Mechanisms of the Repair of DNA Double-strand Breaks
- To Incorporate DSB Repair in the Physical Modeling of Radiation Biological Effects -

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Abstract
Ionizing radiation is thought to exert its pleiotropic biological effects through damages on DNA. DNA double-strand break (DSB) is considered the most critical among various types of DNA damages. DSBs are repaired through homologous recombination (HR) and non-homologous end joining (NHEJ). These processes are in competition with each other but there are also mechanisms to choose the most appropriate one depending on circumstances: species, life cycle phase, cell cycle phase, complexity of DNA damage and chromatin structure. I summarize the current understanding of the molecular mechanisms and interrelationships of these DSB repair processes and also discuss important points of consideration in incorporating DSB repair for the physical modeling of radiation biological effects.

1. Introduction

Ionizing radiation exerts various biological effects, *e.g.*, fatality, infertility, carcinogenesis, on the other hand, cancer cell killing. It is generally thought that these pleiotropic effects of ionizing radiation are mainly mediated through generation of damages on DNA. It is estimated that 1 Gy of low LET radiation produces approximately 500 base damages, 1,000 single-strand breaks and 40 double-strand breaks (DSBs) on DNA. DSB is considered the most critical among various types of DNA damages [1)]. DSBs are repaired through non-homologous end joining (NHEJ) and homologous recombination (HR). These DSB repair mechanisms are essentially conserved among eukaryotic organisms, although there are some differences in the factors involved.

It is important here to recall that, even if the total dose is same, the biological effects of radiation can differ depending on dose rate and fractions. These phenomena are explained by repair of "sublethal" DNA damage between or during irradiation. In addition, the biological effects of radiation are greatly influenced by linear energy transfer (LET). DNA damage caused by high LET radiation are more concentrated spatially, termed cluster damages, and are thought more difficult to be repaired. Thus, DSB repair would have great implication in building a model for biological effects of radiation of low dose, low dose rate or high LET, especially.

2. Mechanisms of DNA Double-strand Break Repair

i. Non-homologous End Joining

NHEJ can be further classified into canonical (or classical) NHEJ (C-NHEJ) and alternative (or atypical) NHEJ (A-NHEJ).

Fig. 1 shows the schematic model of C-NHEJ reaction. In C-NHEJ, a heterodimer of two Ku subunits, which are termed Ku86 (or Ku80) and Ku70, respectively, first binds to DSB. X-ray crystallography revealed that Ku has a

"doughnut-like" structure and binds to DNA through its "pore" [2], giving a clear and simple explanation for the DNA end-specific binding of Ku. Ku in turn, recruits DNA-PKcs (DNA-dependent protein kinase catalytic subunit) [3,4]. When two DNA ends are not readily ligatable, they are processed. This step includes removal of unnecessary overhangs by Artemis nuclease, filling in the gap by DNA polymerase μ or λ, removal of phosphate group attached to 3'-end and addition of phosphate group to 5'end, if absent, by PNKP (polynucleotide kinase/phosphatase). Two DSB ends are finally joined by DNA ligase IV (LIG4), which carries catalytic activity, and XRCC4 and XLF (XRCC4-like factor, also known as Cernunnos) are thought to regulate LIG4. As their names indicate, XRCC4 and XLF share structural similarity and are regarded the relatives (paralogs). XRCC4 is shown to be essential for the stability of LIG4: LIG4 becomes unstable in the absence of XRCC4 [5-8]. XLF is shown to facilitate LIG4 activity in the face of incompatible or mismatched DNA ends [9-12]. XRCC4 and XLF possibly form a fiber to bridge two DNA ends through interaction at their N-terminal globular head domains [13,14]. Most recently, PAXX (paralog of XRCC4 and XLF, also known as XLS for XRCC4-like small protein) was added to the list of essential NHEJ factors [15-17]. PAXX is shown to interact with Ku and to stabilize NHEJ complex.

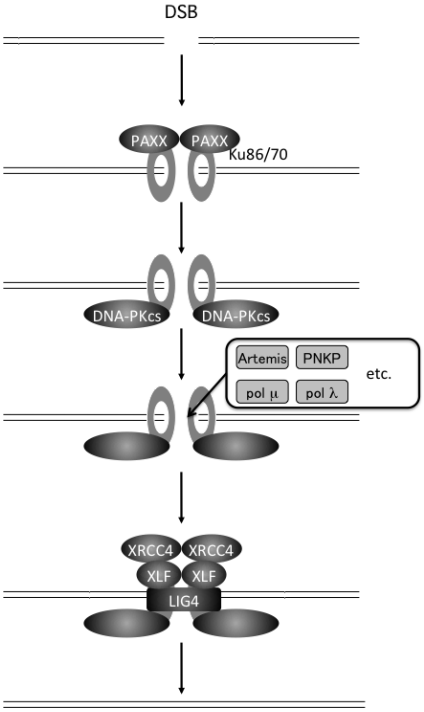

Fig. 1 Schematic model of C-NHEJ reaction.

In immune systems, NHEJ is also involved in V(D)J recombination, to expand the diversity of immunoglobulin and T cell receptor, and in class-switch recombination, to change the type of immunoglobulin. Therefore, mutation in either one of the genes of above factors results in severe combined immunodeficiency (SCID), lacking both of B cells (that produce immunoglobulin) and T cells (that express T cell receptor on its surface). DNA-PKcs is mutated in *scid* mice. Mice deficient for Ku86, Ku70, XRCC4 or LIG4, generated by gene targeting, exhibit immunodeficiency and defects in growth and neuronal development. Human patients, harboring mutation in DNA-PKcs, LIG4 and XLF, were also reported to exhibit dwarfism (extremely short statue) and microcephaly (small-sized brain), which are hallmarks of the defect on growth and neuronal development [18]. Thus, deficiency in NHEJ gene is associated with pleiotropic symptoms in human as well as in other animals. Patients harboring mutation in XRCC4 were reported very recently to show microcephaly and dwarfism but, surprisingly, not overt immunological defects [19-24].

A-NHEJ is a DSB-joining reaction, which is manifested in the absence of C-NHEJ [25]. A-NHEJ might include several types of reactions: some part is driven by micro-homology, called micro-homology-mediated end joining (MMEJ), while others occur independent of micro-homology. In any case, A-NHEJ is considered less ordered and less precise than C-NHEJ. Although A-NHEJ is less understood than C-NHEJ, it might involve single-strand break repair proteins (PARP (poly ADP-ribose polymerase), DNA ligase III and XRCC1), HR proteins (*e.g.*, Mre11 and CtIP, see next section) and other diverse DSB repair proteins (*e.g.*, FANCA, FAAP24 and DNA2) [25,26]. Hereafter "NHEJ" refers to "C-NHEJ".

ii. Homologous Recombination (HR)

Fig. 2 shows the schematic model of HR reaction. The first step of homologous recombination is degradation of one of the strand, which has 5'-end at DSB (denoted as "5'-strand" hereafter), in order to create single-stranded DNA with 3'-overhang (this process is termed "resection"). In this step, Mre11 (human homolog of budding yeast *MRE*11, meiotic recombination 11), which is associated with Rad50 (human homolog of budding yeast *RAD*50, radiation sensitive 50) and Nbs1 (Nijmegen breakage syndrome) and possesses both of endonuclease activity and exonuclease activity, plays a critical role. First, Mre11, by its endonuclease activity stimulated by CtIP, induces a nick on the 5'-strand at some distance from DSB and then, by its exonuclease activity, degrades the strand in 3'-5' direction toward DSB [27,28]. On the other hand, ExoI (exonuclease I) in association with Blm (Bloom's syndrome mutated) proceeds 5'-3' digestion from the nick [29]. The resultant single-stranded DNA region is coated by RPA (replication protein A) and, then, replaced by Rad51 [29]. This replacement is facilitated by BRCA1 (breast cancer susceptibility 1), BRCA2, PALB2 (partner and localizer of BRCA2, also known as FANCN) and Rad51 paralogues [30]. Rad51 promotes strand exchange between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA), *i.e.*, annealing ssDNA and one of the strand of unwound dsDNA, to search for strand with homology [31]. Once the strand with homology was found, it serves as the template for strand synthesis by replicative DNA polymerase δ with the support by PCNA (proliferating cell nuclear antigen) and RFC (replication factor C). Finally, two connected DNA strands (Holliday structure) are separated by Mus81-Eme1 [32], ERCC1/XPF [33] or SLX1-SLX4 [34,35] (this process is termed "resolution").

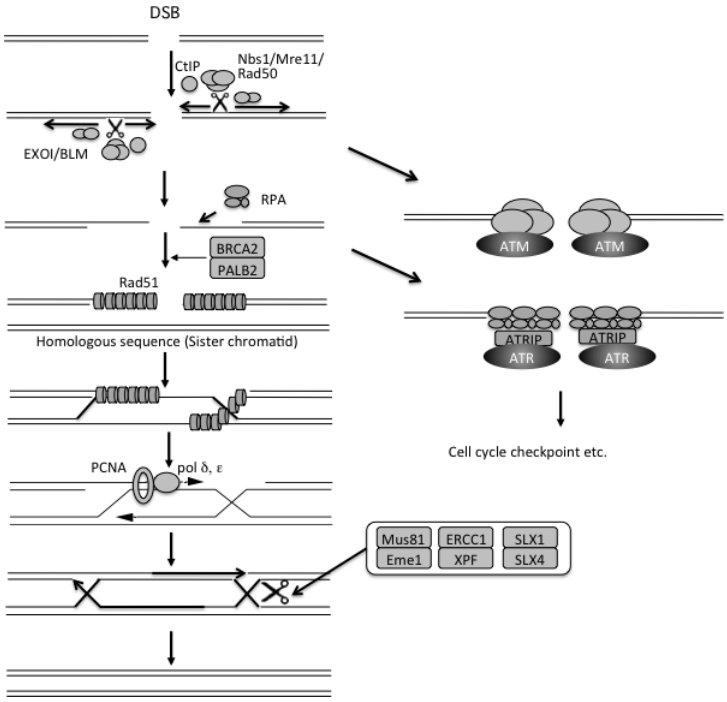

Fig. 2 Schematic model of HR reaction.

Another important function of the complex of Mre11/Rad50/Nbs1 is the recruitment of ATM (ataxia-telangiectasia mutated) to DSB. RPA is associated with ATRIP (ATR-interacting protein), which recruits ATR (ATM- and Rad3-related) to ssDNA. ATM and ATR are protein kinases structurally related to DNA-PKcs. ATM and ATR are shown to phosphorylate (transfer a phosphate group from ATP to serine, threonine or tyrosine residue of a protein) a number of proteins involved in cell cycle regulation, such as Chk1 (checkpoint kinase 1), Chk2 and p53. These protein inhibit CDKs (cyclin-dependent kinase-s), which promote cell cycle progression. Thus, HR is coupled with cell cycle checkpoint.

3. Choice between NHEJ and HR

NHEJ is a reaction to join two DNA ends and, therefore, may sometimes incur nucleotide deletions or insertions at the junction or joining with incorrect partner, leading to chromosomal aberrations like deletions, inversions or translocations. On the other hand, HR is a reaction to reconstitute the sequence around DSB by referring to

homologous chromosome or sister chromatid as the template and, therefore, is considered highly accurate. However, HR might not always be the right choice. Indeed, cells choose the most appropriate DSB repair pathway depending on circumstances as discussed below.

i. Cell cycle and ploidy-dependent choice between NHEJ and HR in budding yeast cells

Budding yeast *Saccharomyces cerevisiae* is one of model organisms, which have been used in cell cycle or DNA repair studies. *S. cerevisiae* exists as haploid or diploid. In haploid, there are two mating types, *i.e.*, a and α. One each of a and α mate to form diploid. In a stressful condition, diploid cell undergoes meiosis to form four spores of haploid.

The homolog of Ku70 in *S. cerevisiae* is called Hdf1 (high affinity DNA-binding factor 1) or Yku70 (yeast Ku70). The radiosensitivity of diploid cell lacking Hdf1 (*hdf1*/*hdf1*) was indistinguishable from that of wild-type cell (Fig. 3A) [36]. On the other hand, diploid cell lacking Rad52 (*rad52*/*rad52*), which is involved in HR, showed increased sensitivity to radiation (Fig. 3A) [36]. The cell lacking both of Hdf1 and Rad52 (*hdf1*/*hdf1 rad52*/*rad52*) was more sensitive than *rad52*/*rad52* cell [36]. Haploid cell lacking Hdf1 (*hdf1*) was radiosensitive in G1 phase, while haploid cell lacking Rad52 (*rad52*) showed normal radiosensitivity (Fig. 3B) [36]. In asynchronous population, there was a radioresistant subpopulation in haploid wild type and *hdf1* but this was not observed in *rad52* or *hdf1 rad52* (Fig. 3C). These observations collectively suggest that HDF1 is required for survival after irradiation only when HR is not available, *e.g.*, in G1 phase of haploid or in HR mutant.

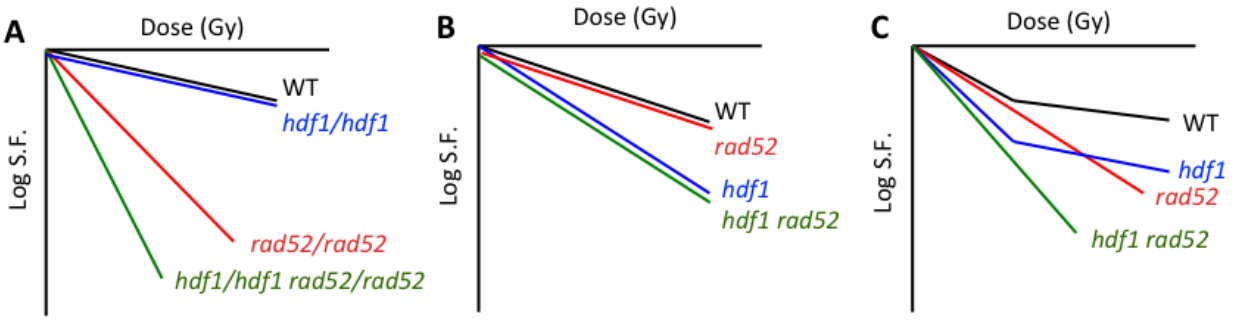

Fig. 3 Survival curves of diploid (A), G1-synchronized haploid (B) and asynchronous haploid (C) budding yeast. S.F.: surviving fraction. These curves are drawn schematically according to 44).

Sae2 (sporulation in the absence of Spo eleven) is the yeast homolog of CtIP (CtBP-interacting protein, also known as RBBP8), stimulating the nuclease activity of Mre11. Sae2 undergoes phosphorylation on serine 267 (Ser267) by

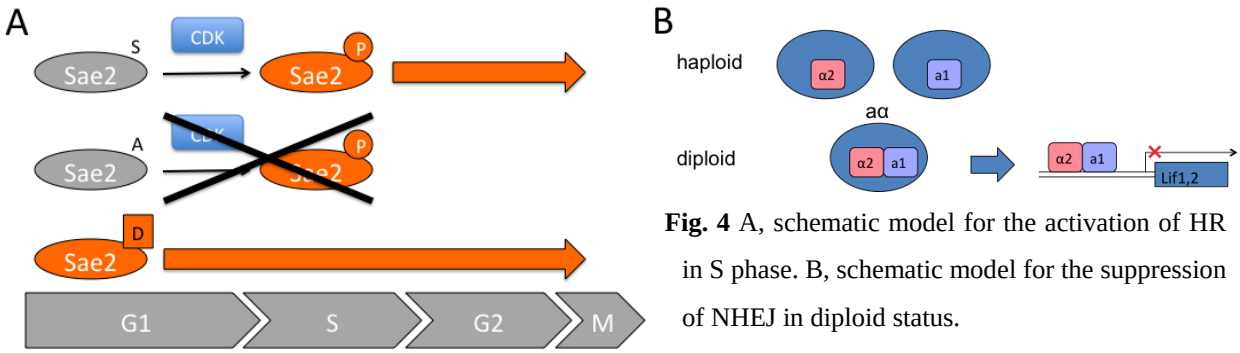

Fig. 4 A, schematic model for the activation of HR in S phase. B, schematic model for the suppression of NHEJ in diploid status.

CDK in S phase (Fig. 4A) [37]. When Ser267 was changed into alanine (S267A), which cannot be phosphorylated, SAE2 showed defective DNA end resection [37]. On the other hand, when Ser267 was changed into glutamic acid (S267E), which was intended to mimic phosphorylation, Sae2 became constitutively active, promoting resection even if CDK was inhibited [37]. These results indicate that phosphorylation by CDK activates the resection function of Sae2 [37]. Moreover, yeast expressing Sae2-S267A showed increased sensitivity to radiation in G2 phase, whereas that expressing Sae2-S267E showed increased sensitivity in G1 phase [37]. The latter observation indicates the importance of silencing HR in G1 phase.

Lif1 (Ligase 4-interacting factor 1) and Lif2 (also known as Nej1 for non-homologous end joining 1) are budding yeast homolog of XRCC4 and XLF, respectively. The a type haploid expresses a1 protein, whereas the α type haploid expresses α2. Upon mating, a1 and α2 associate with each other to form a transcription repressor. The transcription of mRNA of Lif1 and Lif2 is suppressed in diploid by a1/α2 (Fig. 4B) [38-40].

ii. Cell cycle-dependent choice between NHEJ and HR in vertebrate cells

Because of high HR capacity of DT40, knock-out cells for many genes have been established, which significantly contributed to deepening our understanding of interrelationship between NHEJ and HR in vertebrate cells. Fig. 5 shows the survival of wild-type DT40 and mutants lacking DSB repair genes after X-ray irradiation [41-43]. The survival curve of Ku70$^{-/-}$ cell shows a biphasic shape: steep decline in <~1 Gy and shallow decline in >~1 Gy. Ku70$^{-/-}$ cell consists of two subpopulations with distinct radiosensitivity: highly radiosensitive subpopulation, accounting for ~85%, and highly radioresistant subpopulation, accounting for ~15%. Cell cycle synchronization experiment indicated that Ku70$^{-/-}$ cell is highly radiosensitive in G1 phase, while it is radioresistant in G2 phase [41]. Therefore, the radiosensitive subpopulation would represent cells in G1 phase and the radioresistant subpopulation would represent cells in late S and G2 phases. These results indicate that NHEJ is essential in G1 phase but not in late S and G2 phases. The survival curve of Ku70$^{-/-}$ Rad54$^{-/-}$ double-knock-out cell, which is deficient in both of NHEJ and HR, was similar to that of Ku70$^{-/-}$ cell but does not show radioresistant subpopulation. Therefore, the appearance of the radioresistant subpopulation in Ku70$^{-/-}$ might be due to HR. These observations collectively indicate that HR operates only in late S and G2 phases. Although homologous chromosome exists in G1 phase, it

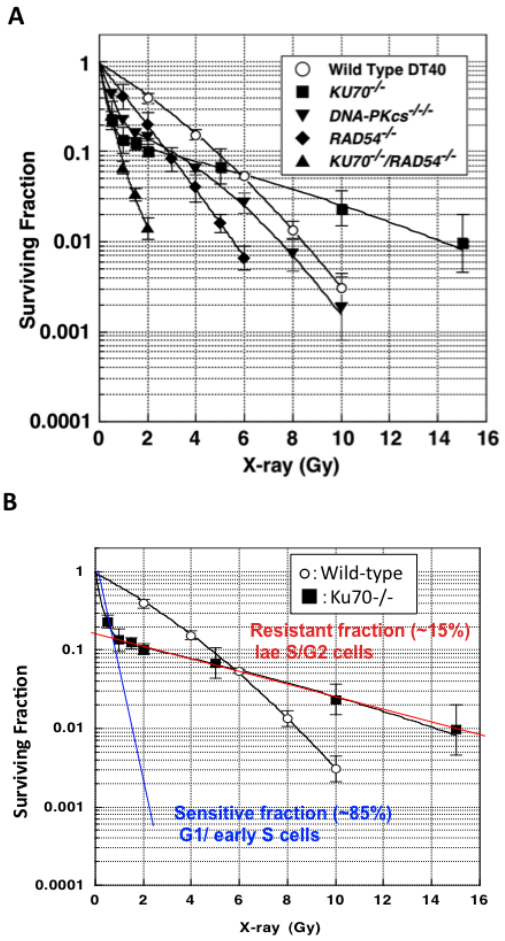

Fig. 5 A, survival curves of wild-type DT40 and DNA repair-deficient mutants. B, decomposition of the survival curve of Ku70$^{-/-}$ into two subpopulations with distinct radiosensitivity. These curves are drawn by using data in 43).

does not serve as the template for HR. DSB repair through HR requires sister chromatid, generated through DNA replication in S phase.

Wild-type DT40 cells also showed cell cycle-related fluctuation in radiosensitivity [41]. The cell survival after irradiation increased toward the late S phase [41]. Such change in radiosensitivity is well documented in mammalian cells and also considered in clinical radiotherapy, although the mechanism has been yet to be clarified. The above change was not observed in Rad54$^{-/-}$ cell. Thus, HR would explain increased radioresistance in late S and G2 phases. It should be also noted that Ku70$^{-/-}$ cell showed higher survival than wild-type DT40 cell in >~6 Gy, indicating that Ku70$^{-/-}$ cell is more radioresistant than wild-type DT40 cell when HR is available. This suggests that NHEJ, which is considered less accurate than HR, is not shut off in late S and G2 phases. The survival curve of DNA-PKcs$^{-/-}$ cell is also biphasic, but less steep in low dose region and less shallow in high dose region than that of Ku70$^{-/-}$ cell [42,43]. This might be because DNA-PKcs plays more auxiliary role in NHEJ than Ku and therefore NHEJ can proceed to some extent without DNA-PKcs. In support of this, the radiosensitivity of DNA-PKcs$^{-/-}$ Ku70$^{-/-}$ cell, lacking both of DNA-PKcs and Ku70, was indistinguishable from that of Ku70$^{-/-}$ cells [42].

There is a significant distinction in the usage of HR in diploid G1 phase, that is, in other words, homologous chromosome as the template, between budding yeast and vertebrate. Although the reason is not clear, it may be related to the size, complexity and organization of genome. Vertebrate genome, as compared to yeast genome, contains much larger extent of non-protein-coding sequence and repeat sequence. In the non-protein-coding region, the addition or deletion of a considerable number of nuclides might be tolerated, as far as it does not change the amino acid sequence in protein. In addition, if the repeat sequence, which appears in different chromosome or different portion of the same chromosome, is mistakenly recognized as the template, HR may incur significant genetic changes, *e.g.*, chromosome aberration.

Similarly to Sae2 in budding yeast, CtIP undergoes phosphorylation on threonine 847 (Thr847) by CDK in S phase and this phosphorylation is essential for the resection function of CtIP [44]. Moreover, mutant CtIP, in which Thr847 had been changed into glutamic acid (T847E), exhibited a constitutive resection function [44]. Thus, phosphorylation of Sae2 and CtIP by CDK might be a conserved mechanism to "license" HR in S phase. Additionally, a very recent study demonstrated that the interaction between BRCA1 and BRCA2/PALB2 is constrained to S/G2 phases, through ubiquitilation of PALB2 on BRCA1-interacting site [45]. Ubiquitilation is catalyzed by the ubiquitin ligase KEAP1 (Kelch-like ECH-associated protein1), in complex with CUL-3-RBX1, whereas deubiquitilation is catalyzed by USP11 [45]. As USP11 (ubiquitin-specific protease 11) is degraded rapidly in G1 phase, PALB2 is kept in ubiquitilated form and, hence, PALB2-BRCA1 interaction is suppressed in G1 phase. Moreover, the combination of CtIP-T847E and siRNA targeting USP11 could restore HR in G1 phase [45].

iii. Choice between NHEJ and HR depending on chromatin status and complexity of DNA damage

As both of NHEJ and HR are operating in late S and G2 phases, which of them will be taken?

To address this question, Shibata and colleagues examined the kinetics of DSB repair in X-ray-irradiated, carbon ion-irradiated and etoposide-treated cells using γ-H2AX foci as the readout [46]. It has been well documented that DSB repair kinetics can be decomposed into two components, *i.e.*, fast component and slow component. The half-life of remaining DSB is 2 hr or less and ~10 hr or more for fast component and slow component, respectively. A number of studies using mutant cells, inhibitors or siRNAs for NHEJ and HR factors, indicate that fast component reflects

NHEJ, whereas slow component reflects HR. In the case of etoposide treatment and, to a lesser extent, X-ray irradiation, majority of DSB was repaired with fast kinetics. On the other hand, greater portion of DSB was repaired with slow kinetics after carbon ion-irradiation. In BRCA2-deficient cells, the delay in slow component was manifested after carbon ion-irradiation and, on the other hand, the repair kinetics was quite similar after etoposide treatment [46]. These observations collectively suggest that complex DNA damages, *e.g.*, those induced by high LET radiation like heavy-ion, are preferentially repaired through HR.

In addition, a part of γ-H2AX foci remains decline with slow kinetics even in the case of etoposide treatment and X-ray irradiation. These protracted γ-H2AX foci overlapped with those of KAP-1 (KRAB-associated protein 1), phosphorylated on serine 824 (Ser824) [46]. It was shown that KAP-1 phosphorylation on Ser824 by ATM occurs in heterochromatic region, inducing chromatin relaxation to facilitate HR. Thus, DNA damages occurred in heterochromatic region might be repaired through HR.

Then, are DNA damages, which are structurally complex or occurred in heterochromatic region, sorted for HR *ab initio* in G2 phase? It was shown that siRNA-mediated knocking down of Ku or DNA-PKcs increased foci of RPA or Rad51, which are considered HR intermediates [46]. In addition, Rad51 foci diminished in the cell expressing mutated DNA-PKcs, lacking six phosphorylation sites clustered between 2609 and 2647 (termed ABCDE cluster) [46]. The phosphorylation of DNA-PKcs at these sites is shown to facilitate its dissociation from DNA [47]. It might be noted that the cell expressing this mutated DNA-PKcs was shown to be more radiosensitive than the cell not expressing DNA-PKcs [47]. In conjunction with this, the mouse harboring this mutation shows more severe phenotype, *i.e.*, embryonic lethality, than the mouse, in which DNA-PKcs is absent [48]. These observations suggest that cells might make an initial attempt to repair DSB in heterochromatic region as well as euchromatic region through NHEJ and that the phosphorylation of DNA-PKcs might be a mechanism of "pathway switch" from NHEJ to HR when it is more appropriate, e.g., complex DNA damage or damage in heterochromatic region (Fig. 6). Without the phosphorylation, DNA-PKcs cannot dissociate from DNA even when NHEJ cannot proceed properly, leading to the "dead-end".

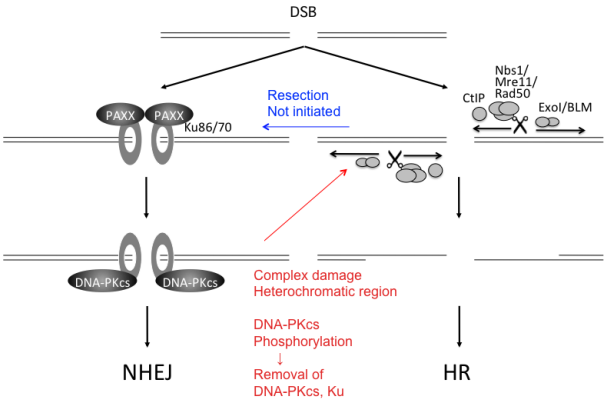

Fig. 6 Possible models for NHEJ/HR pathway switch in G2 phase.

Finally, is there a switch from HR to NHEJ in G2 phase? The siRNA-mediated knocking down of CtIP accelerated DSB repair, in a manner dependent on NHEJ [46]. In addition, siRNA-mediated knocking down of BRCA2 decelerated DSB repair but additional knocking down of CtIP restored DSB repair kinetics [46]. Furthermore, in more recent study they generated Mre11 endonuclease-specific inhibitor (Endo-i) and Mre11 exonuclease-specific inhibitor (Exo-i) [49]. Although both of Endo-i and Exo-i suppressed the resection [49], Exo-i, but not Endo-i, inhibited DSB repair in G2-phase [49]. In the presence of Endo-i, DSB was repaired through NHEJ [49]. Endo-i restored DSB repair kinetics, as in the case of CtIP siRNA [49]. Moreover, siRNA-mediated knocking down of ExoI and BLM rescued DSB repair in the presence of Exo-i [49]. These results suggested that there might be a switch from HR to NHEJ, in a situation in which the resection does not proceed further (Fig. 6).

4. Concluding remark

As discussed above, eukaryotic cells use two major mechanisms, *i.e.*, NHEJ and HR, to repair DSB. There are also the mechanisms to let cell choose the appropriate one depending on circumstances, *e.g.*, cell cycle, ploidy, species, complexity of damage and chromatin structure. These mechanisms might have been acquired and evolved in surviving under the continuous threat of external and internal insult on DNA over billion years since the emergence of life on the earth. Understanding the nature of DSB repair pathways and considering their interrelationship would be important in building the physical and quantitative model of radiation biological effects. The importance would be especially manifested when we consider the effects of radiation of low dose, low dose rate and high LET, especially.

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