Telomeres and Chromosomal Translocations

subtitled

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

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26 **Abbreviations Used:**

27 A-NHEJ   Alternative nonhomologous end joining

28 APOBEC3   Apolipoprotein B editing complex 3

29 BFB   breakage-fusion-bridging

30 BRCA1   Breast cancer allele 1

31 BRCA2   Breast cancer allele 2

32 BLM   Bloom syndrome gene

33 C-NHEJ   Classic nonhomologous end joining

34 CtIP   C-terminal interacting protein

35 DNA2   DNA exonuclease 2

36 DNA-PK$_{cs}$ DNA dependent protein kinase catalytic subunit

37 DNA-PK   DNA dependent protein kinase complex

38 DN-hTERT   Dominant-negative version of human telomerase

39 DSBs   DNA double-strand breaks

40 EME1   Essential meiotic endonuclease 1

41 ERCC1   Excision repair cross-complementing 1

42 EXO1   Exonuclease 1

43 FANCN   Fanconi anemia protein N

44 GEN1   General endonuclease homolog 1

45 HDR   Homology-dependent repair

46 indels   Insertions and/or deletions

47 Ku   Ku70:Ku86 heterodimer

48 LIGI   DNA ligase I

49 LIGIII   DNA ligase III

50 LIGIV   DNA ligase IV

51 MRE11   Meiotic recombination defective 11

52 MRN   MRE11/RAD50/NBS1

53 MUS81   Mutagen sensitive 81

54 NBS1   Nijmegen breakage syndrome 1

55 NRT   Non-reciprocal translocation

56 nt   Nucleotide

57 PARP1   Poly(ADP-ribose) polymerase 1

58 PAXX   Paralog of XRCC4 and XLF

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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

162   – and probably only rarely – aberrant because it is responsible for the stability of the
163   genome.   Thus, it needs to be appreciated and emphasized that chromosomal
164   translocations are by far the exception to the rule of the normally helpful processes
165   (predominately DNA DSB repair) that keep the genome stable.
166   It is a tautology and a fact appreciated by all cancer researchers that a stable
167   genome is highly desirable and is inherently anti-oncogenic.   While this perspective is
168   basically sensible, it is also important to remember that complete stability is antithetical
169   with evolution/life.   That is, perfect immutability is contrary to the process of evolution
170   and thus nature must maintain a balance between accurate DNA repair and the formation
171   of mutations (*i.e.*, the lack of – or mis-repair of – DNA) upon which selection can act.
172   Thus, all DNA repair processes, DNA DSB repair included, have a bit of "sloppiness"
173   inherent in their mechanism.   It is likely that chromosomal translocations are the result
174   of one of these rare sloppy DNA DSB repair events.   Trying to identify how, why and
175   when such events occur; and perhaps most importantly – whether they can be abrogated
176   – is the clinically relevant goal for this field.
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6. DNA Damage

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179
180 As elaborated above, the human genome needs to be nearly (but not completely)
181 immutable in order to ensure the survival of the species. This turns out to be an
182 extremely tall order as the human genome is constantly being chemically assaulted by
183 both endogenous and exogenous factors. The endogenous sources of damage likely
184 vary from cell type to cell type, but can include: lesions associated with aberrant lymphoid
185 gene recombination, DNA replication errors, transcriptional errors, the formation of reactive
186 oxygen species during oxidative phosphorylation, as well as the spontaneous depurination
187 or deamination of nucleotides [nts] due to the proximity of DNA to adjacent water
188 molecules (Tubbs and Nussenzweig 2017) or the aberrant action of cellular deaminases
189 (Swanton et al. 2015). Exogenous sources of DNA damage include, but are certainly
190 not limited to, exposure to ultraviolet light, chemotherapeutic drugs, or ionizing radiation.
191 Indeed, *in toto*, it is estimated that each human cell sustains an astronomical ~70,000
192 lesions per day (Tubbs and Nussenzweig 2017). Importantly, however approximately
193 69,975 of these lesions result in DNA damage on only one strand of the DNA duplex.
194 Thus, although the type of damage can vary extensively and certain types of lesions
195 require discrete DNA repair pathways (expanded upon in the next section) these
196 processes are inherently high fidelity as a consequence of having an undamaged DNA
197 strand upon which to template the repair events. Thus, in human cells there is a
198 surprisingly high level of DNA damage occurring on a daily basis that is nearly completely
199 neutralized by conservative DNA repair pathways that utilize undamaged DNA to restore
200 genome integrity.
201 In contrast to all the other types of lesions combined, human cells suffer only about
202 25 DSB lesions per cell per day (Tubbs and Nussenzweig 2017). Again, the exact
203 cause of the DSB can vary greatly depending upon the cell type. Some likely occur
204 due to aberrant lymphoid recombination processes (Lieber 2016), whereas others may
205 be due to reactive metabolic oxygen production, DNA replication errors (Barnes and
206 Eckert 2017) or the inappropriate action of cytidine deaminases (Knisbacher et al. 2016).
207 Whatever their exact origin, DNA DSBs are uniquely toxic to cells because when both
208 strands of the chromosome are damaged most of the time the only way to restore the
209 chromosome to its original state is if an undamaged homologous chromosome (or sister
210 chromatid if the DSB should occur during S phase of the cell cycle) is available to

211 template the repair event. As a consequence, DSBs are inherently more mutagenic
212 than most other types of lesions because of the difficultly in enacting their proper repair.
213 A second parameter, which is relevant to this chapter, is that for all the other lesions,
214 not only can the undamaged DNA strand help to enact error-free repair, but it also
215 perforce holds the chromosome intact. In contrast, the formation of a DSB generates a
216 window of opportunity, however small, for the two chromosomal fragments to move away
217 from one another. If this happens, the chances of one of those fragments "repairing"
218 itself onto another chromosome (*i.e.*, causing a translocation) rises astronomically.
219   In summary, the vast majority of the DNA lesions that a human cell experiences on
220 a daily basis are generally rapidly and correctly repaired and are likely not relevant for
221 the genesis of chromosomal translocations. Importantly, this is not to say that these
222 types of lesions cannot cause chromosomal translocations. It is just likely that it is not
223 a single single-stranded lesion *per se* that can trigger translocations, but the juxtaposition
224 of two closely spaced single-strand lesions that give rise to a *de facto* DSB that are the
225 culprit. Thus, DSBs and DNA DSB repair (or the lack thereof) have been firmly
226 established as being mechanistically responsible for chromosomal translocations.
227

7. DNA Repair

7.1 DNA Repair Involving only a Single Strand

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

239 When the DNA lesion is bulkier than a single standard nt or when nts are fused
240 together (*e.g.* via the formation of pyrimidine dimers) then a more complicated repair
241 pathway, nucleotide excision repair (Figure 1B), is utilized that is capable of restoring
242 stretches of nts (up to 24 nts in humans) in one event. In nucleotide excision repair,
243 the bulkier lesion is recognized by a multi-subunit protein complex that introduces nicks
244 5' and 3' of the lesion. The offending lesion is then removed as an oligonucleotide by
245 the action of a helicase and the resulting ~20 nt gap is filled in by a polymerase and
246 then sealed by a DNA ligase (Spivak 2015).

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

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

261 further evidenced by the awarding of the 2015 Nobel Prize in Physiology or Medicine to
262 the investigators responsible for the discovery and/or initial characterization of these DNA
263 repair pathways (Kunkel 2015).
264

265 ## 7.2   DNA Double-Strand Break Repair
266 ### 7.2.1   C-NHEJ
267 Although DNA DSB lesions occur proportionately much less frequently than single-
268 stranded lesions, they are so toxic that cells have evolved multiple pathways that utilize
269 hundreds of genes to make sure the DSBs are quickly and (semi)-accurately repaired.
270 The two major pathways are nonhomologous end joining [NHEJ] (Woodbine et al. 2014;
271 Lieber 2010) and homology-dependent repair [HDR] (Jasin and Rothstein 2013).
272   In higher eukaryotes, DNA DSB repair proceeds most frequently by a process that
273 does not require extended regions of homology. Specifically, mammalian cells – and
274 humans in particular – have evolved a highly efficient ability to join nonhomologous DNA
275 molecules together (Roth and Wilson 1985). This pathway is referred to as classic
276 NHEJ [C-NHEJ] and it is generally error-prone. The evolution of a repair pathway that
277 is error-prone may seem paradoxical but is likely due to 1) the increased percentage of
278 non-coding DNA in higher eukaryotes, a feature that more readily tolerates imprecise
279 rejoining (a luxury that bacteria and lower eukaryotes do not have) and 2) the requirement
280 for productive error-prone repair during lymphoid recombination processes to generate a
281 large immune repertoire.
282   Many of the details of C-NHEJ have been worked out, and the process is well
283 (albeit certainly not completely) understood. Following the introduction of a DSB into a
284 chromosome, DNA Ligase IV [LIGIV] will often (if possible) attempt to immediately and
285 precisely rejoin the broken ends to generate a perfect repair event. Exactly how
286 frequently such "error-free" C-NHEJ repair occurs is not known (Betermier et al. 2014),
287 but it is now appreciated that it can occur much more frequently than had been believed
288 (Oh et al. 2014; Waters et al. 2014). At least some fraction of the time, however, the
289 ends cannot be properly rejoined (due, for example, to the loss of nts and/or to aberrant
290 adducts at the break site). In these instances, the ends are bound by the Ku86:Ku70
291 heterodimer [Ku; reviewed by (Hendrickson et al. 2006)], a highly abundant protein
292 complex that binds to the broken DNA ends to prevent unnecessary DNA degradation
293 (Figure 2). The binding of Ku to the free DNA ends subsequently recruits and activates

294   the DNA-dependent protein kinase complex catalytic subunit [DNA-PK$_{cs}$, (Jette and Lees-
295   Miller 2015; Blackford and Jackson 2017)]. DNA-PK$_{cs}$:DNA-PK$_{cs}$ homotypic interactions
296   (one molecule on each end of the DSB), in turn, are the critical feature required for
297   synapsis, which retains the two broken ends near one another (Sibanda et al. 2017;
298   Spagnolo et al. 2006). Once a Ku:DNA-PK$_{cs}$ dimer [also referred to as the DNA
299   dependent protein kinase complex; DNA-PK] is properly assembled at the broken ends
300   it, in turn, activates a tightly-associated nuclease, Artemis (Moshous et al. 2003), to help
301   trim any damaged DNA ends. The extent of deletion is usually only a few nts and
302   generally does not extend much beyond 25 nts with few exceptions (Hendrickson et al.
303   1990; Gauss and Lieber 1996; Lieber 2010). Subsequently, the X family polymerases
304   *mu* and *lambda* fill in missing nucleotides (Lieber 2010). The rejoining of the DNA DSB
305   requires the recruitment (Critchlow et al. 1997b) of LIGIV and accessory factors: Paralog
306   of XRCC4 and XLF [PAXX, (Ochi et al. 2015)], X-ray cross complementing group 4
307   [XRCC4, (Critchlow et al. 1997a; Li et al. 1995)] and/or XRCC4-like factor/Cernunnos
308   [XLF, (Ahnesorg et al. 2006; Buck et al. 2006)] (Figure 2). Finally, it is relevant to note
309   that most of the cells in a human being are either not cycling or in G$_1$ phase of the
310   cell cycle. Because HDR (described below) is predominately restricted to cells in S
311   phase (when a sister chromatid may be available for repair) C-NHEJ is perforce the
312   preferred repair pathway in human cells and this accounts for its frequent usage. In
313   summary, in humans the predominant pathway of DSB repair is C-NHEJ and it first
314   utilizes LIGIV to try and simply re-ligate the ends of a DSB back together. Failing at
315   that, C-NHEJ keeps the ends in proximity, polishes them up by limited resection and
316   polymerization as needed and then uses LIGIV to religate the ends. Due to the nuclease
317   and polymerase action on one or both of the ends, small indels are a classic and
318   frequent hallmark of chromosomal junctions repaired by C-NHEJ.
319
320   **7.2.2 A-NHEJ**
321   It had long been appreciated that the kinetics of C-NHEJ were biphasic – most (~80%)
322   of the ends were rejoined quickly (within 15' to 30' of the chromosome breaking), but
323   some ends could take hours to finally be rejoined. For many years this was interpreted
324   simply as some DSBs being "easier" to repair than others. It was the laboratory of
325   George Iliakis that first suggested that the slow phase of DSB repair may in fact
326   represent a completely separate repair pathway (Wang et al. 2003). This hypothesis

327 dovetailed nicely with earlier work done in yeast, which had genetically documented that

328 in the absence of Ku, DSBs could be repaired by an alternative error-prone end-joining

329 process that utilized microhomology (Boulton and Jackson 1996). Although still somewhat

330 controversial (Pannunzio et al. 2014) significant evidence has accumulated over the past

331 15 years to substantiate the Iliakis hypothesis. The hallmarks of this pathway (generally

332 referred to as alternative nonhomologous end joining [A-NHEJ] to distinguish it from C-

333 NHEJ) are that it is Ku-independent and utilizes small (3 nt[+]) regions of (Frit et al. 2014;

334 Iliakis et al. 2015; Boulton and Jackson 1996) to facilitate end joining. The process of

335 A-NHEJ is mechanistically simple and straightforward: both ends of the DSB are resected

336 to generate 3' overhangs that are intermediate in length from those generated during C-

337 NHEJ (which are a few nucleotides at most) and HDR (which are often hundreds or

338 thousands of nt long). These resected ends can then base pair using now exposed

339 stretches of "microhomology" (probably 3[+] nt). Nucleases are recruited to trim the flaps

340 that are often generated and the nicks/ends are then sealed by a ligase (Frit et al.

341 2014; Iliakis et al. 2015). Thus, A-NHEJ is inherently an error-prone repair process as

342 it always generates deletions including one of the two regions of microhomology and all

343 the DNA in between the two patches of microhomology.

344   Although the intellectual concept of how A-NHEJ occurs is clear, the genetics and

345 biochemistry of the synapsis, processing and ligation of an A-NHEJ DSB repair event

346 are still quite obscure leading to heavy debate by investigators in the field as to the

347 precise mechanism. Several studies have suggested that, like Ku for C-NHEJ, the

348 protein poly(ADP-ribose) polymerase 1 [PARP1] may bind to the DNA ends (Figure 3).

349 Indeed, there is evidence that PARP1 may even compete with Ku for access to the

350 ends thereby determining the choice of the NHEJ pathways used for the repair of specific

351 DSBs (Cheng et al. 2011). Alternatively, the repair complex meiotic recombination

352 11/radiation sensitive 50/Nijmegen breakage syndrome 1 [MRE11/RAD50/NBS1; MRN]

353 has also been implicated as the A-NHEJ end tethering activity (Dinkelmann et al. 2009;

354 Xie et al. 2009). Regardless of whether recognition or tethering of the ends is carried

355 out by either PARP1 or MRN, resection is required to reveal the microhomology that will

356 subsequently be used to mediate the repair event. The initial resection is thought to be

357 carried out by MRN and an associated nuclease, C-terminal interacting protein [CtIP].

358 The short resection mediated by MRN/CtIP is then elongated by DNA exonuclease 2

359 [DNA2] and/or exonuclease 1 [EXO1] (Mimitou and Symington 2008; Bernstein and

13

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

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

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

401

402   **7.2.3 HDR**

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

425 of the intermediates are resolved as non-crossover products by dissolving the
426 interdigitated strands back into their original duplexes after sufficient DNA replication has
427 occurred to restore the genetic information lost at the site of the DSB (Figure 4A). The
428 dissolution process requires the action of the Bloom syndrome gene, topoisomerase 3α
429 and RecQ-mediated genome instability homolog 1 [BLM, TOPO 3α, RMI1, respectively]
430 complex (Wu and Hickson 2003). Less frequently the second end of DNA is captured
431 and a covalently closed "Holliday junction" (Holliday 1964) is formed that can be resolved
432 as either non-crossover products (which are functionally identical to dissolution) or
433 crossover products (Figure 4B). The resolution of Holliday junctions is complicated and
434 in human cells appears to be carried out by at least three partially, redundant resolvases
435 consisting of mutagen sensitive 81/essential meiotic endonuclease 1 [MUS81/EME1,
436 respectively], synthetically lethal with genes of unknown function (X) 1 and 4 [SLX1 and
437 SLX4, respectively], and general homolog of endonuclease 1 GEN1] (Matos and West
438 2014). Finally, LIGI is utilized to covalently seal any nicks left in the DNA.
439   Although HDR is often referred to as error-free repair, that characterization is only
440 partially true. Thus, in the case of non-crossover events in which the repair is templated
441 from a sister chromatid the DSB is in fact repaired in an error-free fashion. However,
442 when a homolog, rather than a sister chromatid, is utilized there is a risk of the loss of
443 heterozygosity and uniparental disomy as observed in several developmental disorders
444 and numerous tumor types (Tuna et al. 2009). In summary, human somatic cells express
445 all of the gene products needed to carry out HDR. These events occur, however, only
446 at very low frequency and usually only in S phase due to the preferred usage of NHEJ.
447   In summary, human cells can repair DNA DSBs by at least three discrete pathways:
448 C-NHEJ, A-NHEJ and HDR. How pathway choice (which pathway is utilized in which
449 cells during which phases in the cell cycle, *etc.*) is biochemically determined is the focus
450 of much research. Regardless, from a logistical perspective, one thing that clearly
451 differentiates these repair pathways is their reliance on different DNA ligases to complete
452 the reaction. Thus, C-NHEJ utilizes exclusively LIGIV, whereas A-NHEJ prefers to use
453 LIGIII (although it can utilize LIGI) and HDR uses exclusively LIGI. In conclusion, until
454 better biochemical or genetic markers become available, ligation is one the most
455 distinguishing features of these repair pathways.
456
457   **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

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

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

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

524   2014), but not in others (Liddiard et al. 2016). This latter observation notwithstanding,
525   the reduction in translocations when C-NHEJ is absent, the infrequent use of
526   microhomology at translocation breakpoints and the lack of an impact on the frequency
527   of translocations when LIGIII is absent, compellingly indicate that most translocations in
528   human cells are LIGIV-dependent and likely mediated by C-NHEJ.

529   Needless to say, these observations raise the question of why a seemingly similar
530   process should be mechanistically so different in these two organisms. To date, there
531   is no clear answer. The most likely explanation has to do with species-specific
532   differences in the factors that make up the DSB repair pathways. For example, DNA-
533   PK_{cs} is the key C-NHEJ factor that tethers the two ends of a DSB together through
534   homodimerization (Sibanda et al. 2017; Spagnolo et al. 2006). Relevantly, DNA-PK_{cs} is
535   more abundant (by ~ an order of magnitude) in human cells than it is in rodent cells
536   (Finnie et al. 1995). Thus, the reduced quantities of DNA-PK_{cs} (and presumably therefore
537   reduced numbers of tethered ends) may provide A-NHEJ in the mouse with additional
538   windows of opportunity for the ends to dissociate and be conscripted by A-NHEJ factors,
539   whereas in human cells, with a superabundance of DNA-PK_{cs} [there are estimated to be
540   between one-half to one million molecules of DNA-PK_{cs} in every human cell; (Meek et
541   al. 2008)] C-NHEJ is the dominant repair pathway. The obvious follow-up question of
542   why human cells should contain so much more DNA-PK_{cs} than rodents is unfortunately
543   not biochemically obvious, but the empirical fact that they do likely provides at least a
544   partial answer for why the two organisms utilize the C-NHEJ and A-NHEJ pathways
545   differentially. In addition, it is well known that chromatin organization and epigenetic
546   modifications can affect the mutation rate across genomes (Schuster-Bockler and Lehner
547   2012; Tubbs and Nussenzweig 2017). Specific chromatin features and epigenetic marks
548   are unlikely to be highly conserved across species and these differences may also
549   impact upon the process of translocations. Finally, it is now appreciated that at least
550   some of the endogenous DSBs generated in vertebrate cells may be due to the aberrant
551   action of apolipoprotein B editing complex 3 [APOBEC3], a cytidine deaminase capable
552   of introducing closely spaced nicks into the DNA (Swanton et al. 2015; Tubbs and
553   Nussenzweig 2017). Importantly, there is a single APOBEC3 gene in the mouse,
554   whereas in humans that locus has been significantly expanded to eight functional
555   isoforms. Thus, differences in APOBEC3 expression could certainly causes significant
556   differences in either the frequency and/or location of DSBs in the genome. Whatever

557 the correct answer(s) may be, it is important to appreciate that particular care must be
558 taken in interpreting or extrapolating experimental results obtained in rodent model
559 systems to humans since some of the basic biology appears to be different [discussed
560 at length as well by (Lieber 2016)].
561
562   **8.1.2. Spatial Karma and Translocations**
563 Regardless of which pathway of end joining (C-NHEJ or A-NHEJ) is used for repair,
564 why are these processes not always faithful?   The correlation of translocations with
565 aberrant A-NHEJ is easiest to reconcile.   Thus, while PARP1 can bind tightly to DNA
566 ends, it is not known to homodimerize.   Moreover, while some studies have suggested
567 that MRN, or subunits thereof, are capable of homodimerization (Williams et al. 2008)
568 there is frankly no A-NHEJ factor comparable to DNA-PK$_{cs}$.   As a consequence of this,
569 it seems likely that the ends of a DSB that are being repaired by A-NHEJ may not be
570 as synaptically as stable as ends being repaired by C-NHEJ and therefore simply stand
571 a statistically higher chance of separating from one another before the repair event is
572 completed.   This model dovetails nicely with the reduced amounts of DNA-PK$_{cs}$ observed
573 in rodents and their correspondingly greater propensity to utilize A-NHEJ in the formation
574 of translocations.   The flip side of this rationalization is more complex.   Thus, in humans,
575 where C-NHEJ apparently predominates, why does the end of DSB ever become capable
576 of joining to an end other than its cognate end?   Indeed, it is well known (albeit mostly
577 from mouse studies) that C-NHEJ is more likely to join DSBs intrachromosomally rather
578 than interchromosomally (Mahowald et al. 2009; Chiarle et al. 2011).   In essence then,
579 when C-NHEJ is utilized it is simply less likely that a translocation will result.   The most
580 compelling explanation for the translocations that do result is that the DSBs may be
581 spatially adjacent to one another.   For example, even early experiments on the spatial
582 organization of the human genome noted that translocations often involved regions that
583 were physically closer to one another than to other regions of the genome (Meaburn et
584 al. 2007).   These observations have been confirmed and extended over the past decade
585 as technology has improved the characterization of the large-scale organization of
586 chromosomes (Roukos and Misteli 2014; Roukos et al. 2013; Bohlander and Kakadia
587 2015).   Thus, nuclear DSBs have a tendency not to move very much (Kruhlak et al.
588 2006; Jakob et al. 2009) and this correlates well with the observation that more than
589 80% of DSBs translocate to regions that are physically located to within 2.5 μm of each

590 other (Roukos and Misteli 2014; Roukos et al. 2013). In conclusion, the current best
591 explanation for why translocations occur in human cells is "bad karma". That is, a
592 translocation likely only occurs when two concomitant DSBs are also spatially close to
593 one another in the nucleus such that a synaptic complex (likely a requirement for repair)
594 can form – albeit in these rare instances between non-cognate ends.
595
596   **8.1.3. Selection, not the Translocation, Drives Cancer**
597 It is well known that particular translocations are the hallmark of certain cancers (Lieber
598 2016; Bunting and Nussenzweig 2013). However, it is important to appreciate that the
599 predominance of a translocation in a tumor is due solely to the subsequent selection
600 that is imposed upon all the translocations that may have occurred during the genesis
601 of that tumor.   That is, if, and only if – and this is a stochastic probability – the
602 translocation generates a novel chromosome that gives the cell a selective growth
603 advantage, will these cells be subsequently amplified to generate the tumor.   Indeed,
604 translocations that are oncogenic have invariably inactivated a tumor suppressor gene,
605 activated an oncogene and/or created a chimeric gene that is acting as an oncogene.
606 This event, however, is independent from the mechanism of the translocation; that is,
607 there is nothing inherently oncogenic about translocations. Both ends of a DSB have a
608 similar propensity to translocate (Chiarle et al. 2011) and although there is a bias towards
609 translocations happening near transcriptional start sites in the mouse (Chiarle et al.
610 2011), this bias is not observed in human cells (Lieber 2016) (yet another difference
611 between mice and humans). Consequently, it is important to appreciate is that there is
612 no evidence of directionality or specificity intrinsic to translocations themselves. Thus,
613 both ends of a broken chromosome likely have the potential to translocate to an infinite
614 number of chromosomal locations and this is likely limited only by the spatial parameters
615 discussed above.
616
617   **8.2 Considerations for when one DSB is a Telomeric End**
618 Up until now, all of the translocations that have been discussed were canonical ones
619 requiring the formation of two DSBs and the generation of four DNA ends.   There is
620 one biologically important scenario, however, where translocations can occur between a
621 DSB and a "single-ended DSB" and hence only involve three DNA ends. This scenario

622 occurs when the end of a chromosome, *i.e.*, a telomere, participates in the translocation
623 reaction.
624
625 ### 8.2.1 Telomeres Stabilize the Genome
626 There are 46 chromosomes in a normal diploid human cell and because each
627 chromosome has 2 ends, there are in principle 92 natural DSBs constitutively present in
628 a cell. Such a scenario, if it truly existed, would be lethal, so evolution has devised an
629 answer in the form of telomeres. Telomeres are specialized nucleoprotein structures
630 that are found at the extreme termini of linear eukaryotic chromosomes. Telomeres
631 "cap" those ends and prevent the recognition of the chromosomal termini as DSBs by
632 the cellular DNA damage response apparatus. Telomeres consist of a repetitive
633 hexameric tract of DNA (TTAGGG) bound by an evolutionarily-conserved complex of
634 proteins collectively called Shelterin (de Lange 2005). Importantly, ongoing cell division
635 (*i.e.*, aging) results in gradual telomere erosion (Harley et al. 1990), and ultimately, the
636 loss of the end-capping function which, in the context of a functional DNA damage
637 response, leads to the induction of a p53-dependent G_1/S cell cycle arrest, known as
638 replicative senescence (d'Adda di Fagagna et al. 2003). This cell-intrinsic limit on
639 replicative lifespan provides a stringent tumor suppressive mechanism. However, in the
640 absence of a fully functional DNA damage checkpoint response, older cells containing
641 short dysfunctional telomeres (which are essentially one-ended DSBs) enter a state of
642 crisis during which telomeres undergo fusion, either between sister chromatids (Figure
643 5A), with interchromosomal telomeres (Figure 5B) or with non-telomeric DSBs, creating
644 dicentric chromosomes and initiating BFB cycles (Counter et al. 1992; Murnane 2012).
645 This, in turn, leads to the creation of genomic rearrangements, including the translocations
646 that are common in cells from many different tumor types (Artandi et al. 2000; Shih et
647 al. 2001). The development of single-molecule approaches to characterize the sequence
648 of telomere fusion events, has revealed that short dysfunctional telomeres are capable
649 of recombining with both telomeric and non-telomeric loci across the genome (Liddiard
650 et al. 2016; Letsolo et al. 2010). Thus, whilst BFB cycles initiated because of telomere
651 dysfunction can lead to chromosomal translocations (Murnane 2012), telomere fusions
652 themselves can also lead directly to translocation events. Intra-chromosomal telomere
653 fusion involving sister-chromatids predominates over inter-chromosomal telomere fusion,
654 which in turn is more frequent than inter-chromosomal fusion between telomeres and

22

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

662   Thus, telomere-dependent crisis is a key event in driving genomic instability and
663   clonal evolution during the progression to malignancy; this is consistent with data and
664   observations of telomere dynamics and fusion in a broad range of human tumor types
665   in which extreme telomere erosion and fusion is observed that correlates with the
666   presence of large-scale genomic rearrangements (Lin et al. 2010; Roger et al. 2013;
667   Meeker et al. 2004). Moreover, patients with tumors that display short dysfunctional
668   telomeres, within the length ranges at which fusion can occur, exhibit a poorer prognosis
669   and response to treatments (Lin et al. 2014; Strefford et al. 2015; Williams et al. 2017).
670   Short dysfunctional telomeres have been identified in the very earliest lesions, including
671   very small adenomatous colorectal polyps (Roger et al. 2013) and in leukemias prior to
672   clinical progression (Lin et al. 2010). Importantly, the short telomeres observed in early
673   stage lesions are identical in length to those observed in more advanced disease clones,
674   indicating that telomere length does not vary considerably during progression. Together
675   these data are consistent with the presence of short telomeres in the cell in which the
676   initiating mutation occurred and that this dictates the telomere length distribution of the
677   developing clone. In this model, if the initiating cell contains short telomeres then the
678   subsequent clone may have a "telomere-mutator" phenotype that drives genomic
679   instability, translocations and clonal progression, whereas a cell with long telomeres gives
680   rise to a clone with a more stable genome, which exhibits slower rates of clonal
681   progression (92). Finally, it is important to note that short dysfunctional telomeres have
682   been observed in the majority of tumor types analyzed (Jones et al. 2012) and thus it
683   appears that a period of telomere-driven genome instability may be a common mechanism
684   underlying the progression to malignancy. Therefore, there is a requirement to
685   understand the mechanisms by which telomere dysfunction can facilitate genome
686   instability.
687

8.2.2 Translocations Involving Telomeres Are Mechanistically Distinct

688

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

702 The view that telomere-mediated translocations may be mechanistically distinct from
703 canonical two DSB-mediated translocations is consistent with the molecular analysis of
704 telomere fusion events directly from human cells undergoing a telomere-driven crisis in
705 culture. These data show that fusion between short telomeres – ones that are almost
706 completely denuded of telomere repeats – is accompanied by deletion and microhomology
707 across the fusion points (Capper et al. 2007). The deletion that accompanies telomere
708 fusion, includes not just the telomere repeat array itself, but extends into the telomere-
709 adjacent DNA, up to the limit of the assays used (6.1 kb), the distribution of fusion
710 points from the start of the telomere repeat arrays, indicates that deletion may be much
711 more extensive. This characteristic profile is also observed at telomere fusion junctions
712 isolated from some human malignancies, including early-stage and pre-malignant lesions
713 (Lin et al. 2010; Roger et al. 2013), as well normal human cells, in which rare stochastic
714 telomeric deletion results in fusion (Capper et al. 2007; Lin et al. 2010; Roger et al.
715 2013). Finally, molecular analysis of fusion events following replicative telomere erosion
716 in human cells carrying hypomorphic MRE11 alleles revealed a change in the mutational
717 spectrum with an increase in insertions at the fusion point (Tankimanova et al. 2012).
718 The reliance on MRE11, the extensive deletion and the high degree of microhomology
719 that accompanied these human telomere fusions was indicative of error-prone processing
720 of short dysfunctional telomeres via the A-NHEJ pathway and suggested that telomere-

24

721 mediated fusions in human cells may be mechanistically fundamentally different than
722 canonical two DSB-mediated translocations, which, as detailed above, appear to be
723 predominately mediated by C-NHEJ.
724

725 **8.2.3 Translocations Involving Human Telomeres can be Mediated by LIGIII or**
726 **LIGIV**

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

25

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

755　　　These experiments beg the question as to why a telomere-mediated translocation
756　(as compared to a interchromosomal DSB-mediated translocation) should be less reliant
757　on C-NHEJ (and/or more reliant on A-NHEJ).　The most obvious difference is simply
758　that while a shortened telomere can bind a single DNA-PK complex, there is no
759　corresponding end to bind a second DNA-PK complex and hence there is a greatly
760　reduced chance of forming a synaptic complex.　The lack of a synaptic complex
761　presumably now permits the recruitment of A-NHEJ factors to the end and/or the
762　displacement of the DNAPK complex from the end such that a higher frequency of A-
763　NHEJ-mediated fusions can occur.　Another factor that might influence the relative
764　activities of A- and C-NHEJ at telomeres may be the nature of a short telomere,
765　compared to a non-telomeric DSB.　The telomeres terminate not with a blunted-ended
766　DSB, but instead have a large (200 to 300 nt) overhang composed of TTAGGG repeats
767　(Wright et al. 1997).　This unique structure has the potential to fold into G-quadruplex
768　structures (Xu et al. 2009) and may represent a non-canonical substrate for DNA repair
769　activity that may favor the slower kinetics of the A-NHEJ pathway over that of C-NHEJ.
770

771　　　**8.2.4　Translocations Involving Human Telomeres can be Mediated by LIGI**
772　The above data strongly suggested that the geometry of the DNA ends and the availability
773　of a requisite DNA ligase controls the type of translocations that can occur in human
774　cells.　To extend these observations a follow-up study was carried out in which the
775　frequency and kind of translocation was quantitated in cells that were genetically
776　engineered to be deficient for both LIGIII and LIGIV, where, presumably, both C-NHEJ
777　and A-NHEJ would be ablated.　In this experimental set-up the telomere was not
778　gradually exposed by the expression of DN-hTERT as before, but was rapidly deleted
779　by the use of a transcription activator-like effector nuclease [TALEN; (Ousterout and
780　Gersbach 2016)].　A TALEN pair was designed to introduce a DSB 14 base pairs from
781　the start of the telomeric TTAGGG repeat on the petite arm of chromosome 17.　Thus,
782　this experimental system is somewhat of a hybrid between those measuring canonical
783　fusions and the system to gradually uncover a telomere end by enforced DN-hTERT
784　expression.　Specifically, the TALEN should generate a DSB with two ends, however,
785　one of those ends is only a couple of kilobases long and consists solely of the telomeric

786 TTAGGG hexameric repeat. It is unclear whether this end can function in a fashion
787 similar to a canonical chromosomal end. With this caveat in mind, it was reassuringly
788 observed that in the absence of LIGIV a greatly decreased frequency of interchromosomal
789 translocations was observed (Liddiard et al. 2016). Very surprisingly, however, in the
790 combined absence of LIGIII and LIGIV significant amounts of both inter-and
791 intrachromosomal translocations were observed inter-chromosomal translocations were
792 also detected albeit at a reduced frequency (Liddiard et al. 2016). Interestingly, whilst
793 the frequency of inter-chromosomal fusion events was decreased in the absence of
794 LIGIV, intra-chromosomal sister chromatid fusion events appeared to be largely
795 unchanged in the different genetic backgrounds tested. Moreover, there were differences
796 in the utilization of microhomology, with significantly greater microhomology observed at
797 intra-chromosomal events compared to inter-chromosomal events. Taken to together
798 these data are consistent with a role for LIGIV-dependent C-NHEJ in driving
799 interchromosomal telomere fusion and A-NHEJ being predominant for intrachromosomal
800 sister chromatid telomere fusion. These data were also important because they provided
801 the first demonstration in human cells that LIGI can facilitate chromosomal translocations
802 – both inter-chromosomal and intra-chromosomal sister chromatid translocations.
803 Moreover, these data revealed considerable redundancy in the utilization of the specific
804 ligases for end-joining, with LIGI being able to facilitate intra-chromosomal fusion as well
805 as inter-chromosomal fusion, albeit less efficiently. This may be discouraging from the
806 clinical perspective, as these data indicate that attempts to inhibit human translocations
807 using small molecule inhibitors to LIGIII and LIGIV (Singh et al. 2014) are destined to
808 fail due to the robust ability of LIGI to compensate for their absence. That said, any
809 intervention that can skew the fusion spectrum towards inter-chromosomal events,
810 creating a larger mutational burden on cells and influencing their ability to escape a
811 telomere-driven crisis, may have clinical utility. A deeper understanding of the key
812 proteins involved in A-NHEJ and telomere fusion may identify additional therapeutic
813 targets that could allow for more selective interventions into these pathways.
814
815   **9. Summary and Future Considerations**
816 In summary, DSBs are normally repaired with high fidelity in the sense that the pieces
817 of DNA that were contiguous before the DSB are contiguous after DNA repair, regardless
818 of the "sloppiness" of the actual join. In order for a canonical chromosomal translocation

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

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

851   repair is regulated there is still significant cause for optimism that windows of opportunity

852   for therapeutic intervention will be uncovered.

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1226

Figure Legends

2

3   Figure 1. Repair of lesions where one DNA strand is still intact. (A) Base Excision

4   Repair. The schematic shows a small piece of double-stranded DNA (colored rectangles

5   represent nucleotides) containing a singly modified nt (red diamond). This lesion is

6   repaired by the action of DNA gylcosylases, endonucleases and phosphodiesterases that

7   ultimately convert the lesion into a single-stranded nick. This nick is then filled in by a

8   DNA polymerase and sealed by a DNA ligase. (B) Nucleotide Excision Repair. The

9   schematic shows two nts (colored lines) that are fused together (red diamond). This

10   lesion is recognized by a multi-subunit protein complex that introduces nicks 5' and 3'

11   of the lesion. The offending lesion is then removed as an oligonucleotide by the action

12   of a helicase and the resulting ~20 nt gap is filled in by a polymerase and then sealed

13   by a DNA ligase. (C) Mismatch Repair. The schematic shows two nucleotides (colored

14   lines) where one base pair is mispaired (red diamonds). The mispaired nts are

15   recognized by mismatch heterodimeric complexes that recruit endonucleases that nick

16   the DNA. Exonucleases then degrade one of the strands resulting in the removal of

17   the offending nt as well as some flanking nts. As before, the resulting gap is

18   subsequently filled in by a DNA polymerase and sealed by a DNA ligase.

19

20   Figure 2. A schematic depicting Classic Non-Homologous End Joining (C-NHEJ). The

21   black lines represent strands of DNA. First, the Ku heterodimer (orange ball) binds onto

22   the ends of the DNA. Ku then recruits DNA-PK$_{cs}$ (blue oval) and the homotypic

23   interactions between two DNA-PK$_{cs}$ molecules tethers the ends together. The nuclease

24   Artemis (yellow PacMan^{TM}), which is physically associated with DNA-PK$_{cs}$, can then

25   remove any mispaired or damaged nucleotides from the ends. Most missing nts are

26   then replaced by the DNA polymerases μ or λ (green pentagon). Finally, a ligase

27   complex, consisting of DNA ligase IV (purple cylinder) and the accessory proteins PAXX

28   (red half oval), XRCC4 (light green tear drop) and XLF (Carolina blue cropped pyramid)

29   then religated the ends back together. This process often results in indels (denoted by

30   the red rectangle) at the site of repair.

31

32   Figure 3. A schematic depicting Alternative Non-Homologous End Joining (A-NHEJ).

33   The black lines represent strands of DNA and the red rectangles blocks of
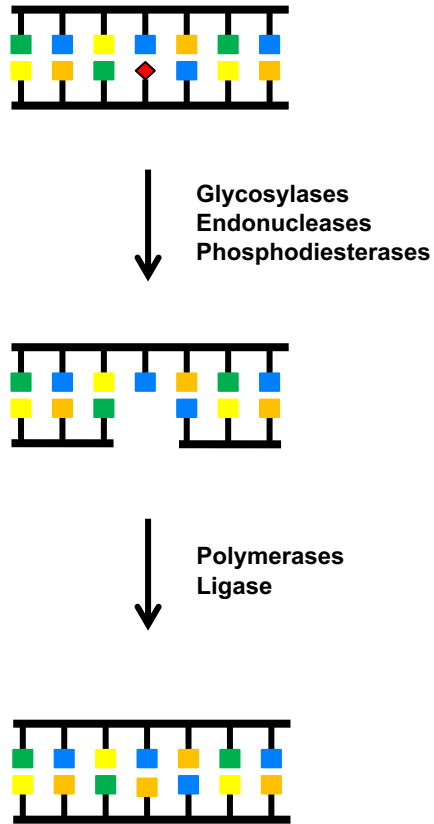
34   microhomology. The broken ends may be held together either by PARP1 (Carolina blue

35   hexagon) or by the MRN complex (orange oval). The initial resection is carried out by

36   MRN and CtIP (crimson PacMan^{TM}). This short resection is then elongated by DNA2

37   and/or EXO1 (light green PacMan^{TM}). Once sufficient 3'-single-stranded DNA [ssDNA]

38   overhangs have been generated the strands can anneal through the exposed

39   microhomology. The presence of microhomology stabilizes the junction. The resulting

40   flaps are likely cleaved off by the ERCC1/XPF heterodimer (yellow PacMan^{TM}). Before

41   ligation occurs it is also likely that POLQ (purple lightning bolt) may act on the DNA

42   ends. Ultimately, the repaired DSB junction is religated using either LIGIII (red cylinder)

43   or LIGI (pink cylinder).

44

45   Figure 4. A schematic depicting Homology-Dependent Repair (HDR). The black lines

46   represent strands of DNA where a DSB has occurred and the blue lines represent an

47   undamaged sister chromatid or a homologue. In HDR, the DNA ends of the DSB are

48   extensively resected to yield 3'-single-stranded DNA overhangs. As described for A-

49   NHEJ, the nuclease(s) responsible for this resection are the MRN:CtIP complex (which

50   generates the initial resection; orange oval and crimson PacMan^{TM}, respectively) followed

51   by the action of DNA2 and EXOI (light green PacMan^{TM}). The resulting overhangs are

52   then coated by RPA (red circles), which removes the secondary structures from the

53   overhangs. A complex of proteins including BRCA1, BRCA2, and FANCN (purple cloud)

54   then help to recruit RAD51 (pink diamond) to the overhangs. Strand invasion into the

55   homologous chromosomal sequence requires RAD54 (yellow moon) and DNA replication

56   (blue-green cloud). Strand exchange generates an interdigitated set of strands that can

57   be resolved into a complicated set of products. (A) In mitotic cells most of the

58   intermediates are resolved as non-crossover products by dissolving the interdigitated

59   strands back into their original duplexes after sufficient DNA replication has occurred to

60   restore the genetic information lost at the site of the DSB. The dissolution process

61   requires the action of the BLM/TOPO 3α/RMI1 complex (green hexagon). (B) Less

62   frequently the second end of DNA is captured and a covalently closed "Holliday junction"

63   is formed that can be resolved as either non-crossover products (which are functionally

64   identical to dissolution) or crossover products. The resolution of Holliday junctions is

65   complicated and in human cells appears to be carried out by at least three partially,

66   redundant resolvases consisting of mutagen sensitive 81/essential meiotic endonuclease
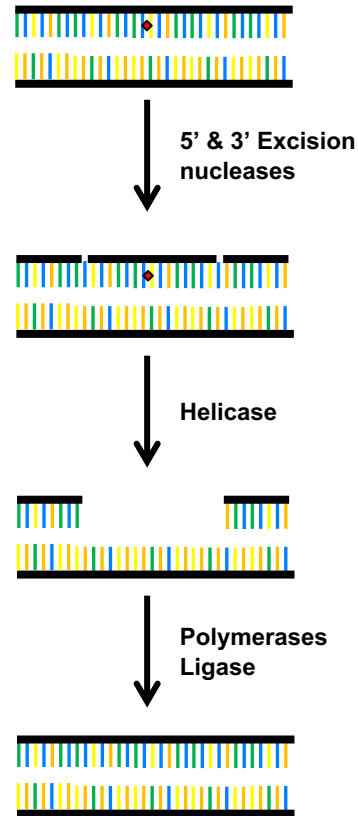
2

67   1 [MUS81/EME1, respectively], synthetically lethal with genes of unknown function (X) 1
68   and 4 [SLX1 and SLX4, respectively], and general homolog of endonuclease 1 GEN1]
69   (1) (light blue cloud). Finally, LIGI (pink cylinder) is utilized to covalently seal any nicks
70   left in the DNA. Note that only the non-crossover product for Holliday Junction resolution
71   is diagrammed.
72
73   Figure 5. Short dysfunctional telomeres can be subjected to sister-chromatid fusion, or
74   inter-chromosomal fusion events to create amplifications, deletion and non-reciprocal
75   translocations. (A) Gradual telomere erosion in the absence of functional DNA damage
76   checkpoints, leads to short telomeres that are no longer protected by the Shelterin
77   complex. Fusion between sister-chromatids results in the formation of a dicentric
78   chromosome, that can form a bridge between daughter cells at anaphase, that be
79   subjected a breakage event. Depending on the position of the break, this can lead to
80   a daughter cell that has lost terminal sequences, or has an addition copy of a gene –
81   in this example, gene B. Further BFB cycles can lead to further amplification and
82   deletion. This process can be stopped by the healing of a DSB via the acquisition of
83   a *de novo* telomere, either by telomerase-mediated extension or by recombination with
84   a pre-existing telomere. Centromeres are depicted as green ovals, telomeres by black
85   and white rectangles and genes in colored squares. (B) inter-chromosomal telomere
86   fusion between short dysfunction telomeres (depicted), or with non-telomeric DSBs, can
87   lead to the formation of dicentric chromosomes and the initiation of BFB cycles that can
88   lead to the formation of non-reciprocal translations (NRT) and deletions. This process
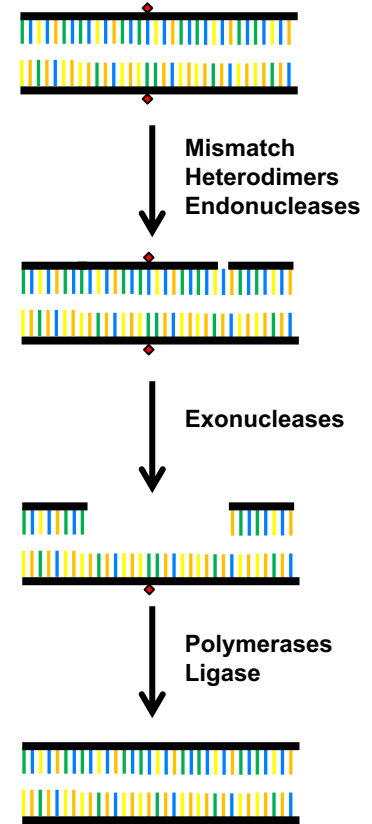89   can be prevented by chromosomal healing via the acquisition of new telomere.
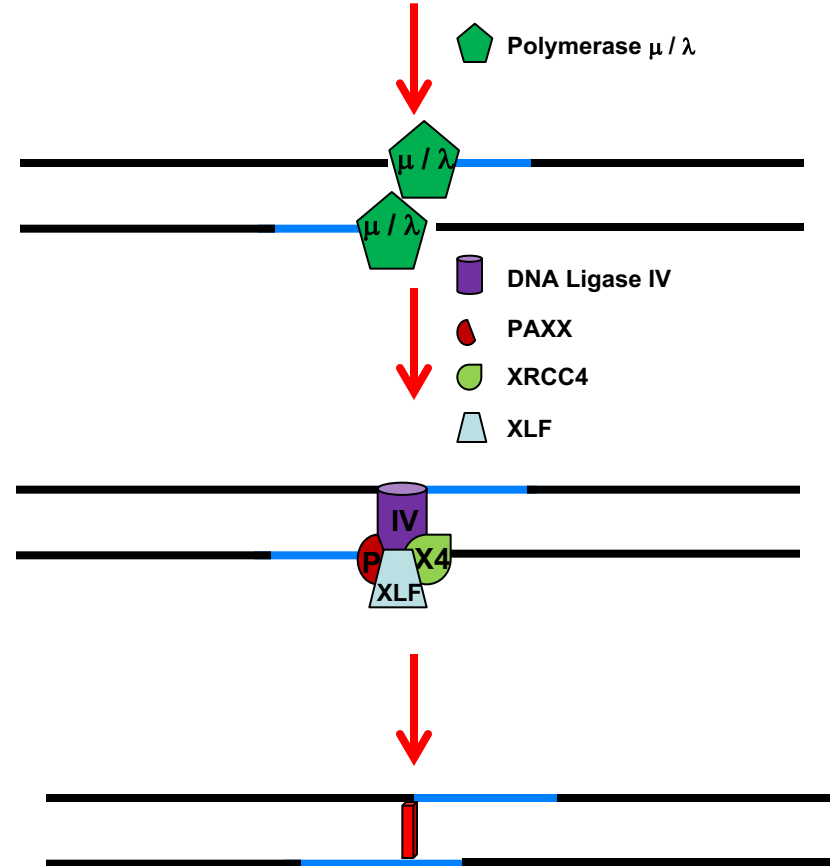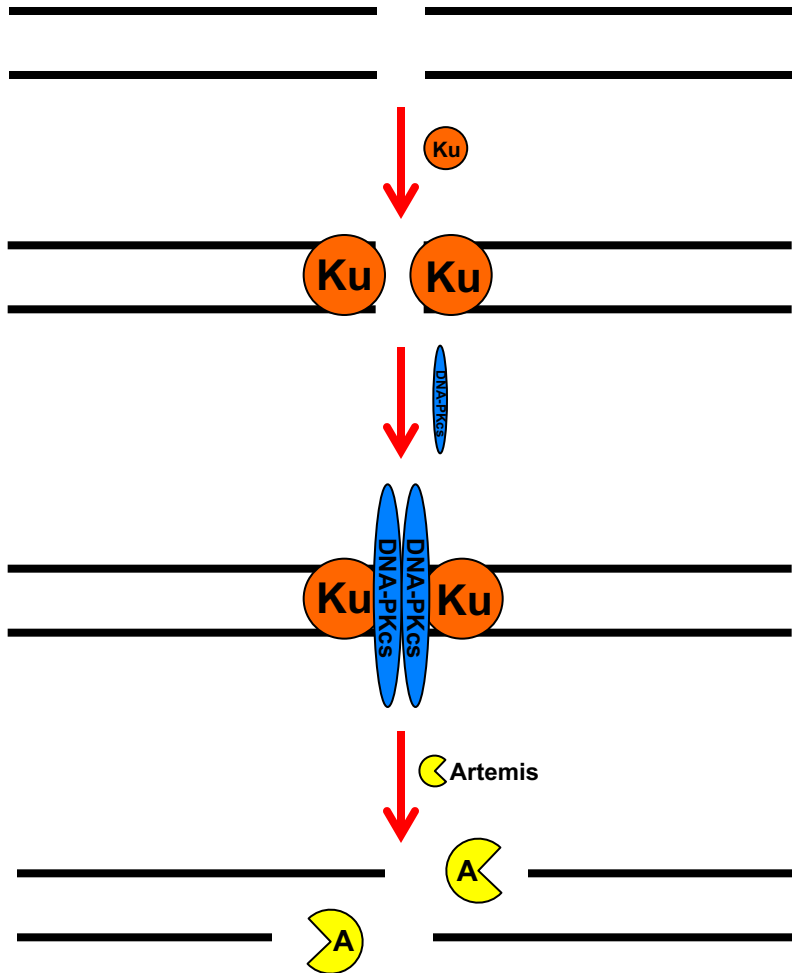90

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
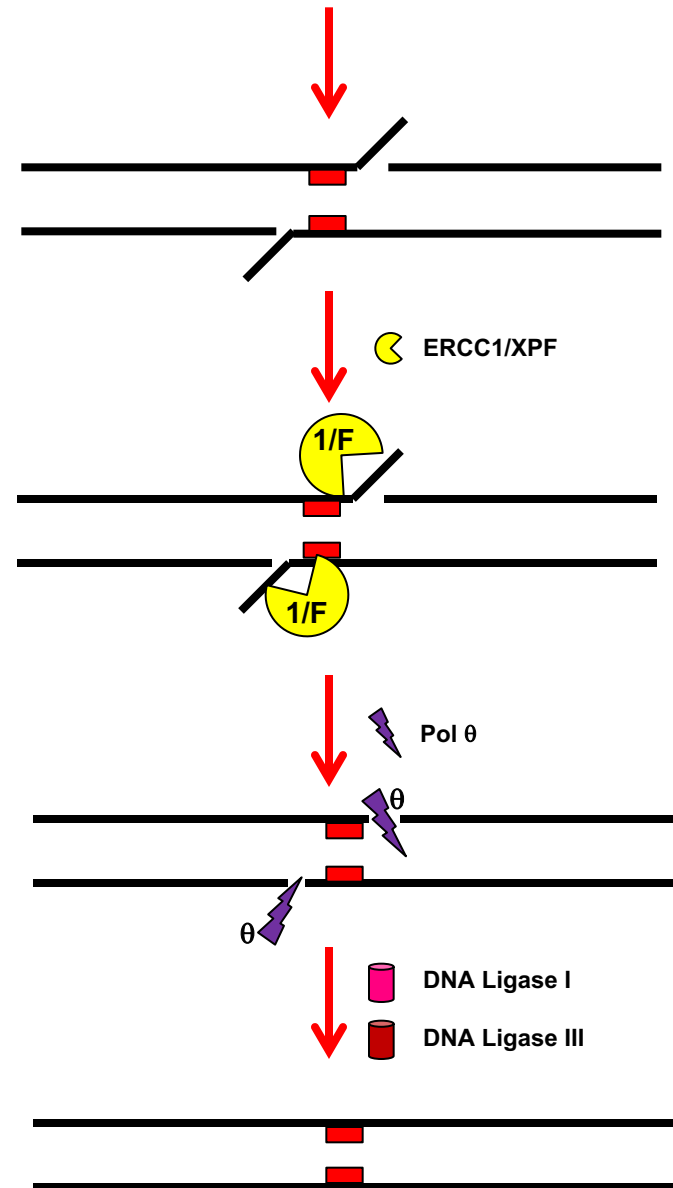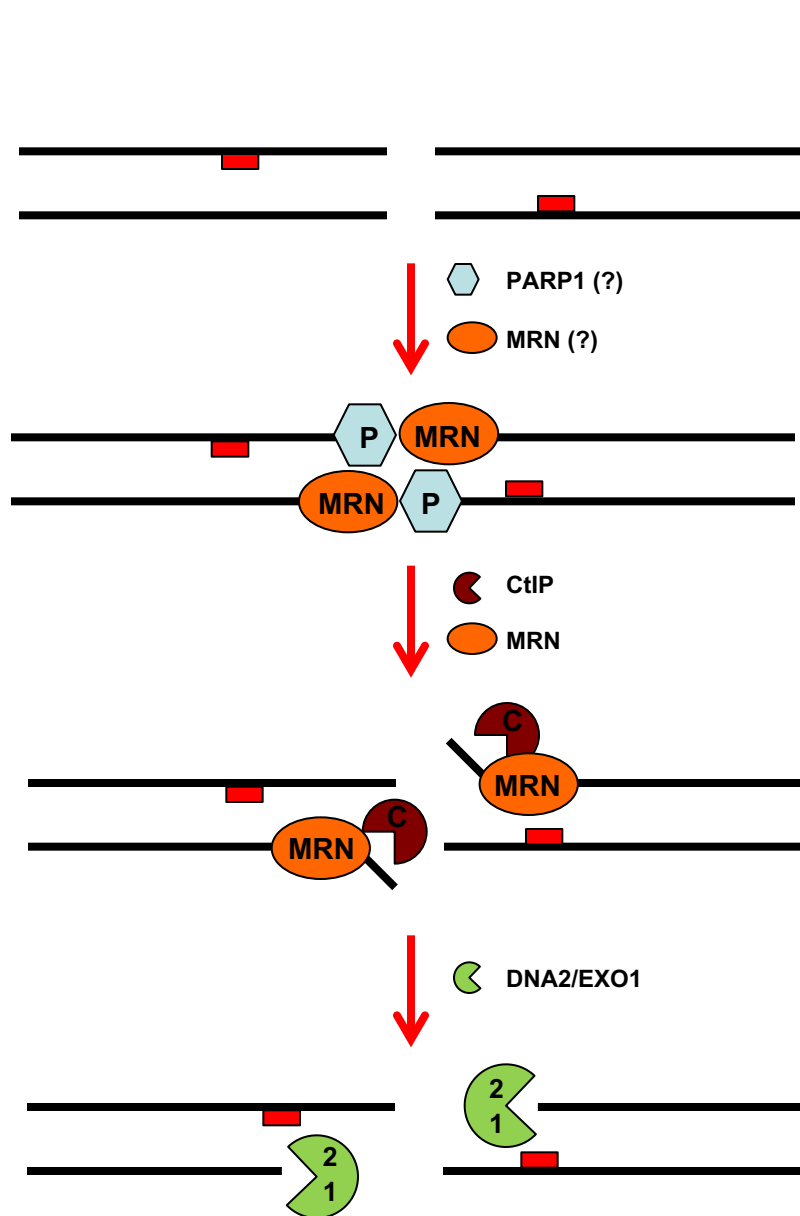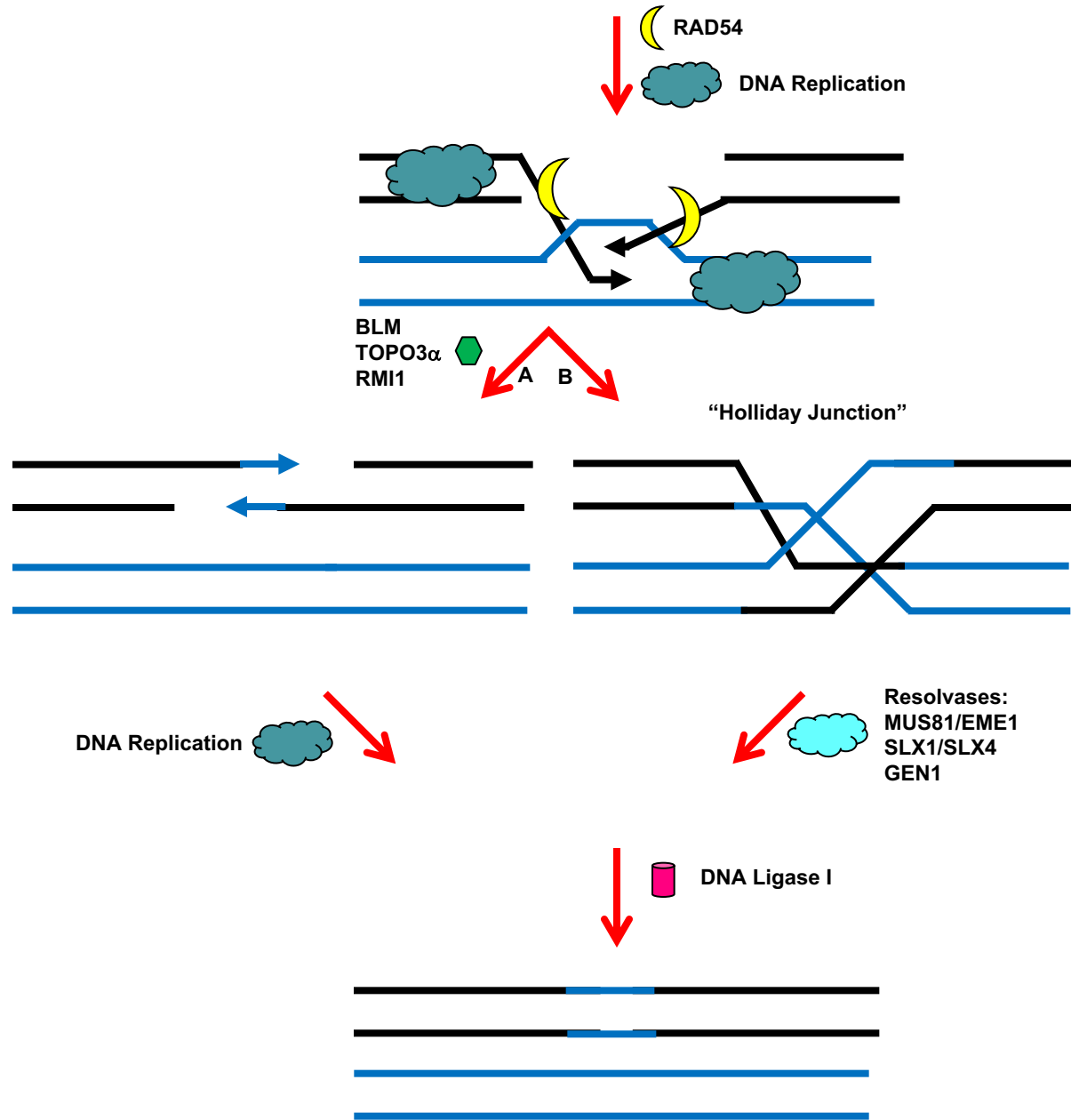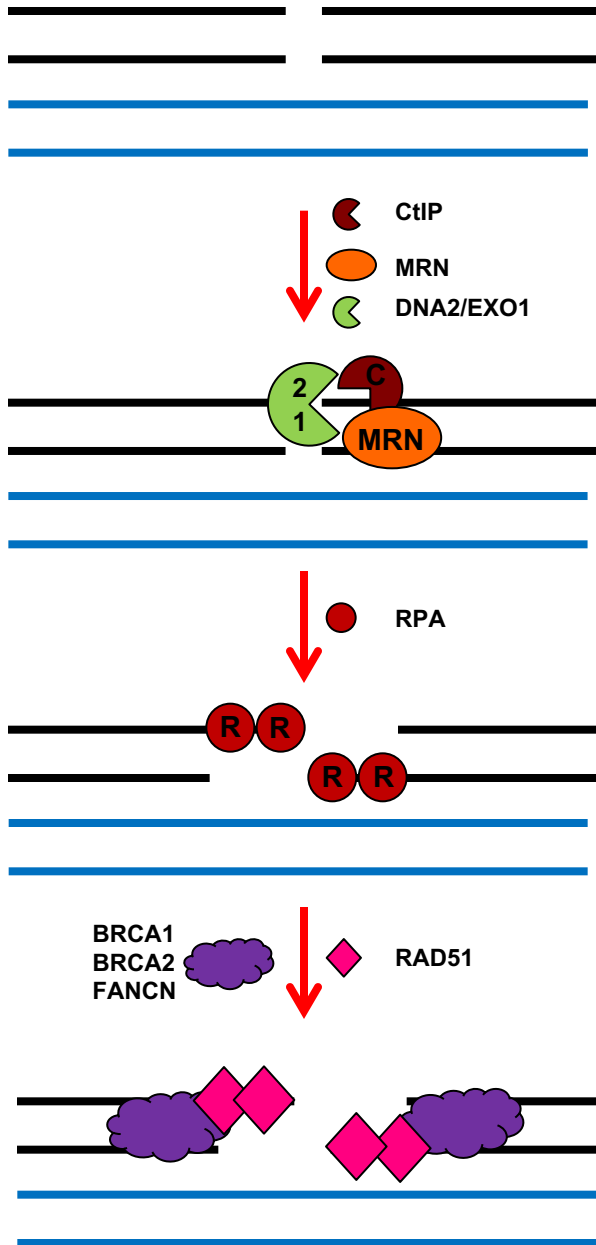
Classic Non-Homologous End Joining (C-NHEJ)

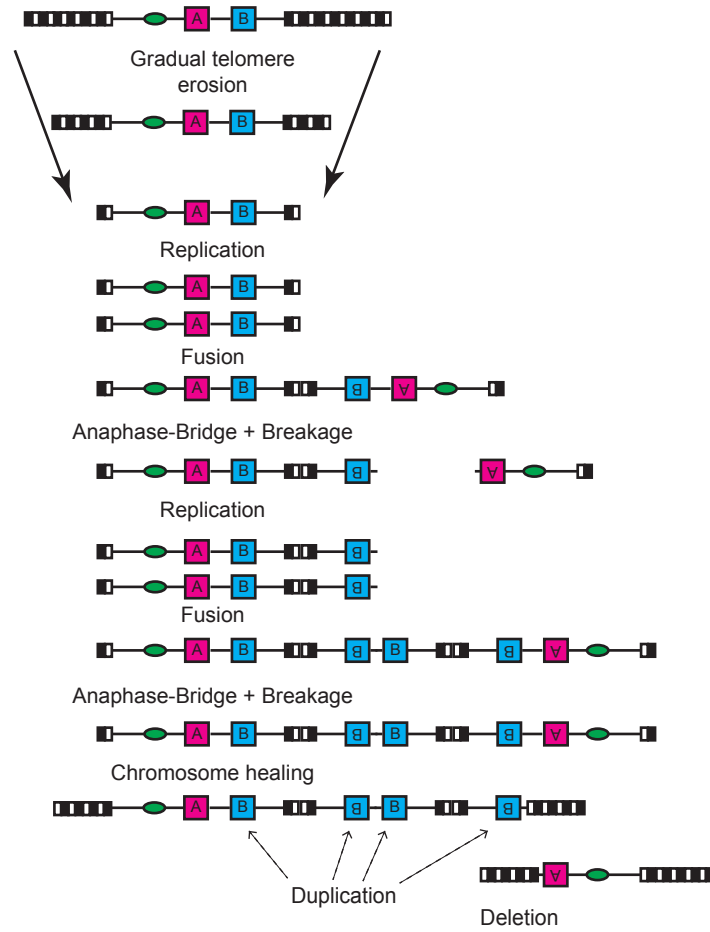# Alternative Non-Homologous End Joining (A-NHEJ)

Homology-Dependent Repair (HDR)

Translocation Mechanisms

A   **Sister chromatid telomere fusion**

Gradual telomere erosion

Replication

Fusion

Anaphase-Bridge + Breakage

Replication

Fusion

Anaphase-Bridge + Breakage

Chromosome healing

Duplication

Deletion

B   **Inter-chromosomal telomere fusion**

Gradual telomere erosion

Gradual telomere erosion

Fusion

Replication

Anaphase-Bridge + Breakage

Chromosome healing

NRT

Deletion

Deletion

NRT