Molecular & Cellular Oncology 1:3, e963438; October 1, 2014; © 2014 Taylor & Francis Group, LLC

How do telomeres and NHEJ coexist?

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Keywords: dicentric, KU, Lig4, Mre11, NHEJ, Rap1, telomere, TRF2

Abbreviations: NHEJ, nonhomologous end joining

The telomeres of eukaryotes are stable open double-strand ends that coexist with nonhomologous end joining (NHEJ), the repair pathway that directly ligates DNA ends generated by double-strand breaks. Since a single end-joining event between 2 telomeres generates a circular chromosome or an unstable dicentric chromosome, NHEJ must be prevented from acting on telomeres. Multiple mechanisms mediated by telomere factors act in synergy to achieve this inhibition.

Telomeres Do Not Fuse

Telomeres are the DNA–protein complexes at the ends of linear chromosomes that solve the problem of replicating chromosome ends by semiconservative DNA replication. They also protect the native chromosome ends from the DNA repair pathways that act on ends generated by double-strand breaks. Evolution has solved this issue differently in prokaryotes and in eukaryotes. In several bacteria and viruses, linear chromosomes have covalently closed hairpin telomere ends. Replication produces inverted repeats that are processed into 2 covalently closed hairpins by a resolvase.[1] In eukaryotes, telomeres are stable open double-strand ends and are therefore at risk of being erroneous substrates for DNA double-strand break repair.

Two pathways efficiently repair double-strand breaks: nonhomologous end joining (NHEJ) and homologous recombination.[2-4] NHEJ is essentially direct relegation between the 2 ends. Homologous recombination is a more complex process that uses a template sequence for repair, most often the sister chromatid in mitotic cells. When these pathways act on telomeres, the consequences are quite different. Since telomeric DNA are tandem arrays of short duplex repeats in the same orientation relative to the chromosome ends, homologous recombination events between telomeres can elongate or shorten telomeres, but cannot fuse them. In other words, a recombination event at telomeres is without immediate consequence on chromosome structure and remains reversible as long as the telomere length stays above a minimum threshold. This length varies between organisms but seems remarkably short in all cases. Homologous recombination at telomeres is not fully repressed in normal cells and may play a physiological role in telomere length homeostasis, in particular when telomere length has shifted far from equilibrium or its usual range.[5,6] In contrast, a single end-joining event mediated by NHEJ between 2 telomeres fuses them, generating either a dicentric chromosome or a circular chromosome, the latter being at risk of being converted into a dicentric through sister chromatid exchange. Dicentrics are unstable and can result in deleterious and potentially oncogenic copy number aberrations through several mechanisms such as breakage-fusion-bridge cycles, chromothrypsis, and polyploidization.[7-11] Thus, the existence of a stable karyotype of linear chromosomes in all cells where the NHEJ pathway is active requires that NHEJ is fully prevented from acting on telomeres.[12-16]

It should be noted that chromosome end fusions can occur with or without telomeric sequences at the fusion. In the first case, telomere function failed despite the native sequence. How such telomere fusions with native telomere sequences are prevented is the topic of this review. In contrast, when all or most of the telomere repeats are missing, the fusion is a secondary consequence of telomere loss.[15-18] In such cases, prevention of fusion relies on the mechanisms ensuring proper telomere sequence maintenance and replication, which will not be covered here.

The term NHEJ can refer to 2 distinct phenomena. The first is often called C-NHEJ (classic or canonical NHEJ) and is an efficient pathway in which a dedicated DNA ligase, LIG4, seals the double-strand ends. The second is often called A-NHEJ (alternative NHEJ). Here, very limited base pairing between single-strand 3′ tails allows ligation by LIG3 (the base excision repair ligase, absent in yeast) or eventual priming of DNA synthesis, the subsequent ligation being carried out by LIG1 (the main replicative ligase, ubiquitous in eukaryotes) but not LIG4. A-NHEJ is inhibited by replication protein A, and is mostly seen in circumstances where C-NHEJ and homologous recombination cannot act (e.g., in mutant contexts).[3,19,20] A-NHEJ might be a back up for these normally efficient pathways or the accidental by-product of various activities acting on DNA in the nucleus, a sort of biochemical noise without a positive function in normal cells. In this review, the terms NHEJ and end joining will refer to LIG4-dependent C-NHEJ.

NHEJ is not always active. For example, it is transiently shut down during mitosis in mammals [21,22] and during S/G2 in the fission yeast *Schizosaccharomyces pombe*.[23] In the budding yeast *Saccharomyces cerevisiae*, NHEJ is an haploid-specific pathway and is repressed in diploid cells.[24-26] In the distantly related yeast

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Submitted: 07/11/2014; Revised: 08/01/2014; Accepted: 08/07/2014
http://dx.doi.org/10.4161/23723548.2014.963438

Lachancea kluyveri, NHEJ was lost during evolution.[27] The problem arising from the coexistence of telomeres and NHEJ is thus relieved in these contexts.

NHEJ Fails to Act on Telomere DNA Ends

The function of end joining is to reseal DNA ends formed by double-strand breaks.[3] Its substrates are double-stranded ends that can be either blunt or possess short single-strand overhangs of a few nucleotides. DNA ends with short and perfectly cohesive overhangs can be repaired very efficiently and accurately by NHEJ. If nucleotides at the ends are damaged (e.g., at breaks generated by ionizing radiation), end joining can still process and repair the broken ends but the native sequences may not be restored. Repair is also slower and in some organisms inefficient.[3] In most eukaryotes, telomeres are composed of an oriented G-rich repeated motif (e.g., $TG_{1–3}$ in *S. cerevisiae* and TTAGGG in vertebrates). They are mostly double-stranded, but display a short terminal 3′ single-strand overhang of approximately 5–10 nt in budding yeast and 20–300 nt in vertebrates.[28,29] Thus, 2 telomeres will not form short cohesive overhangs. If anything else, their fusion by NHEJ would be a relatively slow act involving extensive processing of the ends. This kinetic effect might be the first barrier against fusions with which other mechanisms synergize.

NHEJ acts on double-stranded ends, which are only transiently stable. If the ends remain unrepaired, several nuclease activities degrade the 5′ ends to generate single-strand 3′ tails that are no longer substrates for NHEJ[2,3,30] but are instead committed to homologous recombination. Thus, it should be protective to maintain stable 3′ single-strand overhangs at telomeres that are long enough not to be a substrate for NHEJ. This maintenance results from a regulated equilibrium between 5′ degradation and DNA synthesis priming on the single-strand G-rich tails.[31-35,102]

In mammals, stability of the 5′ strand at the broken ends requires the recruitment of 53BP1 through a series of local histone modifications by ATM, RNF8, and RNF168.[36] 53BP1 protects 5′ ends through at least 2 effectors—RIF1 and PTIP—that either prevent 5′ degradation or reverse it through DNA synthesis.[37-39] 53BP1 is important for NHEJ events requiring relatively complex processing of the ends, in particular the fusions of ends from distinct breaks.[39] The relative slowness of these reactions may explain why they strongly depend on the extensive 5′ stability established by 53BP1. Telomere fusions also require 53BP1.[40] In normal cells, 53BP1 is excluded from telomeres.[41,42] Thus, NHEJ inhibition at telomeres may in part rely on this exclusion.

53BP1 also helps to keep ends mobile. Whether this is a consequence of increased mobility of double-stranded ends versus single-stranded ends is unknown, but since distant ends must meet in order to be fused by NHEJ movement within the nucleus can be a key limiting step in telomere fusions.[40] Through the exclusion of 53BP1, telomeres can attain a relative immobility that protects them against fusion. Similarly, excessive cohesion between sister telomeres favors their fusion by NHEJ.[43]

In addition to a simple 3′ single-strand overhang, the telomere DNA end may adopt unusual conformations to escape NHEJ. In one such case, the 3′ single-strand telomere end loops back to hybridize with the complementary strand of double-stranded telomere repeats, forming an intramolecular D-loop called the T-loop.[44,45] This strand invasion reaction further engages the telomere end into a reversible homologous recombination intermediate. This shifts the issue from preventing NHEJ to preventing further commitment to homologous recombination (that is 3′ extension) and keeping this structure stable (a D-loop is normally transient and unfolded by helicases or cut by nucleases). A likely gain from this structure is that it is less problematic to proceed into a full homologous recombination event once in a while (causing only telomere elongation or shortening) than to generate a single telomere fusion. In other words, it seems best to chose the less harmful of 2 bad events. The 3′ telomere single-strand tails are also sufficiently G rich to form G-quadruplexes that could oppose NHEJ.[46,47] The T-loop and G-quadruplex structures may also help to protect the 3′ telomere single-strand tails from A-NHEJ. In yeast, the 3′ telomere single-strand tails are probably too short to form stable T-loops or G-quadruplexes structures, but they may also be too short for A-NHEJ. Similarly, in *Arabidopsis* a large fraction of telomeres are blunt-ended and unlikely to adopt a secondary structure.[48] In some species, including *S. cerevisiae* and ciliates, A-NHEJ is also prevented by the lack of base pairing between 3′ telomere overhangs.

NHEJ involves a 3 protein complexes containing KU, Mre11, and Lig4.[3,49] KU is a DNA end-binding factor that encircles double-stranded DNA.[50,51] An abundant protein, it binds very rapidly to the broken ends of a double-strand break. Whether more than one KU molecule binds to each end is unclear. At the minimum, the function of KU is to restrain 5′ resection and control the recruitment of Mre11 and Lig4.[52,53] It probably also provides part of the scaffold that might align the ends and bring them in close proximity with the correct phasing to facilitate ligation by Lig4.[54] Unfortunately, this is still speculative and we do not have a complete understanding of the roles of KU during the NHEJ process. The Mre11 complex is involved in several DNA repair processes, including NHEJ.[49,55-57] This complex binds to double-stranded DNA ends and might help to tether the ends together[58,59] As already mentioned, Lig4 is an ATP-dependent DNA ligase dedicated to NHEJ.[53,60] The 2 Lig4 co-factors are Lif1 and Nej1 in budding yeast, and XRCC4 and XLF/CERNU in mammals.[61] XRCC4-XLF can form polymers *in vitro* and may thus act as a splint bridging the 2 broken ends,[62,63] a model that remains to be tested *in vivo*. In addition to these core factors, NHEJ can mobilize a DNA polymerase from the pol-X family to fill in short gaps at the ends prior to ligation: Pol4 in budding yeast; Pol λ and Pol μ in mammals.[64,65] In budding yeast, most telomere fusions by NHEJ require Pol4.[66]

KU is normally present at telomeres, presumably bound to the telomere DNA end.[67,68] Thus, inhibition of NHEJ at telomeres does not involve excluding KU from telomeres. As at double-strand breaks, one function of KU at telomeres is to limit 5′ resection, thus helping to set the size of the 3′ single-strand telomere overhang.[29,33,67] In budding yeast, KU also helps to recruit

telomerase and a heterochromatin factor, Sir4, at telomeres.[69-71] In contrast to KU, the Mre11 complex, Lig4, and its co-factors are absent from telomeres.[72,73] This indicates that NHEJ inhibition is in part based on their exclusion from telomeres, or at least their inability to form a stable complex with telomere DNA ends. An exception is during replication, when Mre11 can be transiently recruited to telomeres.[74,75] At this time, Mre11 is important for telomere elongation by telomerase, at least in budding yeast where the bulk of elongation depends upon Mre11 and its associated checkpoint kinase Tel1^{ATM}. In particular, Mre11 plays an important role in replication-coupled 5′ resection at telomeres, a key step in generating the 3′ single-stranded tails that telomerase can elongate.[76,77] Thus, during telomere replication the inhibition of NHEJ must rely on mechanisms other than Mre11 exclusion.

Multiple Pathways Established by Telomere Factors Synergize to Inhibit NHEJ

The telomere repeated motifs allow the concentration of specialized proteins that recognize them and establish telomeric functions.[78] Perhaps not surprisingly, proteins bound to the double-stranded telomeric repeats and the factors recruited by these proteins are essential to protect telomeres from NHEJ in all species in which this question has been addressed. In the fission yeast *S. pombe*, the Taz1 protein binds telomeric DNA and recruits the Rap1 protein. In the absence of Taz1 or Rap1, telomeres fuse together in a process that requires Lig4, KU, and Mre11.[12,57,79] Thus, both Taz1 and Rap1 are required for NHEJ inhibition, but how these proteins function is unknown.

In mammals, 2 Taz1 orthologs, TRF1 and TRF2, bind double-stranded telomeric DNA. TRF2 also recruits RAP1 to telomeres. In the absence of TRF2, telomeres fuse extensively in a KU, LIG4, and MRE11-dependent manner indicating that TRF2 is essential for NHEJ inhibition.[14,41,55,80,81] In cells lacking RAP1, telomere fusions remain below detection threshold.[82] However, in cells where TRF2 is detached from telomeres, artificial tethering of RAP1 to the telomeres re-establishes NHEJ inhibition independently of TRF2.[83] This suggests that TRF2 protects telomeres from NHEJ through multiple mechanisms, at least one of which involves RAP1 and the others being RAP1-independent. TRF1 also contributes to NHEJ inhibition, perhaps indirectly by favoring TRF2 assembly or function.[84,85]

Details of each inhibitory pathway established by TRF2 are starting to emerge. First TRF2, but not RAP1, is essential to exclude 53BP1 from telomeres through the synergistic inhibition of ATM and RNF168.[42] TRF2 helps to maintain non-compact chromatin at telomeres that disfavors NHEJ.[86] Inhibition of NHEJ by TRF2 in complex with RAP1 can be established *in vitro* on short double-stranded substrates, suggesting that the RAP1-dependent pathway is not related to a structure established by the 3′ telomere single-strand overhang.[87] TRF2 also directly interacts with KU and this interaction might help to inhibit NHEJ by preventing a KU self-association that is predicted to bridge DNA ends during the NHEJ process.[54] In addition, TRF2 protects telomeric 3′ single-strand overhangs from degradation by the nuclease activities of the XPF/ERCC1 and MRE11 complexes.[88,89] TRF2 also generates T-loops independently of RAP1 *in vitro* and *in vivo*, in part through promotion of supercoiling and strand invasion and in part through protection of the Holliday junctions.[45,90,91] It should be noted that T-loops were observed after psoralen crosslinking in isolated nuclei so it remains formally possible that their accumulation is a TRF2-dependent artifact generated during nuclei isolation and that such structures are transient and unfolded by helicases in living cells.

In *S. cerevisiae*, there is no ortholog of Taz1/TRF1/TRF2 and the Rap1 protein directly binds the telomere sequences at a density of approximately 15–20 molcules per telomere. In yeast cells lacking Rap1, the telomeres fuse in a KU, Lig4, and Mre11-dependent manner.[92] Rap1 establishes at least 3 distinct pathways to inhibit NHEJ.[93] One requires the Rap1-interacting factor Rif2, an AAA+ protein originating from a recent duplication of an ORC subunit gene in the yeast lineage. Two insights suggest the mode of action of Rif2. First, Rif2 inhibits NHEJ at a double-strand break in the absence of telomeric DNA when it is artificially targeted there by a domain of Rap1. This suggests that Rif2 acts on protein complexes and not through a telomere-specific DNA structure. It also shows that NHEJ inhibition by Rif2 is a standard cis effect in which the recruitment of a factor locally inhibits a molecular function. Second, Rif2 inhibits not only NHEJ at telomeres, but also all other Mre11-dependent processes (5′ resection, telomerase-mediated telomere elongation and a pathway of homologous recombination between telomeres).[6,33,93-97] The simplest hypothesis is that Rif2 inhibits the Mre11 complex; this single molecular activity would explain the multifunctionality of Rif2. However, for now the mechanism of Rif2 remains unknown. The origin of Rif2 suggests that ORC may play a similar role at telomeres in other eukaryotes, for example in human cells where ORC is recruited to telomeres by TRF2.[98]

Independently of Rif2, in budding yeast Rap1 inhibits NHEJ at telomeres through recruitment of the heterochromatin factor Sir4, a yeast-specific protein of unknown evolutionary origin.[93] Interestingly, Sir4 interacts directly with KU.[69] This interaction is essential for stable Sir4 recruitment at telomeres but may also inhibit NHEJ, for example by disrupting the assembly of Lig4 on KU molecules bound to DNA ends.[53] Although this scenario remains speculative, the parallel with the TRF2-KU interaction in mammals is striking. Interestingly, Sir4 directly interacts with DNA *in vitro*.[99] Whether this property is related to NHEJ inhibition is unknown.

Rap1 in budding yeast also inhibits NHEJ independently of Rif2 and Sir4 through a region of the protein that includes its DNA binding domain.[93] The same region also inhibits 5′ degradation and Mre11 recruitment.[72] DNA binding by Rap1 may directly out-compete the stable assembly of Mre11 on DNA ends, favor a DNA secondary structure that is resistant to NHEJ and 5′ degradation, or recruit unidentified effectors.

We recently found that the yeast translocase and SUMO-dependent ubiquitin ligase Uls1 are essential to maintain NHEJ

inhibition at telomeres.[100] Uls1 does not act in a specific inhibitory pathway; instead, its role is to ensure that Rap1 function is maintained. Specifically, Uls1 eliminates rare non-functional poly-SUMOylated Rap1 molecules bound to telomeres. How poly-SUMOylation cripples the ability of Rap1 to inhibit NHEJ is unknown.

Thus, in both mammals and in yeast, multiple mechanisms cooperate to fully inhibit NHEJ at telomeres. Their synergy is reinforced by the multiplicity of DNA-bound molecules at each telomere and ensures that NHEJ inhibition at telomeres is continuously efficient and resilient to normal telomere length fluctuation. In budding yeast, the protection against telomere fusions goes one step further: dicentrics formed by telomere fusions often break at the fusions during mitosis, a process that restores the parental chromosomes.[101] By allowing reversibility of telomere fusions, this rescue pathway can back up a temporary lapse of NHEJ inhibition at telomeres, further protecting the cell from the deleterious consequences of an unstable karyotype.

Acknowledgments

The author would like to thank Karine Dubrana, Madalena Tarsounas and Pablo Radicella for helpful criticism and advice on this review.

Funding

Studies in the author's laboratory are supported by grants from ARC and ANR (Blanc-SVSE-8-2011-TELO&DICENs).

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