one of the best candidates for such a factor, but recent work has shown that even this enzyme is dispensable for A-NHEJ (Arakawa et al. 2012; Oh et al. 2014). Thus, until this situation is clarified it seems as if the best operational definition of A-NHEJ is: 1) a DNA DSB process that is Ku- and LIGIV-independent but POLQ-dependent, 2) relies upon LIGIII or LIGI and 3) generates repair junctions with 3^{+} nts of microhomology.

7.2.3 HDR

Whereas C-NHEJ is the major DNA DSB repair pathway in human cells, DNA DSBs that occur in S phase of the cell cycle can instead be, and often are, repaired by HDR (Figure 4). In HDR [reviewed by (Jasin and Rothstein 2013)], the DNA ends of the incoming DNA are likely extensively resected to yield 3'-single-stranded DNA overhangs. As described above for A-NHEJ, the nuclease(s) responsible for this resection are the MRN:CtIP complex (which generates the initial resection) followed by the action of DNA2 and EXOI (Mimitou and Symington 2008; Bernstein and Rothstein 2009). The resulting overhangs are then coated by replication protein A [RPA], a heterotrimeric single-stranded DNA binding protein, which removes the secondary structures from the overhangs [reviewed by (Iftode et al. 1999)]. The breast cancer allele 1 and 2 [BRCA1 and BRCA2, respectively] proteins and the Fanconi anemia protein N [FANCN] then help to recruit radiation sensitive 51 [RAD51] to the overhangs (Venkitaraman 2014). RAD51 is the key strand exchange protein in HDR [reviewed by (Thacker 2005)]. In humans, there are at least seven Rad51 family members and almost all of them have been implicated in some aspect of HDR and also in human disease. Strand invasion into the homologous chromosomal sequence requires RAD54 [radiation sensitive 54] and DNA replication. Rad54 is a double-stranded DNA-dependent ATPase that can remodel chromatin and it probably plays critical roles at several steps in the recombination process [reviewed by (Heyer et al. 2006)]. In particular, Rad54 is critical for stabilizing the Rad51-dependent joint molecule formation as well as for promoting the disassembly of Rad51 following exchange (Solinger et al. 2002). Strand exchange generates an interdigitated set of strands that can be resolved into a complicated set of products. In mitotic cells most

of the intermediates are resolved as non-crossover products by dissolving the interdigitated strands back into their original duplexes after sufficient DNA replication has occurred to restore the genetic information lost at the site of the DSB (Figure 4A). The dissolution process requires the action of the Bloom syndrome gene, topoisomerase 3α and RecQ-mediated genome instability homolog 1 [BLM, TOPO 3α, RMI1, respectively] complex (Wu and Hickson 2003). Less frequently the second end of DNA is captured and a covalently closed "Holliday junction" (Holliday 1964) is formed that can be resolved as either non-crossover products (which are functionally identical to dissolution) or crossover products (Figure 4B). The resolution of Holliday junctions is complicated and in human cells appears to be carried out by at least three partially, redundant resolvases consisting of mutagen sensitive 81/essential meiotic endonuclease 1 [MUS81/EME1, respectively], synthetically lethal with genes of unknown function (X) 1 and 4 [SLX1 and SLX4, respectively], and general homolog of endonuclease 1 GEN1] (Matos and West 2014). Finally, LIGI is utilized to covalently seal any nicks left in the DNA.

Although HDR is often referred to as error-free repair, that characterization is only partially true. Thus, in the case of non-crossover events in which the repair is templated from a sister chromatid the DSB is in fact repaired in an error-free fashion. However, when a homolog, rather than a sister chromatid, is utilized there is a risk of the loss of heterozygosity and uniparental disomy as observed in several developmental disorders and numerous tumor types (Tuna et al. 2009). In summary, human somatic cells express all of the gene products needed to carry out HDR. These events occur, however, only at very low frequency and usually only in S phase due to the preferred usage of NHEJ.

In summary, human cells can repair DNA DSBs by at least three discrete pathways: C-NHEJ, A-NHEJ and HDR. How pathway choice (which pathway is utilized in which cells during which phases in the cell cycle, *etc*.) is biochemically determined is the focus of much research. Regardless, from a logistical perspective, one thing that clearly differentiates these repair pathways is their reliance on different DNA ligases to complete the reaction. Thus, C-NHEJ utilizes exclusively LIGIV, whereas A-NHEJ prefers to use LIGIII (although it can utilize LIGI) and HDR uses exclusively LIGI. In conclusion, until better biochemical or genetic markers become available, ligation is one the most distinguishing features of these repair pathways.

8. Translocations

8.1. DSBs and Translocations

As enumerated above, DNA DSBs in human cells can occur either spontaneously or through exposure of the cells to environmental toxins. The vast majority of the time, the two ends of a DSB are rejoined back to one another either by C-NHEJ, A-NHEJ or HDR with a varying loss of genetic information, but in a fashion that almost always restores genome stability. Rarely, one or both ends of a DSB will be incorrectly rejoined to another DSB end resulting in a translocation. The biological consequences of this can be enormous as translocations can inactivate tumor suppressor genes, activate oncogenes or make new chimeric oncogenes (Bunting and Nussenzweig 2013; Bohlander and Kakadia 2015; Lieber 2016). All of these scenarios promote the formation of tumors.

Translocations can occur within a chromosome (an intrachromosomal translocation) which can result in inversions or to another chromosome (interchromosomal translocation). In the latter case, the simplest outcome is a reciprocal translocation where the proximal portion of one chromosome is joined to the distal portion of another chromosome and *vice versa*. Needless to say, since the occurrence of any one DSB is a relatively rare event (only 25 DSBs, per cell, per day) the likelihood of concomitant DSBs existing in the same cell at the same time is quite small and likely explains why translocations occur so much less frequently than other types of mutations. The only situation where two DSBs are not required is when one of the DSBs is the natural end of a chromosome; *i.e.*, a telomere. As we will discuss below, this is a specialized case of translocation. In all other cases, there is a requirement for 2 DSBs to exist simultaneously in order for a translocation to occur. The basic, but as yet still unanswered, question that drives virtually all research in this field is why are these DSBs simply not repaired normally? That is, two DSBs yield four DNA ends: 1 and 2 as well as 3 and 4. In normal repair reactions end 1 would get re-joined to 2 and end 3 would become re-joined to 4. In a translocation, however, 1 joins to 3 (or 4) and 2 joins to 4 (or 3). Why and how the ends of a DSB become available to join with an end other than the one they were normally connected with is key to understanding the genesis of translocations.

8.1.1. Of Men, Mice and Translocations

Before a discussion of the mechanistic aspects of this process can begin however, it is important to understand that the translocations that occur in human cells appear to arise by a different process than translocations that occur in the laboratory workhorse model

organism, the mouse. This appears to be an exceptionally unfortunate biological difference as the mouse is used for a veritable plethora of cancer modeling studies and an enormous amount of pre-clinical cancer research is carried out with the mouse.

In the mouse, it is manifestly compelling that translocations are mechanistically dependent upon A-NHEJ. This conclusion rests upon at least three pieces of evidence. First, in the mouse, when genes involved in C-NHEJ are mutated the translocation frequency actually increases (Zhang et al. 2010; Boboila et al. 2010). This observation is consistent with the interpretation that in the absence of C-NHEJ that there is likely a greater cellular reliance on A-NHEJ. Second, when DNA sequence analyses are utilized to investigate the junctional diversity of translocations in the mouse, the frequency of microhomology – a quasi-hallmark of A-NHEJ – found at the repair site is quite high (Chiarle et al. 2011; Frock et al. 2015). As noted above, however, the appearance of microhomology in and of itself is not unequivocally proof of the use of A-NHEJ. Thus, in one very large study of translocations carried out by the Alt laboratory 75 to 90% of all translocations had microhomologies of 1 to 5 nts at the breakpoint junction. However, only 10% of those same junctions were 5 nts or longer (Chiarle et al. 2011). In conclusion, while clearly not unequivocal, these data are consistent with the use of A-NHEJ. Third, and perhaps the strongest piece of data, comes from a demonstration that genetic ablation of nuclear LIGIII, reduced the occurrence of translocations in the mouse (Simsek et al. 2011). In summary, the increase in translocations when C-NHEJ is absent, the frequent use of microhomology at translocation breakpoints and the reduction of translocations when LIGIII is absent, compellingly indicate that translocations in the mouse are LIGIII-dependent and likely mediated by A-NHEJ.

In contrast (and certainly confusingly), by the same set of criteria it appears as if translocations in human cells are mediated by C-NHEJ. Thus, in contrast to the mouse, mutations in C-NHEJ genes LIGIV and XRCC4 greatly reduce the frequency of translocations in human somatic cells (Ghezraoui et al. 2014; Jones et al. 2014; Liddiard et al. 2016). In addition, although microhomology can be found at translocations breakpoint junctions in human tumors (Tsai et al. 2008), the frequency and amount of it is generally small (Stephens et al. 2009; Berger et al. 2012). Finally, the functional inactivation of LIGIII has little to no impact on translocations in human somatic cells (Ghezraoui et al. 2014). It should be noted, however, that inhibition of PARP1, an A-NHEJ gene, reduced translocations in some human cells (Wray et al. 2013; Byrne et al.

2014), but not in others (Liddiard et al. 2016). This latter observation notwithstanding, the reduction in translocations when C-NHEJ is absent, the infrequent use of microhomology at translocation breakpoints and the lack of an impact on the frequency of translocations when LIGIII is absent, compellingly indicate that most translocations in human cells are LIGIV-dependent and likely mediated by C-NHEJ.

Needless to say, these observations raise the question of why a seemingly similar process should be mechanistically so different in these two organisms. To date, there is no clear answer. The most likely explanation has to do with species-specific differences in the factors that make up the DSB repair pathways. For example, DNA-PK_{cs} is the key C-NHEJ factor that tethers the two ends of a DSB together through homodimerization (Sibanda et al. 2017; Spagnolo et al. 2006). Relevantly, DNA-PK_{cs} is more abundant (by ~ an order of magnitude) in human cells than it is in rodent cells (Finnie et al. 1995). Thus, the reduced quantities of DNA-PK_{cs} (and presumably therefore reduced numbers of tethered ends) may provide A-NHEJ in the mouse with additional windows of opportunity for the ends to dissociate and be conscripted by A-NHEJ factors, whereas in human cells, with a superabundance of DNA-PK_{cs} [there are estimated to be between one-half to one million molecules of DNA-PK_{cs} in every human cell; (Meek et al. 2008)] C-NHEJ is the dominant repair pathway. The obvious follow-up question of why human cells should contain so much more DNA-PK_{cs} than rodents is unfortunately not biochemically obvious, but the empirical fact that they do likely provides at least a partial answer for why the two organisms utilize the C-NHEJ and A-NHEJ pathways differentially. In addition, it is well known that chromatin organization and epigenetic modifications can affect the mutation rate across genomes (Schuster-Bockler and Lehner 2012; Tubbs and Nussenzweig 2017). Specific chromatin features and epigenetic marks are unlikely to be highly conserved across species and these differences may also impact upon the process of translocations. Finally, it is now appreciated that at least some of the endogenous DSBs generated in vertebrate cells may be due to the aberrant action of apolipoprotein B editing complex 3 [APOBEC3], a cytidine deaminase capable of introducing closely spaced nicks into the DNA (Swanton et al. 2015; Tubbs and Nussenzweig 2017). Importantly, there is a single APOBEC3 gene in the mouse, whereas in humans that locus has been significantly expanded to eight functional isoforms. Thus, differences in APOBEC3 expression could certainly causes significant differences in either the frequency and/or location of DSBs in the genome. Whatever

the correct answer(s) may be, it is important to appreciate that particular care must be taken in interpreting or extrapolating experimental results obtained in rodent model systems to humans since some of the basic biology appears to be different [discussed at length as well by (Lieber 2016)].

8.1.2. Spatial Karma and Translocations

Regardless of which pathway of end joining (C-NHEJ or A-NHEJ) is used for repair, why are these processes not always faithful? The correlation of translocations with aberrant A-NHEJ is easiest to reconcile. Thus, while PARP1 can bind tightly to DNA ends, it is not known to homodimerize. Moreover, while some studies have suggested that MRN, or subunits thereof, are capable of homodimerization (Williams et al. 2008) there is frankly no A-NHEJ factor comparable to DNA-PK_{cs}. As a consequence of this, it seems likely that the ends of a DSB that are being repaired by A-NHEJ may not be as synaptically as stable as ends being repaired by C-NHEJ and therefore simply stand a statistically higher chance of separating from one another before the repair event is completed. This model dovetails nicely with the reduced amounts of DNA-PK_{cs} observed in rodents and their correspondingly greater propensity to utilize A-NHEJ in the formation of translocations. The flip side of this rationalization is more complex. Thus, in humans, where C-NHEJ apparently predominates, why does the end of DSB ever become capable of joining to an end other than its cognate end? Indeed, it is well known (albeit mostly from mouse studies) that C-NHEJ is more likely to join DSBs intrachromosomally rather than interchromosomally (Mahowald et al. 2009; Chiarle et al. 2011). In essence then, when C-NHEJ is utilized it is simply less likely that a translocation will result. The most compelling explanation for the translocations that do result is that the DSBs may be spatially adjacent to one another. For example, even early experiments on the spatial organization of the human genome noted that translocations often involved regions that were physically closer to one another than to other regions of the genome (Meaburn et al. 2007). These observations have been confirmed and extended over the past decade as technology has improved the characterization of the large-scale organization of chromosomes (Roukos and Misteli 2014; Roukos et al. 2013; Bohlander and Kakadia 2015). Thus, nuclear DSBs have a tendency not to move very much (Kruhlak et al. 2006; Jakob et al. 2009) and this correlates well with the observation that more than 80% of DSBs translocate to regions that are physically located to within 2.5 μm of each

other (Roukos and Misteli 2014; Roukos et al. 2013). In conclusion, the current best explanation for why translocations occur in human cells is "bad karma". That is, a translocation likely only occurs when two concomitant DSBs are also spatially close to one another in the nucleus such that a synaptic complex (likely a requirement for repair) can form – albeit in these rare instances between non-cognate ends.

8.1.3. Selection, not the Translocation, Drives Cancer

It is well known that particular translocations are the hallmark of certain cancers (Lieber 2016; Bunting and Nussenzweig 2013). However, it is important to appreciate that the predominance of a translocation in a tumor is due solely to the subsequent selection that is imposed upon all the translocations that may have occurred during the genesis of that tumor. That is, if, and only if – and this is a stochastic probability – the translocation generates a novel chromosome that gives the cell a selective growth advantage, will these cells be subsequently amplified to generate the tumor. Indeed, translocations that are oncogenic have invariably inactivated a tumor suppressor gene, activated an oncogene and/or created a chimeric gene that is acting as an oncogene. This event, however, is independent from the mechanism of the translocation; that is, there is nothing inherently oncogenic about translocations. Both ends of a DSB have a similar propensity to translocate (Chiarle et al. 2011) and although there is a bias towards translocations happening near transcriptional start sites in the mouse (Chiarle et al. 2011), this bias is not observed in human cells (Lieber 2016) (yet another difference between mice and humans). Consequently, it is important to appreciate is that there is no evidence of directionality or specificity intrinsic to translocations themselves. Thus, both ends of a broken chromosome likely have the potential to translocate to an infinite number of chromosomal locations and this is likely limited only by the spatial parameters discussed above.

8.2 Considerations for when one DSB is a Telomeric End

Up until now, all of the translocations that have been discussed were canonical ones requiring the formation of two DSBs and the generation of four DNA ends. There is one biologically important scenario, however, where translocations can occur between a DSB and a "single-ended DSB" and hence only involve three DNA ends. This scenario

occurs when the end of a chromosome, *i.e.*, a telomere, participates in the translocation reaction.

8.2.1 Telomeres Stabilize the Genome

There are 46 chromosomes in a normal diploid human cell and because each chromosome has 2 ends, there are in principle 92 natural DSBs constitutively present in a cell. Such a scenario, if it truly existed, would be lethal, so evolution has devised an answer in the form of telomeres. Telomeres are specialized nucleoprotein structures that are found at the extreme termini of linear eukaryotic chromosomes. Telomeres "cap" those ends and prevent the recognition of the chromosomal termini as DSBs by the cellular DNA damage response apparatus. Telomeres consist of a repetitive hexameric tract of DNA (TTAGGG) bound by an evolutionarily-conserved complex of proteins collectively called Shelterin (de Lange 2005). Importantly, ongoing cell division (*i.e.*, aging) results in gradual telomere erosion (Harley et al. 1990), and ultimately, the loss of the end-capping function which, in the context of a functional DNA damage response, leads to the induction of a p53-dependent G_1/S cell cycle arrest, known as replicative senescence (d'Adda di Fagagna et al. 2003). This cell-intrinsic limit on replicative lifespan provides a stringent tumor suppressive mechanism. However, in the absence of a fully functional DNA damage checkpoint response, older cells containing short dysfunctional telomeres (which are essentially one-ended DSBs) enter a state of crisis during which telomeres undergo fusion, either between sister chromatids (Figure 5A), with interchromosomal telomeres (Figure 5B) or with non-telomeric DSBs, creating dicentric chromosomes and initiating BFB cycles (Counter et al. 1992; Murnane 2012). This, in turn, leads to the creation of genomic rearrangements, including the translocations that are common in cells from many different tumor types (Artandi et al. 2000; Shih et al. 2001). The development of single-molecule approaches to characterize the sequence of telomere fusion events, has revealed that short dysfunctional telomeres are capable of recombining with both telomeric and non-telomeric loci across the genome (Liddiard et al. 2016; Letsolo et al. 2010). Thus, whilst BFB cycles initiated because of telomere dysfunction can lead to chromosomal translocations (Murnane 2012), telomere fusions themselves can also lead directly to translocation events. Intra-chromosomal telomere fusion involving sister-chromatids predominates over inter-chromosomal telomere fusion, which in turn is more frequent than inter-chromosomal fusion between telomeres and

non-telomeric loci (Liddiard et al. 2016). The characteristics of the non-telomeric loci involved in telomere fusion have yet to be fully characterized, but thus far it is apparent that they occur predominantly within coding regions of the genome; indicating a potential role for chromatin structure and replication timing in conferring sensitivity to fusion (Liddiard et al. 2016). Larger datasets documenting the specific loci involved in telomere fusion are required before a definition of these fusagenic loci can be provided and potential hot spots identified.

Thus, telomere-dependent crisis is a key event in driving genomic instability and clonal evolution during the progression to malignancy; this is consistent with data and observations of telomere dynamics and fusion in a broad range of human tumor types in which extreme telomere erosion and fusion is observed that correlates with the presence of large-scale genomic rearrangements (Lin et al. 2010; Roger et al. 2013; Meeker et al. 2004). Moreover, patients with tumors that display short dysfunctional telomeres, within the length ranges at which fusion can occur, exhibit a poorer prognosis and response to treatments (Lin et al. 2014; Strefford et al. 2015; Williams et al. 2017). Short dysfunctional telomeres have been identified in the very earliest lesions, including very small adenomatous colorectal polyps (Roger et al. 2013) and in leukemias prior to clinical progression (Lin et al. 2010). Importantly, the short telomeres observed in early stage lesions are identical in length to those observed in more advanced disease clones, indicating that telomere length does not vary considerably during progression. Together these data are consistent with the presence of short telomeres in the cell in which the initiating mutation occurred and that this dictates the telomere length distribution of the developing clone. In this model, if the initiating cell contains short telomeres then the subsequent clone may have a "telomere-mutator" phenotype that drives genomic instability, translocations and clonal progression, whereas a cell with long telomeres gives rise to a clone with a more stable genome, which exhibits slower rates of clonal progression (92). Finally, it is important to note that short dysfunctional telomeres have been observed in the majority of tumor types analyzed (Jones et al. 2012) and thus it appears that a period of telomere-driven genome instability may be a common mechanism underlying the progression to malignancy. Therefore, there is a requirement to understand the mechanisms by which telomere dysfunction can facilitate genome instability.

8.2.2 Translocations Involving Telomeres Are Mechanistically Distinct

Telomere fusion is clearly an important and physiologically relevant, mutational event. Key to the function of mammalian telomeres is the Shelterin complex that plays a fundamental role in protecting the natural chromosomal termini from aberrant NHEJ-mediated joining events (de Lange 2005). For example, in the mouse, the abrogation of telomere recognition factor 2 [TRF2], a core component of Shelterin, confers a widespread telomere fusion phenotype (van Steensel et al. 1998) that is dependent upon the activity of LIGIV. In contrast, fusions were readily detected in telomerase-deficient mice, with short dysfunctional telomeres, despite the absence of core components of C-NHEJ pathway, including DNA-PK_{cs} or LIGIV (Maser et al. 2007; Rai et al. 2010). Thus, in the mouse and in the context of short dysfunctional telomeres, which is likely the most biologically relevant form of telomere dysfunction, telomeres are no longer fully recognized by the Shelterin complex and the processing of telomere fusion appears to be mediated by either C-NHEJ or A-NHEJ.

The view that telomere-mediated translocations may be mechanistically distinct from canonical two DSB-mediated translocations is consistent with the molecular analysis of telomere fusion events directly from human cells undergoing a telomere-driven crisis in culture. These data show that fusion between short telomeres – ones that are almost completely denuded of telomere repeats – is accompanied by deletion and microhomology across the fusion points (Capper et al. 2007). The deletion that accompanies telomere fusion, includes not just the telomere repeat array itself, but extends into the telomere-adjacent DNA, up to the limit of the assays used (6.1 kb), the distribution of fusion points from the start of the telomere repeat arrays, indicates that deletion may be much more extensive. This characteristic profile is also observed at telomere fusion junctions isolated from some human malignancies, including early-stage and pre-malignant lesions (Lin et al. 2010; Roger et al. 2013), as well normal human cells, in which rare stochastic telomeric deletion results in fusion (Capper et al. 2007; Lin et al. 2010; Roger et al. 2013). Finally, molecular analysis of fusion events following replicative telomere erosion in human cells carrying hypomorphic MRE11 alleles revealed a change in the mutational spectrum with an increase in insertions at the fusion point (Tankimanova et al. 2012). The reliance on MRE11, the extensive deletion and the high degree of microhomology that accompanied these human telomere fusions was indicative of error-prone processing of short dysfunctional telomeres via the A-NHEJ pathway and suggested that telomere-

mediated fusions in human cells may be mechanistically fundamentally different than canonical two DSB-mediated translocations, which, as detailed above, appear to be predominately mediated by C-NHEJ.

8.2.3 Translocations Involving Human Telomeres can be Mediated by LIGIII or LIGIV

To experimentally test this idea, a study was undertaken utilizing human cell lines in which either nuclear LIGIII (Oh et al. 2014) or LIGIV (Oh et al. 2013) (and presumably A-NHEJ or C-NHEJ, respectively) had been inactivated by gene targeting. A dominant-negative version of human telomerase [DN-hTERT; (Hahn et al. 1999)] was then expressed in these cells to cause gradual telomere shortening and the status of the telomere stability was assessed by a single telomere length analysis [STELA; (Baird et al. 2003)] and single-molecule telomere fusion analyses. These approaches allow one to either 1) quantitate the length of a single telomere, 2) detect and characterize the DNA sequence of translocations or 3) detect and quantitate sister chromatid:sister chromatid fusions/translocations. These experiments demonstrated that translocations involving telomeres occurred in either LIGIII- or LIGIV-null cells (Jones et al. 2014). Thus, unlike canonical translocations, which are heavily dependent upon LIGIV, a high frequency of telomere-mediated translocations was still observed in LIGIV-null cells. There were, however, some parallels with canonical translocations. Thus, the majority of the translocations that occurred in LIGIII-null cells (*i.e.*, translocations perforce mediated by LIGIV) were biased 3:1 towards interchromosomal translocations, as is observed for canonical translocations. Similarly, in LIGIV-null cells (*i.e.*, translocations perforce mediated by LIGIII) while there were still interchromosomal translocations, telomere fusions were now biased 52:1 towards intrachromosomal sister chromatid fusion events (Jones et al. 2014). These biases were so significant that they had a profound biological effect – cells that were LIGIII-null were not able to survive the DN-hTERT-induced crisis whereas those that were either wild type or LIGIV-null readily survived. A parsimonious interpretation of this data is that the LIGIV-mediated interchromosomal translocations were predominately toxic und ultimately lethal for cells whereas the LIGIII-mediated intrachromosomal fusions provided a growth advantage that could be selected for during crisis. This interpretation is consistent with the gene duplications and localized

amplifications that are associated with sister:sister fusion events that are not observed with interchromosomal translocations (Murnane 2012).

These experiments beg the question as to why a telomere-mediated translocation (as compared to a interchromosomal DSB-mediated translocation) should be less reliant on C-NHEJ (and/or more reliant on A-NHEJ). The most obvious difference is simply that while a shortened telomere can bind a single DNA-PK complex, there is no corresponding end to bind a second DNA-PK complex and hence there is a greatly reduced chance of forming a synaptic complex. The lack of a synaptic complex presumably now permits the recruitment of A-NHEJ factors to the end and/or the displacement of the DNAPK complex from the end such that a higher frequency of A-NHEJ-mediated fusions can occur. Another factor that might influence the relative activities of A- and C-NHEJ at telomeres may be the nature of a short telomere, compared to a non-telomeric DSB. The telomeres terminate not with a blunted-ended DSB, but instead have a large (200 to 300 nt) overhang composed of TTAGGG repeats (Wright et al. 1997). This unique structure has the potential to fold into G-quadruplex structures (Xu et al. 2009) and may represent a non-canonical substrate for DNA repair activity that may favor the slower kinetics of the A-NHEJ pathway over that of C-NHEJ.

8.2.4 Translocations Involving Human Telomeres can be Mediated by LIGI

The above data strongly suggested that the geometry of the DNA ends and the availability of a requisite DNA ligase controls the type of translocations that can occur in human cells. To extend these observations a follow-up study was carried out in which the frequency and kind of translocation was quantitated in cells that were genetically engineered to be deficient for both LIGIII and LIGIV, where, presumably, both C-NHEJ and A-NHEJ would be ablated. In this experimental set-up the telomere was not gradually exposed by the expression of DN-hTERT as before, but was rapidly deleted by the use of a transcription activator-like effector nuclease [TALEN; (Ousterout and Gersbach 2016)]. A TALEN pair was designed to introduce a DSB 14 base pairs from the start of the telomeric TTAGGG repeat on the petite arm of chromosome 17. Thus, this experimental system is somewhat of a hybrid between those measuring canonical fusions and the system to gradually uncover a telomere end by enforced DN-hTERT expression. Specifically, the TALEN should generate a DSB with two ends, however, one of those ends is only a couple of kilobases long and consists solely of the telomeric

TTAGGG hexameric repeat. It is unclear whether this end can function in a fashion similar to a canonical chromosomal end. With this caveat in mind, it was reassuringly observed that in the absence of LIGIV a greatly decreased frequency of interchromosomal translocations was observed (Liddiard et al. 2016). Very surprisingly, however, in the combined absence of LIGIII and LIGIV significant amounts of both inter-and intrachromosomal translocations were observed inter-chromosomal translocations were also detected albeit at a reduced frequency (Liddiard et al. 2016). Interestingly, whilst the frequency of inter-chromosomal fusion events was decreased in the absence of LIGIV, intra-chromosomal sister chromatid fusion events appeared to be largely unchanged in the different genetic backgrounds tested. Moreover, there were differences in the utilization of microhomology, with significantly greater microhomology observed at intra-chromosomal events compared to inter-chromosomal events. Taken to together these data are consistent with a role for LIGIV-dependent C-NHEJ in driving interchromosomal telomere fusion and A-NHEJ being predominant for intrachromosomal sister chromatid telomere fusion. These data were also important because they provided the first demonstration in human cells that LIGI can facilitate chromosomal translocations – both inter-chromosomal and intra-chromosomal sister chromatid translocations. Moreover, these data revealed considerable redundancy in the utilization of the specific ligases for end-joining, with LIGI being able to facilitate intra-chromosomal fusion as well as inter-chromosomal fusion, albeit less efficiently. This may be discouraging from the clinical perspective, as these data indicate that attempts to inhibit human translocations using small molecule inhibitors to LIGIII and LIGIV (Singh et al. 2014) are destined to fail due to the robust ability of LIGI to compensate for their absence. That said, any intervention that can skew the fusion spectrum towards inter-chromosomal events, creating a larger mutational burden on cells and influencing their ability to escape a telomere-driven crisis, may have clinical utility. A deeper understanding of the key proteins involved in A-NHEJ and telomere fusion may identify additional therapeutic targets that could allow for more selective interventions into these pathways.

9. Summary and Future Considerations

In summary, DSBs are normally repaired with high fidelity in the sense that the pieces of DNA that were contiguous before the DSB are contiguous after DNA repair, regardless of the "sloppiness" of the actual join. In order for a canonical chromosomal translocation

to occur there needs to be two contemporaneous DSBs within a cell (which is a low frequency event) and the ends that were contiguous with one another before the DSBs, need to be rejoined aberrantly. This (mis)rejoining of the ends is likely mediated, at least in part, by their spatial proximity within the nucleus with the closer that the DSB ends are to each other the greater the likelihood of a translocation occurring. In the mouse, these events are predominately mediated by LIGIII/A-NHEJ whereas in human cells they are mediated by LIGIV/C-NHEJ. When a telomere shortens or when it loses its protective proteinaceous cap, the Shelterin complex, it is treated by the cell as a one-ended DSB and can engage in the formation of translocations as well. In this instance, however, both A-NHEJ and C-NHEJ seem to play an active role in mediating the resulting translocations. Layered over all of this is an additional layer of complexity provided by the recent demonstration that LIGI can fully compensate for translocations that were previously exclusively or predominately ascribed to LIGIII/A-NHEJ or LIGIV/C-NHEJ.

As is often the case in biology, reality is often much more complex than first envisioned. In the beginning, most models of chromosomal translocations invoked the aberrant use of either LIGIII or LIGIV. It is now clear that the situation is significantly more complicated with all three DNA ligases capable of generating translocations in a fashion that likely depends upon the state of the cell cycle, the level of expression of the various ligases within a given cell type and whether one of the translocating ends is telomeric or not. As a consequence, simplistic approaches of inhibiting a single ligase [and such specific inhibitors are not even currently available; (Singh et al. 2014)] are likely destined to fail. Nonetheless, it is clear that in a human cell where all three ligases are expressed that inhibiting LIGIV will significantly decrease interchromosomal translocations, which could potentially be used to therapeutic benefit. What is clinically perhaps more relevant however, is trying to inhibit the intrachromosomal sister chromatid:sister chromatid fusions as these appear to be critical for cells to escape crisis and thus become oncogenic (Jones et al. 2014). In this scenario, inhibition of both LIGI and LIGIII will likely be necessary to achieve a therapeutic outcome. Given that LIGI also has important functions in DNA replication (an essential cellular process) it is likely that such approaches will have significant toxic side effects. Nonetheless, as more is learned about all three DNA ligases, and especially about how pathway choice for DSB

repair is regulated there is still significant cause for optimism that windows of opportunity for therapeutic intervention will be uncovered.

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Figure Legends

Figure 1. Repair of lesions where one DNA strand is still intact. (A) Base Excision Repair. The schematic shows a small piece of double-stranded DNA (colored rectangles represent nucleotides) containing a singly modified nt (red diamond). This lesion is repaired by the action of DNA gylcosylases, endonucleases and phosphodiesterases that ultimately convert the lesion into a single-stranded nick. This nick is then filled in by a DNA polymerase and sealed by a DNA ligase. (B) Nucleotide Excision Repair. The schematic shows two nts (colored lines) that are fused together (red diamond). This lesion is recognized by a multi-subunit protein complex that introduces nicks 5' and 3' of the lesion. The offending lesion is then removed as an oligonucleotide by the action of a helicase and the resulting ~20 nt gap is filled in by a polymerase and then sealed by a DNA ligase. (C) Mismatch Repair. The schematic shows two nucleotides (colored lines) where one base pair is mispaired (red diamonds). The mispaired nts are recognized by mismatch heterodimeric complexes that recruit endonucleases that nick the DNA. Exonucleases then degrade one of the strands resulting in the removal of the offending nt as well as some flanking nts. As before, the resulting gap is subsequently filled in by a DNA polymerase and sealed by a DNA ligase.

Figure 2. A schematic depicting Classic Non-Homologous End Joining (C-NHEJ). The black lines represent strands of DNA. First, the Ku heterodimer (orange ball) binds onto the ends of the DNA. Ku then recruits DNA-PK_{cs} (blue oval) and the homotypic interactions between two DNA-PK_{cs} molecules tethers the ends together. The nuclease Artemis (yellow PacMan^{TM}), which is physically associated with DNA-PK_{cs}, can then remove any mispaired or damaged nucleotides from the ends. Most missing nts are then replaced by the DNA polymerases μ or λ (green pentagon). Finally, a ligase complex, consisting of DNA ligase IV (purple cylinder) and the accessory proteins PAXX (red half oval), XRCC4 (light green tear drop) and XLF (Carolina blue cropped pyramid) then religated the ends back together. This process often results in indels (denoted by the red rectangle) at the site of repair.

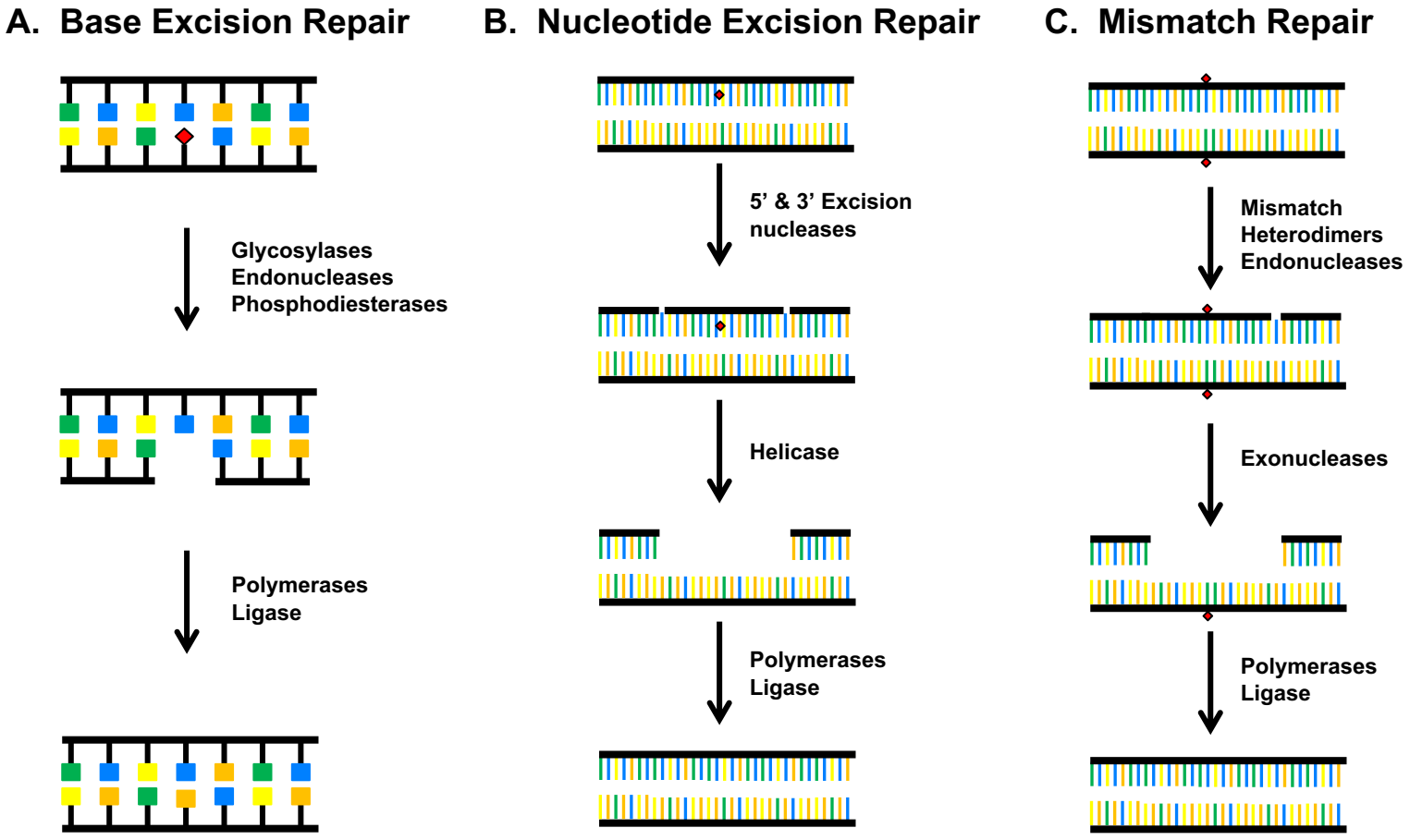
Figure 3. A schematic depicting Alternative Non-Homologous End Joining (A-NHEJ). The black lines represent strands of DNA and the red rectangles blocks of

microhomology. The broken ends may be held together either by PARP1 (Carolina blue hexagon) or by the MRN complex (orange oval). The initial resection is carried out by MRN and CtIP (crimson PacMan™). This short resection is then elongated by DNA2 and/or EXO1 (light green PacMan™). Once sufficient 3'-single-stranded DNA [ssDNA] overhangs have been generated the strands can anneal through the exposed microhomology. The presence of microhomology stabilizes the junction. The resulting flaps are likely cleaved off by the ERCC1/XPF heterodimer (yellow PacMan™). Before ligation occurs it is also likely that POLQ (purple lightning bolt) may act on the DNA ends. Ultimately, the repaired DSB junction is religated using either LIGIII (red cylinder) or LIGI (pink cylinder).

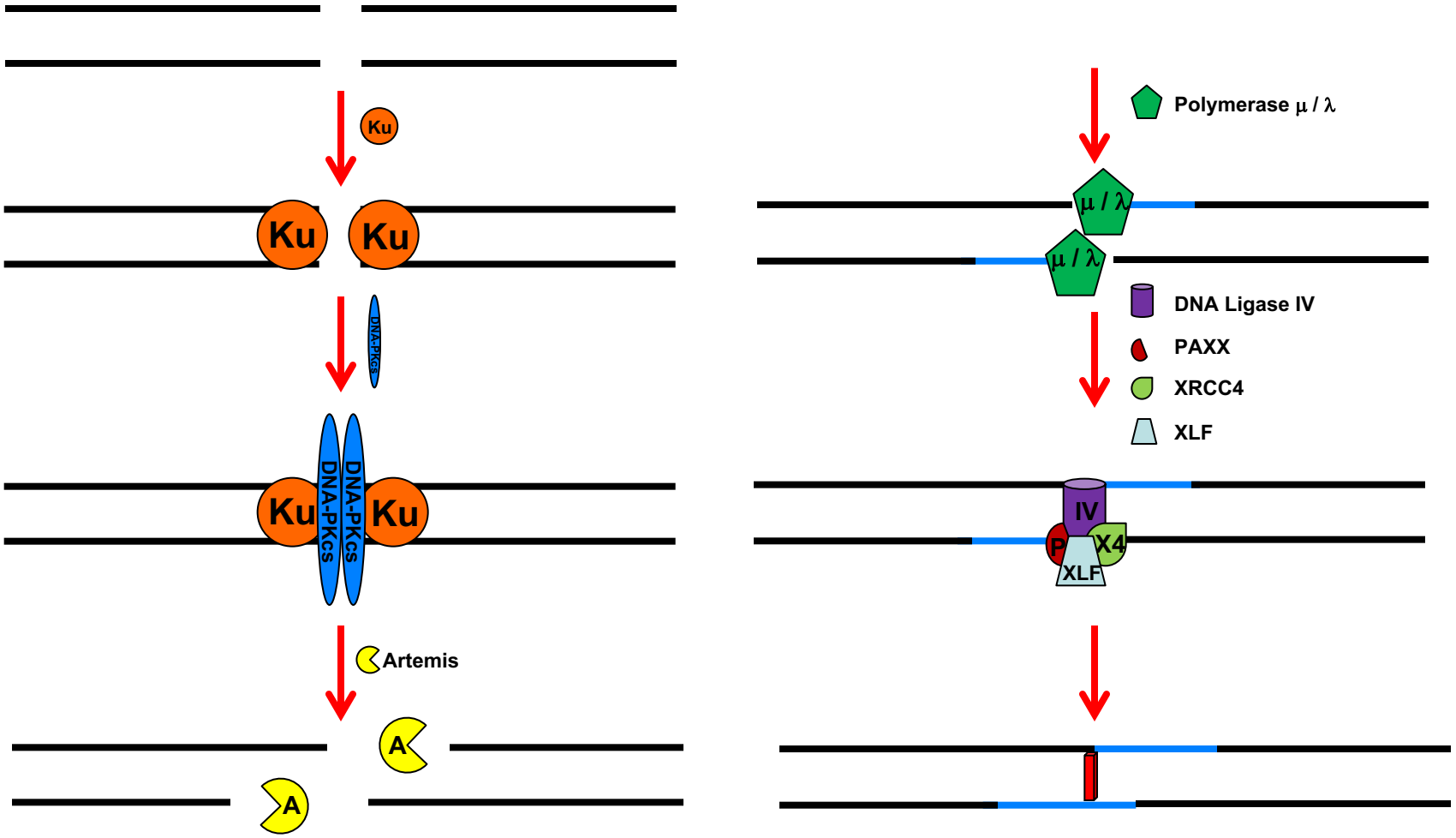
Figure 4. A schematic depicting Homology-Dependent Repair (HDR). The black lines represent strands of DNA where a DSB has occurred and the blue lines represent an undamaged sister chromatid or a homologue. In HDR, the DNA ends of the DSB are extensively resected to yield 3'-single-stranded DNA overhangs. As described for A-NHEJ, the nuclease(s) responsible for this resection are the MRN:CtIP complex (which generates the initial resection; orange oval and crimson PacMan™, respectively) followed by the action of DNA2 and EXOI (light green PacMan™). The resulting overhangs are then coated by RPA (red circles), which removes the secondary structures from the overhangs. A complex of proteins including BRCA1, BRCA2, and FANCN (purple cloud) then help to recruit RAD51 (pink diamond) to the overhangs. Strand invasion into the homologous chromosomal sequence requires RAD54 (yellow moon) and DNA replication (blue-green cloud). Strand exchange generates an interdigitated set of strands that can be resolved into a complicated set of products. (A) In mitotic cells most of the intermediates are resolved as non-crossover products by dissolving the interdigitated strands back into their original duplexes after sufficient DNA replication has occurred to restore the genetic information lost at the site of the DSB. The dissolution process requires the action of the BLM/TOPO 3α/RMI1 complex (green hexagon). (B) Less frequently the second end of DNA is captured and a covalently closed "Holliday junction" is formed that can be resolved as either non-crossover products (which are functionally identical to dissolution) or crossover products. The resolution of Holliday junctions is complicated and in human cells appears to be carried out by at least three partially, redundant resolvases consisting of mutagen sensitive 81/essential meiotic endonuclease

1 [MUS81/EME1, respectively], synthetically lethal with genes of unknown function (X) 1 and 4 [SLX1 and SLX4, respectively], and general homolog of endonuclease 1 GEN1] (1) (light blue cloud). Finally, LIGI (pink cylinder) is utilized to covalently seal any nicks left in the DNA. Note that only the non-crossover product for Holliday Junction resolution is diagrammed.

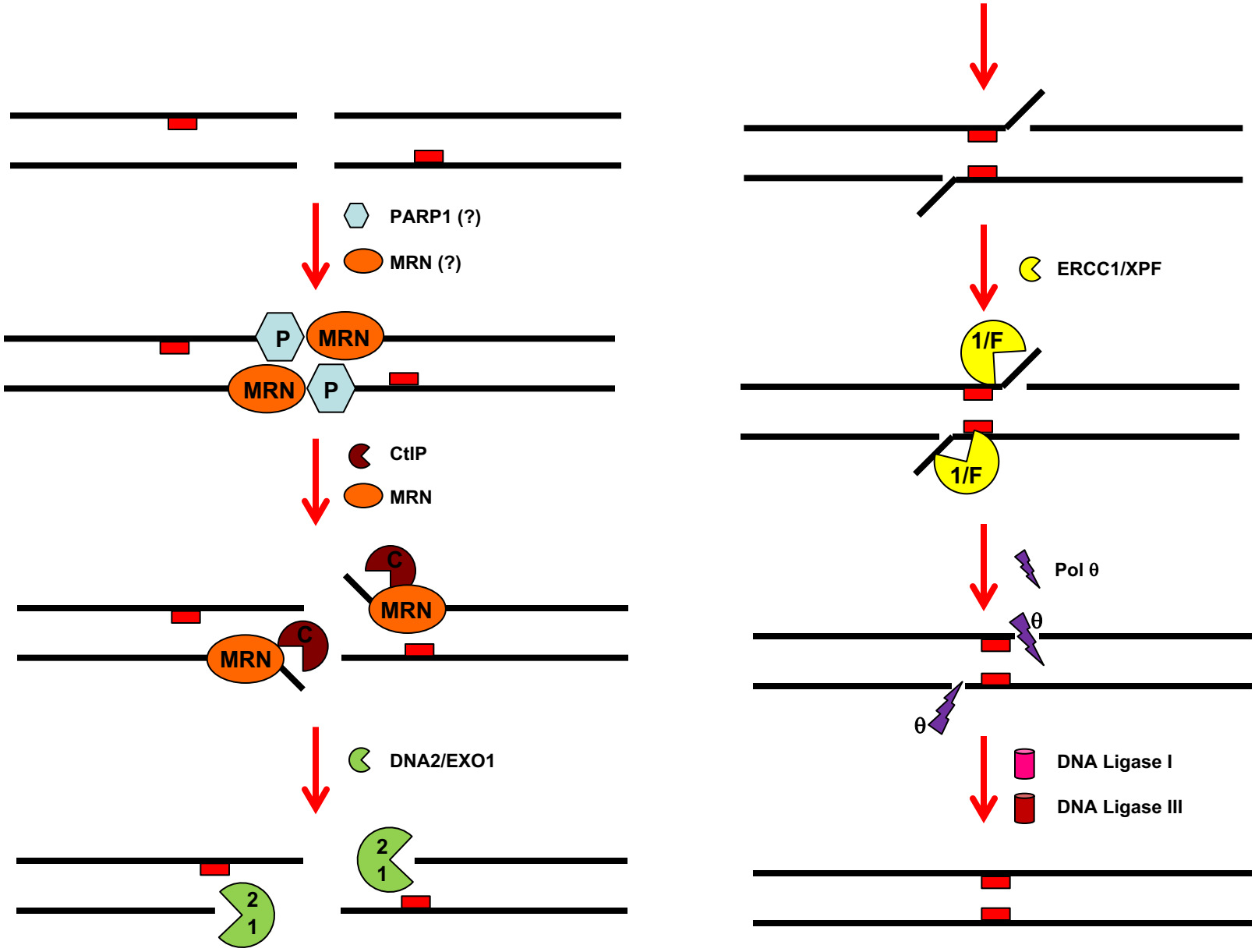
Figure 5. Short dysfunctional telomeres can be subjected to sister-chromatid fusion, or inter-chromosomal fusion events to create amplifications, deletion and non-reciprocal translocations. (A) Gradual telomere erosion in the absence of functional DNA damage checkpoints, leads to short telomeres that are no longer protected by the Shelterin complex. Fusion between sister-chromatids results in the formation of a dicentric chromosome, that can form a bridge between daughter cells at anaphase, that be subjected a breakage event. Depending on the position of the break, this can lead to a daughter cell that has lost terminal sequences, or has an addition copy of a gene – in this example, gene B. Further BFB cycles can lead to further amplification and deletion. This process can be stopped by the healing of a DSB via the acquisition of a *de novo* telomere, either by telomerase-mediated extension or by recombination with a pre-existing telomere. Centromeres are depicted as green ovals, telomeres by black and white rectangles and genes in colored squares. (B) inter-chromosomal telomere fusion between short dysfunction telomeres (depicted), or with non-telomeric DSBs, can lead to the formation of dicentric chromosomes and the initiation of BFB cycles that can lead to the formation of non-reciprocal translations (NRT) and deletions. This process can be prevented by chromosomal healing via the acquisition of new telomere.


A. Base Excision Repair
Glycosylases
Endonucleases
Phosphodiesterases
Polymerases
Ligase
B. Nucleotide Excision Repair
5' & 3' Excision
nucleases
Helicase
Polymerases
Ligase
C. Mismatch Repair
Mismatch
Heterodimers
Endonucleases
Exonucleases
Polymerases
Ligase

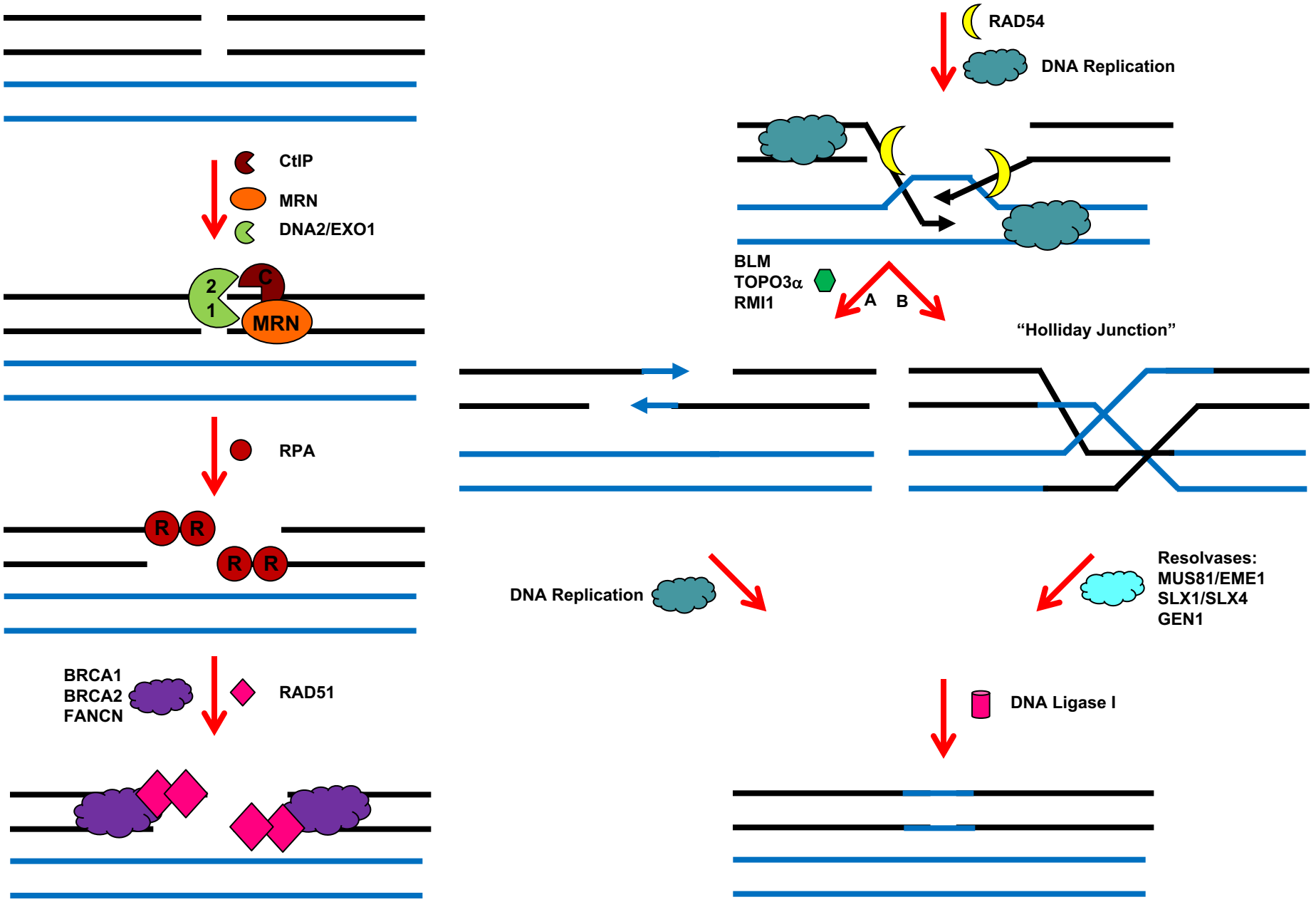
Classic Non-Homologous End Joining (C-NHEJ)



Alternative Non-Homologous End Joining (A-NHEJ)

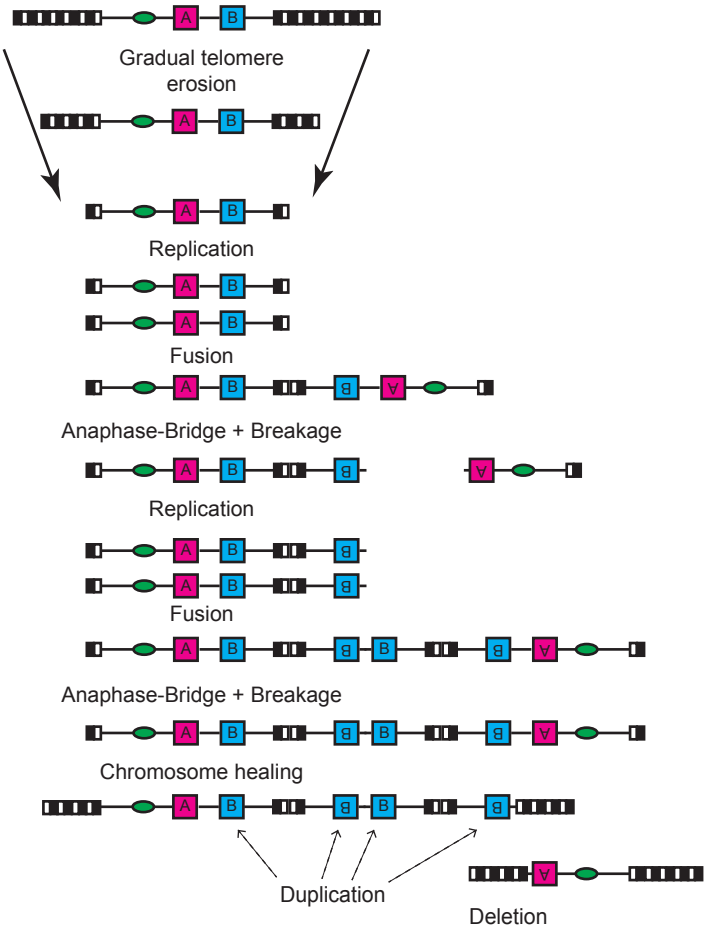


Homology-Dependent Repair (HDR)



Translocation Mechanisms

A Sister chromatid telomere fusion



B Inter-chromosomal telomere fusion