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Non-homologous end-joining factors of *Saccharomyces cerevisiae*

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Abstract

DNA double-strand breaks (DSB) are considered to be a severe form of DNA damage, because if left unrepaired, they can cause a cell death and, if misrepaired, they can lead to genomic instability and, ultimately, the development of cancer in multicellular organisms. The budding yeast *Saccharomyces cerevisiae* repairs DSB primarily by homologous recombination (HR), despite the presence of the KU70, KU80, DNA ligase IV and XRCC4 homologues, essential factors of the mammalian non-homologous end-joining (NHEJ) machinery. *S. cerevisiae*, however, lacks clear DNA-PKcs and ARTEMIS homologues, two important additional components of mammalian NHEJ. On the other hand, *S. cerevisiae* is endowed with a regulatory NHEJ component, Nej1, which has not yet been found in other organisms. Furthermore, there is evidence in budding yeast for a requirement for the Mre11/Rad50/Xrs2 complex for NHEJ, which does not appear to be the case either in *Schizosaccharomyces pombe* or in mammals. Here, we comprehensively describe the functions of all the *S. cerevisiae* NHEJ components identified so far and present current knowledge about the NHEJ process in this organism. In addition, this review depicts *S. cerevisiae* as a powerful model system for investigating the utilization of either NHEJ or HR in DSB repair.
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Keywords: Non-homologous end-joining; *Saccharomyces cerevisiae*; Yku70/80; Mre11/Rad50/Xrs2; Lig4/Lif1; Nej1

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1. Introduction

DNA double-strand breaks (DSB), which can be induced by a number of endogenous and exogenous agents, are considered the most lethal form of DNA damage. If unrepaired or misrepaired, they can initiate processes leading to mutagenesis, tumorigenesis and cell death. Therefore, efficient repair of DSB is essential to maintain genome stability and cell viability. Two main mechanisms, homologous recombination (HR) and non-homologous end-joining (NHEJ), have evolved for the repair of DSB in eukaryotic cells (for reviews, see [1–6]).

NHEJ was initially discovered in mammalian cells. The proteins identified as the key players in mammalian NHEJ are the DNA-dependent protein kinase (DNA-PK), the DNA ligase IV/XRCC4 complex and ARTEMIS (reviewed in [4,7–15]). DNA-PK is a nuclear serine/threonine protein kinase that belongs to the phosphatidyl-inositol-3 family of protein kinases. It consists of three subunits: a catalytic subunit (DNA-PKcs) and the two DNA end-binding subunits, KU70 and KU80, which form a stable heterodimer (KU70/80) (for reviews, see [11,16–23]). DNA-PKcs forms a complex with ARTEMIS [24], a function of which is important for nucleolytic end-processing step in NHEJ. DNA ligase IV is the DSB repair ligase that physically associates with XRCC4, forming a complex of 1:2 (DNA ligase IV:XRCC4) stoichiometry [25–27]. XRCC4 is a nuclear phosphoprotein that modulates stability and activity of DNA ligase IV and targets the enzyme to the site of DSB (for reviews, see [12,28,29]) (as the mammalian NHEJ is outside the scope of this review, the reader is referred to references listed in Table 1 as well as the reviews listed above).

According to current models of the mammalian NHEJ (Fig. 1), KU70/80 is the first component to bind to broken DNA ends. Subsequently, DNA end-bound KU70/80 recruits DNA-PKcs, resulting in a stable complex formation between DNA-PKcs and KU70/80. This complex presumably maintains the proximity of the two broken DNA ends and thus serves as an end-to-end synapsis factor [12,30]. Subsequently, the kinase activity of DNA-PKcs, being activated after binding of DNA-PKcs to DNA ends, mediates several phosphorylation events (for a review, see [11]), whose importance is not fully understood. Autophosphorylation of DNA-PK potentially triggers remodelling of the DNA-PK–DNA end complex, thereby allowing access of DNA ends to further processes without disrupting synapsis [30]. DNA-PK also phosphorylates and activates the endonuclease activity of ARTEMIS [24], which is likely necessary for the nucleolytic DNA end-processing step of NHEJ. The end-processing stage of NHEJ further involves gap-filling. The prevailing evidence suggests that DNA polymerase μ or λ functions in this capacity [31–34]. NHEJ is completed by the ligation reaction carried out by the DNA ligase IV/XRCC4 complex (reviewed in [4,8–12]).

The NHEJ components have also been revealed in other organisms including bacteria and yeasts [35–49], suggesting that NHEJ has been conserved through evolution and operates from bacteria to man. Therefore, investigating NHEJ in simple unicellular organisms such as the budding yeast *Saccharomyces cerevisiae*, which has already proven to be a powerful model system for studying DNA repair, might contribute to a better understanding of the process in complex multicellular organisms. The present review is focused on NHEJ factors of *S. cerevisiae*. Its purpose is to comprehensively describe the functions of all the *S. cerevisiae* NHEJ components and to summarize knowledge about the process in this organism.

2. The *S. cerevisiae* non-homologous end-joining factors

2.1. *The synapsis factors of the* S. cerevisiae *non-homologous end-joining*

2.1.1. *The Yku70/80 complex*

The KU70/80 heterodimer is one of the most abundant DNA end-binding proteins in mammalian cells. It was originally identified as an autoantigen in patients with polymyositis-scleroderma overlap syndrome [50] and is now known to bind to a variety of discontinuities in double-stranded DNA (dsDNA), including single-stranded gaps and bubbles of non-complementarity. Its highest affinity, however, is to blunt, 5′ or 3′ overhanging and hairpin dsDNA ends [51,52]. At the site of DSB, KU70/80 acts as a bridging complex. In the end-to-end fusion process, KU70/80 functions as an alignment, recruitment and stimulating factor (Table 1) [53–57].

A yeast homologue of the KU70/80 autoantigen, Yku70/80 (also referred to as Hdf1/Hdf2) has been identified (Table 2) [35,37,39,58]. The amino acid sequence of Yku70 predicts a 70.647 kDa protein that shares significant homology with the human KU70 protein [35]. Similarly, Yku80, having a molecular weight of 71.240 kDa, displays a high degree of homology with its human counterpart [39]. As expected, the Yku70/80 heterodimer binds specifically to DNA ends in a sequence-independent manner [58,59], a function analogous to its mammalian equivalent.

Disruption of either the *YKU70* or *YKU80* gene affects mating-type switching and spontaneous mitotic recombination [38]. In addition, it leads to sensitivity to bleomycin [38,39] and methyl methanesulfonate (MMS) [39,58], agents which lead directly or indirectly to the induction of DSB [60]. Since yeast employs HR

Table 1
The mammalian NHEJ factors and their function in the NHEJ process

Factor	Structure/biochemical property/biological function/interacting partners in NHEJ	References
KU70/80	Crystal structure of the KU70/80 complex shows a dyad-symmetrical molecule with a preformed ring that encircles duplex DNA DNA ends-binding complex DNA-dependent ATPase ATP-dependent DNA helicase Protects DNA ends from degradation Important synapsis factor in NHEJ recruiting DNA-PKcs when bound to DNA ends As a part of DNA-bound DNA-PK complex recruits DNA ligase IV/XRCC4 complex to site of DSB Translocates along the DNA and thereby stimulates DNA ligase IV/XRCC4-mediated ligation Interacts with DNA-PKcs, DNA ligase IV and DNA polymerase μ	[31,52,55,56,81,184,185,197–211]
DNA-PKcs	3D structure shows head, palm and arm domains in the protein. The head encloses a cavity surrounded by protein density and two openings in the front of the molecule. Upon DNA binding, closure of the cavity between the head and palm domains occurs and the palm domain clips the enzyme onto DNA. This correlates with the DNA-PKcs kinase activity activation ATP-dependent serine/threonine protein kinase of the phosphatidylinositol 3-kinase family Binds to DNA ends as well as to DNA ends-bound KU70/80 Important synapsis factor in NHEJ Presumably possesses also regulatory function in NHEJ via phosphorylation of the downstream targets Interacts with KU70/80, ARTEMIS and XRCC4	[24,30,184–186,201–205,209,210,212–228]
ARTEMIS	Consists of the metallo-β-lactamase, metallo-β-lactamase-associated CPSF ARTEMIS Snm1/Pso2 (β-CASP) and C-terminal domains Possesses 5′ to 3′ exonuclease activity on its own; however, upon complex formation with, and subsequent phosphorylation by, DNA-PKcs it acquires endonucleolytic activity on 5′ and 3′ overhangs, as well as hairpins Involved in nucleolytic end-processing step of NHEJ Interacts with DNA-PKcs	[24,191,229,230]
XRCC4	XRCC4 constitutes dimers and tetramers, the two forms that exist in an equilibrium. An XRCC4 monomer consists of a globular N-terminal head domain followed by a long helical C-terminal tail. Dimerization occurs via association of two head domains and the initial parts of helical tails. Tail to tail interaction between the two XRCC4 dimers, forming a four-helix packet, leads to tetramerization Crystal structure of DNA ligase IV-XRCC4 complex reveals that a single DNA ligase IV molecule interacts asymmetrically with one XRCC4 dimer. In the complex, the two helical tails of the XRCC4 dimer remain in contact with each other over their entire length. In two specific regions they form a left-handed, parallel coiled coil, a platform to which DNA ligase IV adheres Binds to DNA cooperatively Modulates stability and activity of DNA ligase IV Targets DNA ligase IV to site of DSB Interacts with DNA ligase IV and DNA-PKcs	[25–27,148–151,201,202,216,231–234]
DNA ligase IV	For details on DNA ligase IV/XRCC4 structure, see row above Contains an ATP-dependent DNA ligase domain and two BRCT domains ATP-dependent double-stranded DNA ligase Binds to DNA in complex with XRCC4 Interacts with XRCC4 and KU70/80	[26,27,55,149–151,201,231,235,236]

to repair DSB under most circumstances, loss of Yku70 and Yku80 activity significantly hypersensitizes yeast cells to ionising radiation (IR) only when the HR is debilitated [37,59]. Consistent with this are the findings showing normal DSB repair and cell cycle arrest after IR exposure in the *yku70* mutant strain [59]. Similarly to the IR sensitivity, the MMS sensitivity of the *rad52* mutant strain is elevated significantly when either the

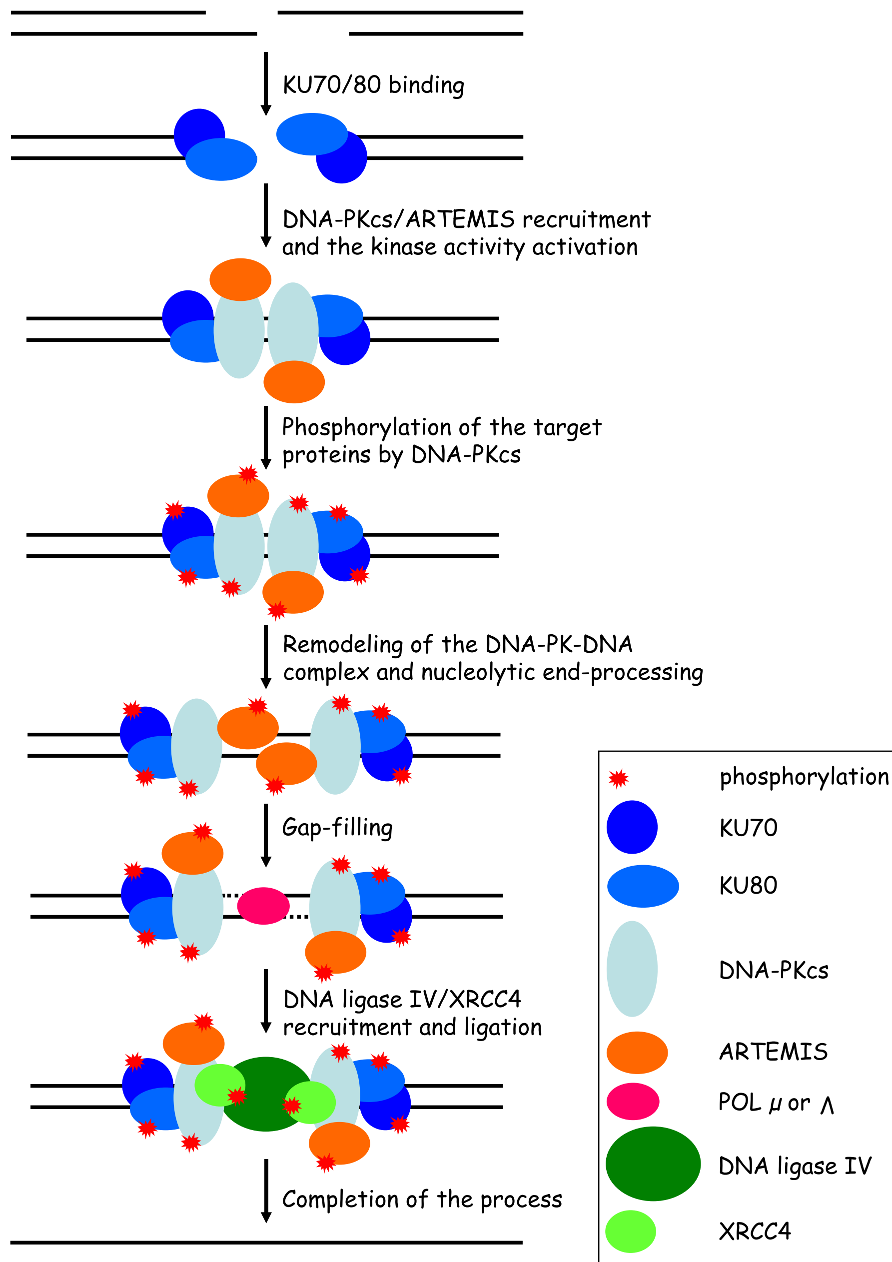

Fig. 1. A simple model of the mammalian NHEJ. Following DSB formation, broken DNA ends are firstly bound by the KU70/80 heterodimer and subsequently brought to the proximity and aligned by the DNA-dependent protein kinase (DNA-PK), which is reconstituted at the site of the DSB after recruitment of its catalytic subunit (DNA-PKcs) by DNA-bound KU70/80. This step is referred to as synapsis. As a functional consequence of interaction, DNA-PKcs brings ARTEMIS to the broken DNA ends. After associating with DNA-bound KU70/80, DNA-PKcs kinase activity, triggering several phosphorylation events, is activated. Autophosphorylation of DNA-PK presumably causes remodelling of the DNA-PK–DNA end complex, resulting in accessibility of DNA ends to further processes without disrupting synapsis. Phosphorylation of ARTEMIS triggers nucleolytic DNA ends-processing, followed by gap-filling, a step mediated most likely by DNA polymerase μ or λ. The process is completed by simple ligation carried out by the DNA ligase IV/XRCC4 complex.

YKU70 [38] or *YKU80* [37] genes are inactivated. The hypersensitive phenotype of the *yku70 rad52* double mutant was also observed after exposure to bleomycin [38]. In contrast, inactivation of *YKU70* or *YKU80* does not result in any detectable increase in sensitivity towards agents such as ultraviolet (UV) light [37] and hydroxyurea (HU) [59].

The *yku70* or *yku80* disruption mutant also shows a temperature-sensitive phenotype for growth at 37 °C [35–37,59,61]. Interestingly, strains lacking Yku70/80 do not die immediately upon transfer to the restrictive temperature but continue dividing for several generations [61]. After several hours, cells arrest growth and appear as enlarged single-budded cells with abnormally

Table 2
The *Saccharomyces cerevisiae* NHEJ factors and phenotype of the corresponding mutants

Yeast factor	Mammalian homologue	Mutant phenotype
Yku70/80	KU70/80	Sensitivity to bleomycin and MMS No UV and HU sensitivity IR sensitivity observed only when coupled with a *rad52* mutation Normal IR-induced cell cycle arrest Normal DSB repair after IR exposure at chromosomal level Mating-type switching and spontaneous mitotic recombination defect No growth defect at 30 °C No growth at 37 °C No gross chromosomal rearrangements Telomere length shortening No silencing at telomeres Defect in plasmid repair assay
Mre11/Rad50/Xrs2	MRE11/RAD50/NBS1	Sensitivity to many DSB-inducing agents Impaired meiosis Reduced integration of transforming DNA Telomere length shortening No silencing at telomeres Poor mitotic growth Defective checkpoint activation Defect in plasmid repair assay and in assay that measures NHEJ at the chromosomal level Elevated rates of spontaneous mitotic recombination Defects in HR Premature senescence Gross chromosomal rearrangements
Lig4/Lif1	DNA ligase IV/XRCC4	No sensitivity to IR and MMS of dividing cells; a slight sensitivity to these agents of non-dividing cells No obvious growth defects at 30 °C Growth at 37 °C could be affected (for details, see Section 2.3) No telomere length shortening No gross chromosomal rearrangements Meiosis could be affected (for details, see Section 2.3) Defect in plasmid repair assay
Nej1	–	Defect in plasmid repair assay and in an assay that measures NHEJ at the chromosomal level No UV and MMS sensitivity No growth defect at 30 and 37 °C No defect in postdiauxic/stationary-phase stimulation of NHEJ
Pol4	DNA polymerase μ or λ	No UV, MMS, EMS, MNNG, IR and H_2O_2 sensitivity, although a weak sensitivity to MMS and IR was observed in another study (see text for details) No mitotic growth defect No sporulation nor spore viability defect Normal levels of spontaneous mitotic recombination Increased levels of meiotic recombination Normal levels of UV-induced recombination and mutagenesis No chromosome loss Increased frequency of illegitimate mating
Rad27	FEN-1	Increased sensitivity to MMS, EMS, MNU and ENU Moderate UV, H_2O_2 and IR sensitivity Increased spontaneous and UV-induced mutagenesis Increased plasmid loss Elevated short-sequence recombination No growth defects Temperature-sensitive phenotype Premature aging Instability of telomeric repeats Defects in Ty1 mobility Gross chromosomal rearrangements Viability dependent on HR proteins, but not on those involved in NHEJ

For references, see text.

high DNA content, indicating a defect in the regulation of DNA replication coupled with, or causing, an arrest in G_2 phase of the cell cycle [35,61]. The *yku70 yku80* double mutant displays no additional growth defects. This temperature sensitivity cannot be complemented by the expression of either the single subunits or the human KU70/80 heterodimer. Yku70/80 or KU70/80 corresponding DNA binding activity is not detectable in the *yku70*- or *yku80*-deficient strains transformed with plasmids expressing human KU70 or KU80, respectively. Thus, Yku70 and KU80 or Yku80 and KU70 cannot form functional heterodimers. Furthermore, at the permissive temperature, the *yku70* or *yku80* mutant strain has greatly shortened telomeres, corresponding to loss of ~65% of the $C_{1–3}A$ terminal telomeric repeat sequences [37,62–64]. Strikingly, whereas telomere length is not affected when wild type strains are incubated at 37 °C, the transfer of the *yku70* or *yku80* mutant strains to 37 °C leads to a further dramatic loss of telomeric repeats. Therefore, it appears that the death of Yku70/80-deficient yeast strains at 37 °C is a consequence of the loss of telomeric repeats [65].

Since cells expressing a functional Yku70/80 can precisely join cohesive ends of a transformed linearized plasmid, while cells deficient for one of the Yku70/80 subunits display reduced recircularization efficiency and an increased frequency of imprecisely joined products, Yku70/80 is considered to be an essential part of the *S. cerevisiae* NHEJ [36,37,58,64]. The recircularization efficiency (a measure of the ability of yeast cells to repair restriction enzyme generated DSB in vivo) can be established using the transformation-based plasmid repair assay. In this assay, a *S. cerevisiae* strain is transformed with a yeast – *E. coli* shuttle plasmid that has been linearized by treatment with a restriction enzyme. To normalize for differences in transformation efficiency between strains and between repeats of the same experiment, a supercoiled version of the same plasmid is transformed into the yeast strain in parallel. Since the plasmid must be recircularized in order to be propagated, the number of transformants obtained with the linear plasmid normalized to the number obtained with the supercoiled plasmid provides a quantitation of the ability of the yeast strain to mediate repair of the restriction enzyme-generated DSB. To prevent the DSB from becoming repaired by HR with the yeast genome, the sites for restriction enzyme cleavage of the plasmid are within regions that are not homologous to chromosomal sequences. DSB with cohesive ends are repaired with high efficiency in wild type yeast strains in that transformant yields are over 70% of the values obtained with supercoiled plasmid. In marked contrast, strains debilitated in *YKU70* or *YKU80* show a dramatic 40–100-fold decrease in transformants recovered with linearized DNA. This is not affected by the presence or absence of Rad52. Expectedly, strains mutated in both *YKU70* and *YKU80* are no more impaired in plasmid repair than strains mutated for either *YKU70* or *YKU80* alone. Additionally, in Yku70/80-deficient cells, plasmids are not repaired accurately but in an error-prone way, yielding molecules that underwent losses of up to several hundred base pairs at the joining site, with junctional overlapping sequences of 3–15 bp. Surprisingly, Yku70/80 does not play a positive role in the rejoining of plasmid molecules bearing blunt DNA termini [37].

As mentioned above, inactivation of *YKU70* [62,64] or *YKU80* [65] leads to telomeric shortening, showing that Yku70/80 plays a crucial role in telomere length maintenance. Moreover, Yku70/80 represses the transcription of RNA polymerase II genes in close proximity to telomeres, a process called telomere position effect (TPE) [66]. In these end-regions of chromosomes, the chromatin is in a unique condensed structure that does not permit access by the transcriptional apparatus and the introduced gene is, therefore, silent [67,68]. If the telomeric chromatin structure is then disrupted, telomeric silencing is relieved, allowing the gene to be expressed, and telomeric silencing is lost [65,69,70]. TPE is severely diminished in Yku70/80-deficient cells, although the repression of the silent mating type loci, at an internal chromosome site, is maintained normally in the same cells [65,70,71], indicating that silencing in general is not affected [69]. The association of Yku70/80 with telomeric silencing is in accord with the fact that Yku70/80 binds directly to telomeric DNA [71,72] and that Yku70 interacts with Sir4 [73]. Yku80 also interacts with Sir4 and this interaction is mediated by the C- and N-terminal regions of Yku80 and Sir4, respectively [74]. Sir4 is constitutively bound to Sir2, forming a Sir2/Sir4 complex, but the Sir3 association with this complex is prevented by the intramolecular interaction within Sir4 [75]. At telomeres, Yku80 interacts with the N-terminus of Sir4, thereby inducing a conformational change within Sir4 that facilitates the recruitment of Sir3 to the Sir4 C-terminus [74]. Therefore, it appears that Yku70/80 following binding to telomeric DNA ends and, through its interaction with Sir4, helps to recruit the Sir2/Sir3/Sir4 complex to the telomere [65,73]. Moreover, the Yku80/Sir4 interaction is likely to play a vital role in the assembly of telomeric heterochromatin, and thus in the establishment of TPE [74]. The association of Yku70/80 with TPE is consistent with studies in vertebrate systems, which has revealed that KU70/80 is a potent inhibitor of transcription [76,77].

The dual requirement of Yku70/80 at site of DSB, where it promotes end-to-end fusion, and at telomeres, which are specifically protected from end-joining, presents a paradox. A possible resolution is that Yku70/80 performs different activities at these two different classes of DNA ends. Moreover, Yku70/80 has probably multiple separable functions at the telomere. It may be that

Yku70/80 associates with subtelomeric chromatin, where it influences the formation of heterochromatin. Independently, Yku70/80 probably associates with the chromatin terminus, where it mediates telomere length regulation via interactions with telomerase and telomere end protection via inhibition of an end-processing activity [57]. However, prior support for separable functions for Yku70/80 has come from the identification of the C-terminal truncation mutants. One mutation in Yku70, a deletion of 30 C-terminal amino acids (Yku70-c30), that abolishes DNA-binding activity, causes a Yku70 deletion phenotype; telomeres are very short, displaying G-tails, and NHEJ is not functional. On the other hand, when the C-terminal 25 amino acids of the Yku70 protein are deleted (Yku70-c25), DNA-binding capacity is indistinguishable from the wild type protein and NHEJ is fully functional. However, these same cells still display shortened telomeres and clearly detectable ssDNA overhangs of the 3′-ends. Taken together, deletion of only 5 amino acids more from the Yku70-c25 construct completely abolishes DNA-binding of the Yku70/80 complex and renders it non-functional. Thus, the extreme C-terminal domain of Yku70 is probably specifically involved in maintaining telomere integrity, but not in DNA-binding or end-joining activity. Interestingly, the terminal 25 amino acids of Yku70 contain 8 lysine residues. Deletion of 3 of these lysine residues in Yku70 with 9 amino acids deleted causes a weak decrease in telomere length when compared with wild type cells. This decrease is more pronounced in Yku70 with deletion of 20 amino acids, where 6 of the 8 lysine residues are deleted. All 8 lysine residues are removed in the Yku70-c25 mutant, and shortening of telomeres is almost as severe as observed in the *yku70*-deficient strain. Thus, this lysine-rich domain at the C-terminus may be important for interacting with yet unidentified protein(s) [72].

As stated above, Yku70/80 is associated with the ends of chromosomes in vivo [71,78,79]. Since there is enhanced degradation of broken chromosomes in the absence of Yku70/80, a role for Yku70/80 in protection of DNA ends from nucleolytic processing was proposed [80]. Such a role is in line with crystal structure of KU70/80 (Table 1), since this heterodimer encircles the duplex DNA like the thread of a screw [81]. Furthermore, Yku70/80 appears to be also involved in the clustering of yeast telomeres at peripheral sites in the nucleus because mutations in the *YKU70* or *YKU80* gene affect the subnuclear organization of yeast telomeres and thus, abolish the clustered distribution of telomeric foci. In wild type diploid yeast cells, the 64 telomeres are usually found in 6 or 7 clusters around the nuclear periphery, whereas cells mutated in either Yku70/80 subunit have around 9 clusters that seem to be located more randomly throughout the whole nucleus [69]. Taken together, it appears that Yku70/80 is somehow involved in clustering the telomeres of several chromosomes and tethering them at sites in the nuclear periphery [51].

The *TEL1* gene encodes a protein that shares homology with phosphatidyl-inositol-3 family of protein kinases and therefore could represent a DNA-PKcs equivalent in *S. cerevisiae* [82]. Interaction between Tel1 and Yku70/80 was examined by comparing the phenotype of the *tel1 yku70* or *tel1 yku80* double mutant with the phenotype of the respective single mutants. Such analysis showed different roles of Tel1 and Yku70/80 in several cellular processes [39,62,65]. Moreover, the phenotype of the *yku70* or *yku80* mutant significantly differs from that of the *tel1* mutant for TPE, repair of plasmid-based DSB, and growth and further dramatic loss of telomeric repeats at 37 °C [65], strengthening the notion that Tel1 and Yku70/80 operate independently from one another and, accordingly, that Tel1 is not an essential component of the *S. cerevisiae* NHEJ and does not represent a functional homologue of the DNA-PKcs in this organism. Indeed, Tel1 is probably the *S. cerevisiae* ATM homologue (for further details, see Section 5) [83].

2.1.2. The Mre11/Rad50/Xrs2 complex

In contrast to mammalian systems, where satisfactory evidence for the involvement of the MRE11/RAD50/NBS1 complex in NHEJ is still missing, the genetic and biochemical studies carried out in *S. cerevisiae* unequivocally implicate the Mre11/Rad50/Xrs2 complex, a yeast homologue of MRE11/RAD50/NBS1, in NHEJ [58,65,84–86]. All individual components of the Mre11/Rad50/Xrs2 complex were cloned and shown to encode 692, 1312 and 854 amino acid proteins with a molecular weight of 77.650, 152.568 and 96.364 kDa, respectively [87–90]. By comparing their sequences with those of mammals, it has been revealed that while human MRE11 and RAD50 represent the clear structural and functional homologues of their yeast counterparts, NBS1 is a functional rather than a structural analogue of Xrs2 [91–95].

Stoichiometry of the purified Mre11/Rad50/Xrs2 complex is 2:2:1 [86]. The complex possesses Mn^{2+}-dependent 3′ to 5′ dsDNA and ssDNA endonuclease, ssDNA exonuclease and hairpin cleavage activities, all of which are specified by four phosphoesterase motifs residing in the N-terminal part of Mre11 [64,86,96–99]. Moreover, the complex binds and hydrolyzes ATP via Rad50 [100,101]. ATP binding and hydrolysis was shown to enhance nuclease activity of the complex in vitro [99] in agreement with in vivo findings that a mutant allele of the *RAD50* gene that prevents ATP binding confers a null phenotype with respect to DNA repair [100]. Finally, Mre11/Rad50/Xrs2 possesses a DNA-binding activity, which is, interestingly, exhibited by all the individual subunits separately [96,98,99,102].

It has been shown that Rad50 contains Walker A and B motifs in the N- and C-terminal parts of the protein, respectively, which are responsible for ATP binding and hydrolysis. These motifs are separated by a long coiled-coil region, in the center of which, a hinge motif possessing a sequence Cys–X–X–Cys (CXXC), is present. Crystal structure of Rad50 reveals that CXXC motif promotes Zn^{2+}-dependent dimerization of Rad50, as the two CXXC motifs form interlocking hooks that bind one Zn^{2+} ion. The functional importance of the CXXC motif for the whole complex assembly and DSB repair has clearly been demonstrated in vivo using strains that were mutated individually in the two cysteine residues. These strains were IR sensitive and display impaired Mre11-binding, demonstrating that the structure of the Rad50 dimer has consequences for the structure and biological function of whole complex. Coiled-coil regions extend from the Zn^{2+}-binding site in opposite directions and this allows them to link two distinct Mre11 dimers. The Mre11 dimer binds to the coiled-coils of two Rad50 molecules adjacent to the ATPase domain, forming a globular head, which is responsible for the DNA-binding and/or DNA end-processing activities of the complex. Hence, one Mre11/Rad50 heterodimer possesses two DNA-binding and end-processing active sites. These two active sites could bind two separate broken DNA ends at a time and, subsequently, could act as a bridging factor during NHEJ [103–108] (see text below for further details).

Participation of the Mre11/Rad50/Xrs2 complex in NHEJ in the budding yeast stemmed from the findings that showed 100-fold reduced frequency of integration of transforming DNA that shares no homology with the host genome in the *rad50* mutant [109]. Moreover, monocentric [85] and dicentric [110] plasmid systems developed for quantitative analysis of a deletion formation as a consequence of NHEJ disclosed 10- and 50-fold defects, respectively, in strains debilitated in Mre11/Rad50/Xrs2. Further in vivo experiments [58,65,84], exploiting an HO endonuclease-induced chromosomal DSB assay and a transformation-based plasmid repair assay (see Section 2.1.1), definitively established a role of the Mre11/Rad50/Xrs2 complex in the *S. cerevisiae* NHEJ. Plasmid repair assays showed that strains, from which *MRE11*, *RAD50*, or *XRS2* was deleted, exhibited a dramatic (up to 40-fold) drop in NHEJ compared to the wild type strain [58,64,65] and that the *mre11 yku70*, *rad50 yku70*, *xrs2 yku70*, *mre11 lig4*, *rad50 lig4* or *xrs2 lig4* double mutants were not appreciably more debilitated in NHEJ than were the single mutants [64,65]. These data therefore provided clear in vivo evidence for the involvement of Mre11/Rad50/Xrs2 in NHEJ. Moreover, they indicated that Mre11/Rad50/Xrs2 functions epistatically with Yku70/80 and Lig4/Lif1 in NHEJ. When the nature of the residual plasmid repair events taking place in the *mre11*, *rad50* and *xrs2* mutants was analyzed, it was found that virtually all NHEJ products arising in these mutants were accurate [65]. This is in sharp contrast with the situation for Yku70/80-deleted strains (see Section 2.1.1), indicating different roles for Yku70/80 and Mre11/Rad50/Xrs2 in NHEJ. Furthermore, the HO endonuclease-induced chromosomal DSB assay has revealed that there are distinct mechanisms of NHEJ producing either insertions or deletions and that these two pathways are differently affected by cell cycle stage when HO is expressed. This assay uses *MATα* strain, which lacks *HML* and *HMR* donor sequences and carries the *URA3*-based plasmid pGAL-HO, in which the *HO* gene is under the control of a galactose-inducible promoter. Upon galactose induction, a high level of *HO* expression and subsequent HO cleavage throughout the cell cycle can be achieved. Importantly, DSB repair in *MATα* locus by HR is prevented in this strain because of the *HML* and *HMR* deletions, so that virtually all repair events scored are due to either insertions or deletions arising during NHEJ. As demonstrated, the overall NHEJ efficiency of this strain was dramatically reduced (up to 70-fold) by inactivation of the *RAD50*, *MRE11* or *XRS2* genes. Moreover, inactivation of these genes markedly reduced insertions, while significantly increasing the proportion of deletions [84]. Thus, the Mre11/Rad50/Xrs2 complex exerts an important role in the insertion-producing pathway of NHEJ and this tempts speculation that there might be a physical link between synapsis and gap-filling in NHEJ (for details on the NHEJ gap-filling factors, see text below).

In vivo NHEJ assays mentioned above [65,84,85,110] suggested that the nuclease activity of the Mre11/Rad50/Xrs2 complex may not play a role in NHEJ and that the function of this complex may rather be to bring together broken DNA ends and/or to recruit other NHEJ factors. To support this, it has been shown that two strains, each expressing different nuclease-defective Mre11 protein, were not deficient in the plasmid repair assay [64,96] and that overexpression of Exo1, 5′ to 3′ exonuclease exhibiting also flap endonuclease activity, failed to rescue NHEJ deficiency of an *mre11* mutant [111]. Furthermore, recent biochemical data [86] strongly supported this notion by demonstrating that Mre11/Rad50/Xrs2 serves as an end-bridging factor in NHEJ that links DNA ends and provides the scaffold upon which the NHEJ machinery is assembled. No nuclease activity of Mre11/Rad50/Xrs2 was required for this function. In vitro experiments further showed that Mre11/Rad50/Xrs2 interacts with (interaction between Mre11/Rad50/Xrs2 and Lig4/Lif1 is mediated via Xrs2 and Lif1, respectively) and specifically stimulates intermolecular ligation by Lig4/Lif1, a process that is additionally stimulated by Yku70/80.

As is the case with strains deficient in Yku70/80, those debilitated in Mre11/Rad50/Xrs2 also suffer dramatic telomeric attrition, losing approximately 65% of

the terminal repeats at permissive temperature. Since no further telomere shortening was observed in the *mre11 yku70*, *rad50 yku70* or *xrs2 yku70* double mutants under these conditions, Yku70/80 and Mre11/Rad50/Xrs2 function epistatically in telomere length maintenance [64,65]. Notably, shifting of the *yku70* or *yku80* mutant strain from permissive to restrictive temperature led to a further dramatic loss of telomeric repeats, presumably representing the mechanism by which these mutant strains die at 37 °C (see Section 2.1.1). Surprisingly, a further striking reduction in telomere length upon transfer of strains disrupted for any of the Mre11/Rad50/Xrs2 subunit to 37 °C could be detected only in the *rad50* mutant, which correlates with severely impaired growth of this mutant at the restrictive temperature [65]. The reason why the *mre11* and *xrs2* mutants do not show the same growth defects and telomere shortening at the restrictive temperature is unknown, but this might indicate that there are also separate functions for each individual subunit of Mre11/Rad50/Xrs2. Similarly, the lack of observable NHEJ defects displayed by the nuclease-defective *mre11* mutants (see text above) was paralleled by the lack of any telomere shortening in these mutants [64], although the other did have impaired telomere length maintenance [96]. The basis of this discrepancy is unknown and calls for further examination. As already mentioned, another phenotype related to telomeric DNA is transcriptional silencing of the genes in close proximity to telomeres, a process called TPE [66]. This process has been markedly diminished in the *yku70* or *yku80* mutants [65,70,71] (see Section 2.1.1). Astonishingly, TPE analysis in the *mre11*, *rad50*, or *xrs2* mutants showed no essential role of Mre11/Rad50/Xrs2 in this process [65]. This suggests partially separate functions of the Yku70/80 and Mre11/Rad50/Xrs2 complexes at the telomeres and provides evidence for a different composition of the protein complexes operating at these regions of the *S. cerevisiae* genome.

In addition to their abnormal phenotypes for NHEJ or telomere metabolism, cells debilitated in Mre11/Rad50/Xrs2 are defective in meiotic recombination, display premature senescence, suppression of gross chromosomal rearrangements, elevated rates of spontaneous mitotic recombination, and delayed mating-type switching ([64,65,89,96–98,112–116], reviewed in [6]) (Table 2). Importantly, they also display a remarkable defect in HR, the main DSB repair mechanism in the budding yeast. Part of the difficulty in identifying the role of Mre11/Rad50/Xrs2 in the particular DSB repair mechanism arises from the fact that several processes can affect the efficiency of HR and NHEJ in vivo, for example the loss of checkpoint activity in the budding yeast resembles the loss of the *MRE11/RAD50/XRS2* complex with regard to its effects on NHEJ. This raises the possibility that inactivation of the Mre11/Rad50/Xrs2 complex affects HR and NHEJ, at least in part, through defects in checkpoint functions [117–119].

2.2. The end-processing factors of the S. cerevisiae *non-homologous end-joining*

As shown in Fig. 1, DNA end-processing mediated by the nucleases and DNA polymerases may be required beyond simple ligation, particularly when the DNA termini are damaged or not fully compatible. NHEJ at such DNA ends proceeds via intermediates, the generation of which depends on the use of terminal microhomologies [120]. Microhomologies are short tracts of DNA sequence homology close to the break site that facilitate DNA ends alignment [121].

In *S. cerevisiae*, the gap-filling polymerase is likely to be Pol4, a member of the functionally diverse Pol X family of nucleotidyl transferases. Within this family, two mammalian DNA polymerases (DNA polymerase μ and λ) possess the same domain structure (BRCA1 carboxyl terminus – BRCT, lyase, and nucleotidyl transferase domains) as Pol4 [121] and function in the gap-filling step in NHEJ [31–34]; consequently, Pol4 and DNA polymerase μ or λ could have similar functions in the *S. cerevisiae* and mammalian NHEJ, respectively.

It has been shown that the *pol4* mutant displays no altered sensitivity to a variety of DNA damaging agents, including UV light, IR, MMS, ethyl methanesulfonate (EMS), *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) and hydrogen peroxide (H_2O_2) [122,123], although another study demonstrated its weak sensitivity towards MMS and IR [124]. Moreover, *pol4* mutation does not affect UV-induced recombination and mutagenesis, mitotic growth, sporulation and spore viability, spontaneous mitotic recombination and chromosome loss. In contrast, it confers increased levels of meiotic recombination, probably due to elevated levels of meiosis-specific DSB, and an increased frequency of illegitimate mating [122–124] (Table 2). A weak MMS and IR sensitivity suggested possible involvement of Pol4 in DSB repair. Therefore, further experiments specifically analyzed the DSB repair phenotype of the *pol4* mutant and showed that unlike the core NHEJ mutants such as *lig4* (see text below), the *pol4* mutant displays an essentially normal rate of religation of complementary ends in transformation-based plasmid repair assay (see Section 2.1.1) relative to the wild type strain, demonstrating that it does not have a generalized NHEJ defect. However, when specifically designed plasmid substrates with only single nucleotide microhomology were used, the *pol4* mutant showed a defect in rejoining of one type of junction, mixed joins. A defect of *pol4* cells in mixed joining was also demonstrated in HO endonuclease-induced chromosomal DSB assay. Therefore, it seems that Pol4 is required for NHEJ only if efficient gap-filling polymerization at mixed joins in conjunction with

the removal of 5′- and 3′-terminal mismatches is needed [120]. In vitro experiments investigating the DNA synthesis activity of Pol4 on different DNA substrates are in line with in vivo data [120,122]. In conclusion, the DNA substrate specificity of Pol4 corresponds to its role in NHEJ because small gaps associated with non-complementary flaps are predicted intermediates in a subset of DNA joining events, which involve microhomology-mediated alignment of the broken DNA ends [121]. To support a role of Pol4 in NHEJ, Tseng and Tomkinson [121] showed a direct interaction of N-terminal BRCT domain of Pol4 with the Lig4 subunit of the Lig4/Lif1 complex. This interaction stimulates the DNA synthesis activity of Pol4 and, to a lesser extent, the ligation activity of Lig4/Lif1, and thus couples two steps of the microhomology-dependent sub-pathway of NHEJ, gap-filling and ligation, in order to allow ligation to occur immediately upon completion of the minimum synthesis required to form a ligatable substrate.

As mentioned above, DNA polymerase μ also contains a BRCT domain [121]. Notably, this DNA polymerase associates with the DNA ligase IV/XRCC4 complex and stimulates its ligation activity [31], strengthening the notion that Pol4 and DNA polymerase μ could be the functional homologues in process of NHEJ, even though these two DNA polymerases are also employed by other DNA repair pathways and thus have different functions.

Another protein thought to be involved in the end-processing step of the *S. cerevisiae* NHEJ is Rad27 (FEN-1 in mammals), a structure-specific nuclease that possesses flap endonuclease and 5′ to 3′ exonuclease activities [125]. The *rad27* mutant cells are highly sensitive to MMS, EMS, methyl nitrosourea (MNU) and ethyl nitrosourea (ENU), but only moderately sensitive to UV light, H_2O_2 and IR [126–129]. Moreover, these cells display increased levels of spontaneous and UV-induced mutagenesis as well as plasmid loss events [127,128]. The *rad27* mutant is viable and shows no growth defects in case that HR, but not NHEJ, is not debilitated [128,130,131], suggesting that the DNA lesions that accumulate in *rad27* strain are processed by HR or that Rad27 could be an integral part of NHEJ, or its sub-pathway(s). Consistent with this, *rad27* cells display greatly inhibited completion of DSB repair [132]. In addition, the Rad27 protein plays a role in short-sequence recombination [133–140], instability of telomeric repeats [141], Ty1 mobility [142], gross chromosomal rearrangements [116], life span [143], temperature-sensitivity and, most importantly, processing of the Okazaki fragments during normal DNA replication (reviewed in [144,145]) (Table 2). Regarding NHEJ, genetic assays developed by Wu et al. [146] showed that the Rad27 protein acts at a subset of NHEJ events that require processing of 5′ flaps, in a manner independent upon the exonucleolytic processing that can also occur during NHEJ.

2.3. *Ligation factors of the* S. cerevisiae *non-homologous end-joining*

Lig4 and Lif1 are considered the *S. cerevisiae* counterparts of the mammalian DNA ligase IV and XRCC4, respectively, because Lig4 is an ATP-dependent DSB repair DNA ligase and Lif1 is its stabilizing, stimulating and targeting co-factor [40–44,147] (Table 2). In a similar manner to DNA ligase IV and XRCC4, Lig4 and Lif1 form a highly stable complex, in which a region between the two BRCT domains of Lig4 interacts with the central region of Lif1 [43,147]. The yeast complex consists of 944 and 421 amino acid subunits, with molecular weight of 108.514 and 48.259 kDa, respectively [40–44]. The crystal structure of the mammalian complex has been revealed and shows a 1:2 (DNA ligase IV:XRCC4) stoichiometry [26]. The complex possesses DNA-binding and ATP-dependent dsDNA ligase activities [148–151] (Table 1).

Inactivation of the *LIG4* or *LIF1* gene does not lead to increased sensitivity of dividing cells to agents inducing DSB such as IR and MMS. In non-dividing cells, however, it results in a slight sensitization toward these agents, a phenotype also observed in a strain, in which loss of Lig4/Lif1 is coupled with impaired HR (i.e. in *lig4 rad52* double mutant). This could be explained by a lack of homologous sequence (homologous chromosome or sister-chromatid) in non-dividing haploid cells. In such cells, homology-driven repair is impossible and therefore NHEJ may be required. This is consistent with the findings that showed that NHEJ efficiency is increased in stationary [152] and haploid cells [153] (see sections below) compared to exponentially and diploid cells, respectively. Notably, the *lig4* or *lif1* mutant is not UV sensitive [40–42,44]. Regarding cell growth, these mutants do not show any obvious growth defects at 30 °C, although growth at 37 °C could be affected. The temperature-sensitivity phenotype requires, however, re-examination, because growth defects at 37 °C were observed uniquely in a single study [40–44]. Since growth defects at 37 °C were linked to the loss of telomeric repeats in some NHEJ-deficient strains [65] (see Sections 2.1.1 and 2.1.2), investigating the telomere length maintenance at restrictive temperature in the *lig4* or *lif1* mutant should address whether or not these mutants are temperature-sensitive. Notably, it has already been found that telomere length shortening in these mutants at 30 °C is not affected, so that one can also expect the same phenotype at 37 °C. Nonetheless, Lig4/Lif1 does not seem to have an essential function in telomere length maintenance and in protection of telomere ends from degradation under normal physiological conditions [41,43,65]. Further discrepancy for meiotic defects of the *lig4* and *lif1* mutants has been noted by the Tomkinson [44] and the Lindahl groups [40], who showed that the *lig4* mutation has no effect on sporulation or that sporulation in the *lig4* strain is less efficient compared to the wild type, respectively.

The most striking phenotype of the *lig4* or *lif1* mutant is observed in a transformation-based plasmid repair assay (for details on assay, see Section 2.1.1) [40–44]. In this assay, disruption of the *LIG4* or *LIF1* gene results in a dramatic reduction in the ability to repair restriction enzyme-generated cohesive as well as blunt-ended DSB in plasmid DNA. When the types of the DSB repair events in the *lig4* or *lif1* mutant were analyzed precisely, correct religation of the plasmid represented only a small fraction of events. Therefore, in the absence of Lig4 or Lif1 plasmid-based DSB are repaired by error-prone mechanism. Notably, a significant fraction of repair products was generated mostly by Rad52-dependent HR process (gap repair, genomic integration and gene conversion), an estimate made using the *lig4 rad52* double mutant [40,41].

2.4. *Additional regulatory factor of the* S. cerevisiae *non-homologous end-joining*

Recently, an additional regulatory NHEJ gene has been revealed in *S. cerevisiae* [154–157]. Existence of this gene was already assumed from the findings showing that NHEJ process is controlled by a Mata1–Matα2-repressed component in *S. cerevisiae* [153,158]. Indeed, an additional regulatory NHEJ component, designated Nej1 (NHEJ regulator 1) [155–157] or Lif2 (Lif1-interacting factor) [154] (from now on referred to as only Nej1), has been shown to be encoded by a haploid-specific gene, expression of which is virtually undetectable in the presence of the Mata1–Matα2 repressor [155,157]. This is in agreement with the presence of a consensus-binding site for the Mata1–Matα2 repressor in the promoter region of the *NEJ1* gene [155,157]. The reason why NHEJ is enhanced in haploid cells via Nej1-dependent mechanism might be that these cells lack a homologous donor chromosome to repair DSB by HR during some phases of cell cycle.

The *NEJ1* gene encodes a protein of 342 amino acid residues with a molecular weight of 39.115 kDa [154]. There is no obvious domain structure within Nej1, except two putative transmembrane helices in the N-terminal part of the protein [155,157], and therefore it is not surprising that homology searches did not reveal any Nej1 homologue in other organisms [155]. However, Nej1 is highly conserved within the genus *Saccharomyces*: at least four other *Saccharomyces* species, namely *S. paradoxus*, *S. bayanus*, *S. mikatae* and *S. kudriavzevii*, contain an Nej1 orthologue ([159], http://www.yeastgenome.org), suggesting a genus specific regulation of NHEJ.

No obvious biochemical function has yet been assigned to Nej1. The Nej1 protein has been shown to interact physically with Lif1 [154–157,160], but not with any other NHEJ factor [155]. Nej1/Lif1 interaction is mediated by the C- and N-terminal parts of Nej1 and Lif1, respectively [154,156]. As a functional consequence of the interaction, Nej1 could target Lif1 into the nucleus, demonstrated by a comparison of the localization of Lif1 in the isogenic wild type and *nej1* mutant cells [157]. However, this would require further examination since the Åström group [155] showed no role of the Nej1 protein in regulating the stability or localization of Lif1.

At present, it is clear that Nej1 functions in promoting DSB repair by NHEJ because the *nej1* mutant displays a defect in plasmid repair assay (see Section 2.1.1) [155–157]. Furthermore, the mutant is defective in HO endonuclease-induced chromosomal DSB assay (see Section 2.1.2) [84,154,155]. In contrast, it is not sensitive to UV light and MMS [157], shows no obvious growth defect at 30 °C and 37 °C [154] and is not affected in postdiauxic/stationary-phase stimulation of NHEJ. Postdiauxic/stationary-phase stimulation of NHEJ means that NHEJ efficiency is consistently induced several fold when cells left the exponential phase of fermentative growth and made the transition to respiratory metabolism and stationary phase [160].

Recently, another role of Nej1, antithetical to one in promoting end fusion at the site of DSB, has been revealed: Nej1 protects telomeres from NHEJ-dependent end fusions in the absence of telomerase [162]. This implies a possible involvement of Nej1 in maintenance of genomic stability in *S. cerevisiae*, since end fusions at telomeres result in dicentric chromosomes and classic breakage-fusion-breakage cycles [163].

3. Non-homologous end-joining versus homologous recombination: what can *S. cerevisiae* tell us?

Eukaryotic cells can repair DSB by HR and NHEJ (reviewed in [1–3,6,164–167]). The process of HR involves a resection of the 5′ ends at a DSB to create 3′ nucleoprotein filaments, followed by strand exchange with a homologous donor duplex, synthesis from the broken 3′ termini, and ultimately resolution of the extended D-loop [6,168]. In the absence of a donor duplex and in the presence of tandem repetitive sequences flanking the break, the direct annealing of the two resected 3′ ends can be catalyzed, creating a deletion in an alternative pathway of HR, known as single-strand annealing (SSA) [169–171]. In contrast, DSB repair by NHEJ represents a mechanism that does not require extensive sequence homology [4,7–14].

The utilization of either NHEJ or HR can be regulated by the nature of the DSB and by cell type. In *S. cerevisiae*, NHEJ is efficient at rejoining DNA ends with cohesive overhangs but not blunt ends or ends with noncohesive overhangs, which must be left to HR [36,120,154,158,172,173]. Regarding cell type, NHEJ and HR are both active in haploid cells, as opposed to diploids, where NHEJ is suppressed by a repression of Nej1 as a consequence of the *MAT*a/*MAT*α mating-type (for details,

see Section 2.4) [153–155,157,158,161]. Furthermore, cell cycle and growth phase can influence DSB repair, favoring NHEJ in G_1 and postdiauxic/stationary phase [152]. This is in very good agreement with data obtained for vertebrate cells [174,175]. This suggests that favoring NHEJ and HR in cells lacking and possessing a homologous donor sequence to repair DSB, respectively, is conserved from unicellular to multicellular organisms.

To explore the basis of pathway utilization more thoroughly, Karathanasis and Wilson [152] developed the chromosomal assay, called suicide deletion assay, in which the coding sequence of the I-*Sce*I endonuclease was placed under the control of the *GAL1* promoter and inserted into the *ADE2* gene just downstream of its start codon. Two I-*Sce*I cleavage sites flank the expression cassette, which also includes a *URA3* marker gene to allow for positive selection and maintenance of the allele. On transfer to galactose medium, the induced I-*Sce*I endonuclease is expected to cleave both sites, resulting in a 3.3-kb interstitial deletion of the chromosome and concomitant loss of the fragment containing the I-*Sce*I expression cassette. The result is thus immediate termination of I-*Sce*I expression; thereby ending the futile cleavage cycle that otherwise prevents detection of simple religation events. Outside of the I-*Sce*I cleavage sites, the allele configuration mimics the plasmid repair assay [152], i.e., with *ADE2* status indicating whether repair occurred by NHEJ via a 4-bp 3′ overhangs or SSA via a 28-bp terminal direct repeat. By using this assay, it has been observed that the mutation of the *RAD52* gene decreased SSA by 16-fold, but led to no corresponding increase in NHEJ. Conversely, mutation of either *YKU70* or *LIG4* gene led to the expected large decrease in NHEJ efficiency but no apparent increase in SSA. Interestingly, mutation of *YKU70* or *RAD52* not only did not enhance the remaining pathway, but in fact led to a mild 1.5-fold decrease in SSA [152] and NHEJ events [152,176], respectively. These results show that a simple competition model [158,177] between NHEJ and HR (represented by SSA) does not seem adequate to account for DSB repair pathway utilization [152].

Further insight into pathway utilization has been obtained by Frank-Vaillant and Marcand [178] who examined in vivo stability of dsDNA ends and the balance between NHEJ and HR. The stability of an unprocessed DNA ends generated by the HO endonuclease in the budding yeast is surprisingly high with a half-life of about 2 h in cells blocked in G_1 or in G_2 and about 1 h in cells growing exponentially with a doubling time of 4 h. Afterwards, the 5′ strand of DNA ends generated by the HO endonuclease is progressively degraded, producing long 3′ ssDNA tails [179,180]. In vivo, Yku70/80 interacts with intact DNA ends, as opposed to the Rad52 protein that interacts with processed DNA ends (Rad52 loading on DNA is coincident or posterior to the initiation of the 5′ resection). Thus, the 5′ strand processing suppresses NHEJ but not HR [178] that is initiated by arising 3′ ssDNA tails [164]. An estimation of the 5′ to 3′ resection rate is approximately 4 kb/h, i.e., about 1 nt/s, in exponentially growing cells [181]. In the absence of Yku70, the overall 5′ resection rate is about 2-fold faster [80], suggesting that Yku70/80 lowers the 5′ processing velocity but does not affect the initiation of this processing [178]. In contrast, if either *RAD50* or *MRE11* are inactivated, this rate is decreased [80,180]. Moreover, since entry into S phase destabilizes the ends of a DSB created in G_1, it is possible that DNA replication through the ends induces the initiation of the 5′ resection, thereby suppressing DSB repair by NHEJ and forcing repair by HR. Ultimately, it appears that NHEJ precedes HR temporally, and that the availability of substrate dictates the particular pathway used [178]. The fact that NHEJ acts before HR might be conserved through evolution. However, since NHEJ in mammals accommodates cohesive and non-cohesive ends and thus could overlap and compete with HR on partially resected ends [182], the situation in higher eukaryotes is presumably more complex and the transition from NHEJ to HR may not be as sharp as in *S. cerevisiae* [178].

4. Current model of the *S. cerevisiae* non-homologous end-joining

Based on existing genetic and biochemical data (cited above), a current model for the *S. cerevisiae* NHEJ is presented (Fig. 2(a)). The first NHEJ component to bind to a DSB is Yku70/80 heterodimer. Binding of Yku70/80 to a DSB presumably protects the broken DNA ends from nucleolytic degradation and marks the damaged site in order to be recognized by further NHEJ components. Consistent with the later, the Mre11/Rad50/Xrs2 complex is subequently recruited to the site of DSB. This complex mediates DNA end-bridging and, in combination with Yku70/80, targets the Lig4/Lif1/Nej1 complex to the DNA ends. The NHEJ process is completed by DNA end-joining mediated by the combination of Lig4/Lif1/Nej1 and Mre11/Rad50/Xrs2, a reaction which is further stimulated by Yku70/80. In case that the DNA ends are damaged or not fully complementary, DNA end-processing reactions are likely to be required (Fig. 2(b)). End-processing reactions generate ligatable structure and therefore must be carried out prior DNA end-joining. They are dependent on the sequence homology surrounding the break site, as sequence homology facilitates alignment of DNA ends. A NHEJ sub-pathway that requires DNA end-processing proceeds basically the same way as that already described and presented (see text above and Fig. 2(a)). The only difference is the involvement of the nucleolytic and gap-filling factors, which are likely to be Rad27 and Pol4, respectively. Once DNA end-processing is completed, NHEJ proceeds by DNA end-joining.

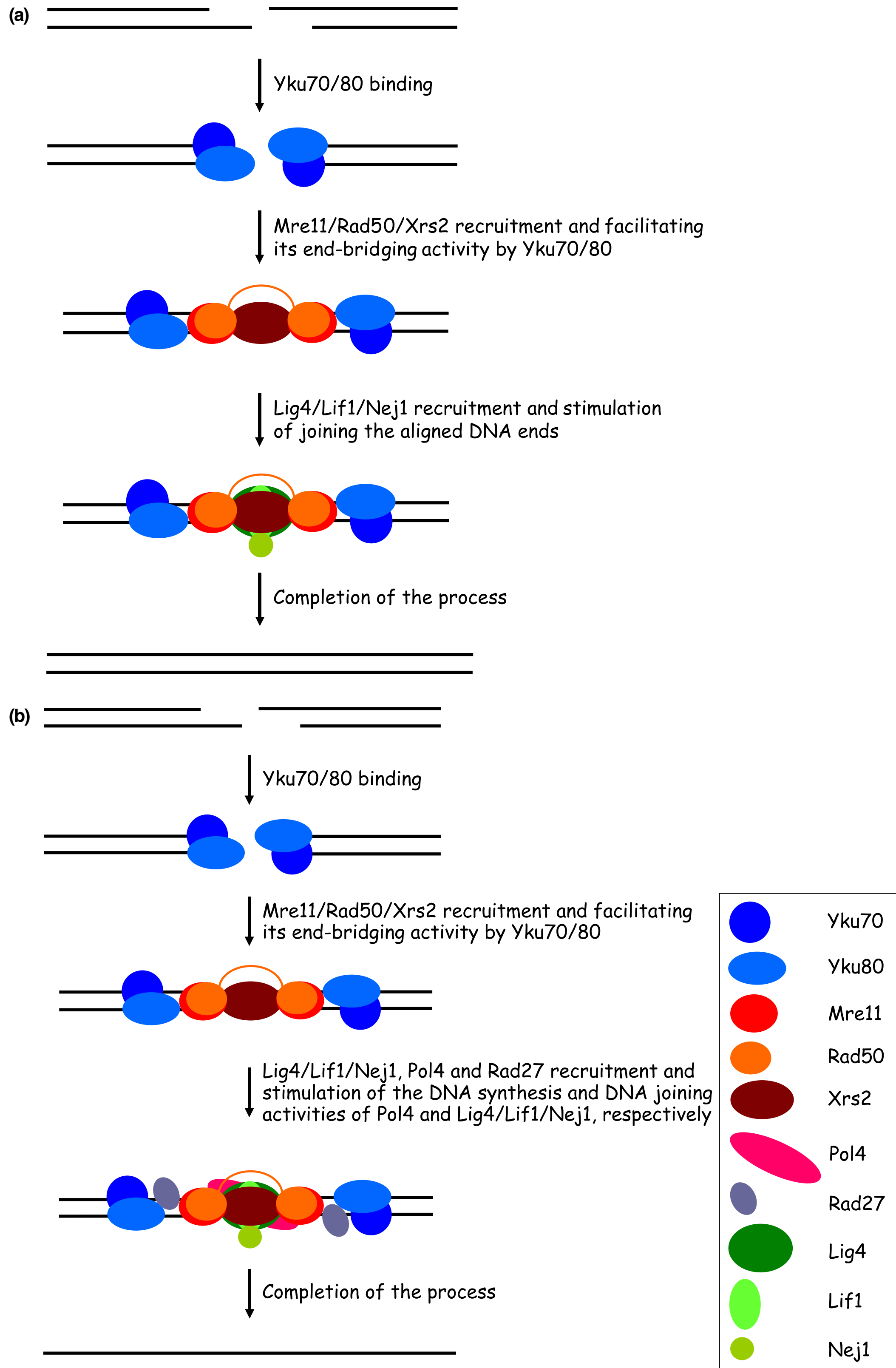

Fig. 2. Current model of the *Saccharomyces cerevisiae* NHEJ. (a) *Simple religation*. In a similar manner to the process of mammalian NHEJ, Yku70/80 heterodimer is the first NHEJ factor to bind to broken DNA ends. Subsequently, DNA-bound Yku70/80 recruits the Mre11/Rad50/Xrs2 complex and facilitates its end-bridging activity. This activity enables Lig4/Lif1/Nej1 complex to join Yku70/80-bound DNA ends. (b) *DNA-ends-processing-dependent NHEJ*. If broken DNA ends are non-complementary, DNA end-processing reactions, generating ligatable structure and occurring prior simple ligation, are required. These reactions include nucleolytic end-processing and gap-filling mediated by Rad27 and Pol4, respectively.

5. Conclusions

It is clear now that NHEJ has been conserved throughout evolution and operates from bacteria to man, although contribution of this pathway to DSB repair significantly depends on the particular organism [35–49]. Nevertheless, investigating NHEJ in simple unicellular organisms, particularly in the budding yeast, might considerably contribute to a better understanding of the process in complex multicellular organisms, because the core NHEJ factors, such as KU70/80 and DNA ligase IV/XRCC4 (Yku70/80 and Lig4/Lif1 in *S. cerevisiae*, respectively) [35–44], highly preserved their basic functions and structures.

In *S. cerevisiae*, Yku70/80 is the first protein that binds to the DNA ends, subsequently attracting other NHEJ proteins and/or complexes. The next factor to reach the broken DNA ends is presumably Mre11/Rad50/Xrs2, recruitment of which could be mediated by its direct interaction with Yku70/80, as might be inferred from a situation in mammalian cells, where the C-terminal part of KU70 interacts with the N-terminal part of MRE11 [183]. Although mammalian MRE11/RAD50/NBS1 has not yet been shown to participate in NHEJ, one cannot rule out the possibility that the Yku70/Mre11 interaction occurs in lower eukaryotes and that in these organisms it mediates protein assembly at the site of DBS during NHEJ. Our preliminary two-hybrid results for the full-length proteins indeed show a weak Yku70/Mre11 interaction (our unpublished results), which might reflect a transient complex formation between Yku70/80 and Mre11/Rad50/Xrs2. However, further experiments focused on Yku70/Mre11 interaction are required to reveal its biological consequence.

Undoubtedly, the Mre11/Rad50/Xrs2 complex does play an important role in assembly of other NHEJ factors, as it further interacts with Lig4/Lif1 and this interaction significantly enhances the end-joining efficiency of Lig4/Lif1 by juxtaposing linear DNA fragments via their ends [86]. Moreover, the findings by Moore and Haber [84] that showed eliminated gap-filling events in strains debilitated in Mre11/Rad50/Xrs2 suggest link between synapsis and gap-filling steps of NHEJ. Consequently, Mre11/Rad50/Xrs2 may also perform an important role in recruiting Pol4 through a direct physical association.

In mammalian cells, KU70/80 significantly enhances the DNA binding and kinase activity of DNA-PKcs [184] and this is due to a direct and highly specific interaction between 12 extreme C-terminal amino acid residues of KU80 and DNA-PKcs. Interestingly, the sequence alignment of these 12 amino acid residues showed high sequence conservation among KU80 homologues within vertebrate organisms. Yku80, however, lacks this sequence [185], and this could be one of the reasons why no clear orthologue of DNA-PKcs in the *S. cerevisiae* genome has been found yet, although DNA-PKcs orthologues have already been identified in a wide variety of other organisms [186–190]. In this regard, it is noteworthy that *S. cerevisiae* possesses Tel1 and Mec1, the proteins which are related to DNA-PKcs in sequence [83]. However, as shown in Section 2.1.1, Tel1 is not an essential component of the *S. cerevisiae* NHEJ and does not represent a functional homologue of DNA-PKcs. Although we are not aware of evidence proving that Mec1 does not function in NHEJ, one can assume that the *mec1* mutant would behave similarly to *tel1* in NHEJ phenotype. Tel1 and Mec1 therefore rather represent kinases involved in DNA damage signalling and are presumably ATM and ATR homologues, respectively [83]. Anyway, it would be interesting to find out whether there is any kinase in *S. cerevisiae* that would have an equivalent function in NHEJ as DNA-PKcs does in mammalian NHEJ or whether there is no need for such a function in this lower eukaryote.

DNA-PKcs forms a physical complex with ARTEMIS that alone is a 5′ to 3′ exonuclease, but following association with, and phosphorylation by, DNA-PKcs, it acquires hairpin opening and overhang endonuclease activities [24]. Although the role of ARTEMIS/DNA-PKcs as an overhang nuclease in NHEJ could be theoretically substituted by other factors, such as FEN-1 or MRE11/RAD50/NBS1, it is clear that these are unable to complement IR sensitivity of ARTEMIS- and DNA-PKcs-null mammals [12]. Thus, ARTEMIS/DNA-PKcs complex seems to play a unique role in hairpin opening and overhang processing in mammals [12]. Although, it appears that a clear homologue of ARTEMIS does not exist in *S. cerevisiae* [191], the Pso2 (also referred to as Snm1) protein could be a potential candidate, as suggested by significant sequence similarity (mainly within metallo-β-lactamase domain) shared by these two proteins [14,191,192]. This is supported by in vivo findings by Yu et al. [193] who showed that Pso2 may recognize DNA hairpin structures and bind them. However, further experiments, mainly biochemical ones using the purified protein, are required to reasonably address the question whether there is functional homology between Pso2 and ARTEMIS. At present, it is evident that Pso2 is not deficient in HO endonuclease-induced chromosomal DSB repair, but is necessary for repair of DSB generated during interstrand cross-link repair [194]. Moreover, contrary to mammalian MRE11/RAD50/NBS1, the yeast Mre11/Rad50/Xrs2 complex is likely to function also as a DNA hairpins opening factor, because genetic evidence suggests that Pso2 cannot be exclusive DNA hairpins opening factor [193,195].

In *S. cerevisiae*, the additional protein, Nej1 (see Section 2.4), involved in the regulation of NHEJ, has been revealed [154–157]. It is encoded by a haploid-specific gene, which is regulated by mating-type [154,155,157]. Since Nej1 interacts with Lif1 [154–156,160], Nej1 may

regulate the Lif1 subcellular distribution, thereby controlling the formation of Lig4/Lif1 complex in the nucleus [154,157,196]. Although database searches have so far failed to identify Nej1 homologue in other organisms, Nej1 appears to be highly conserved within genus *Saccharomyces* (see Section 2.4). However, given the high degree of functional conservation of other NHEJ components between *S. cerevisiae* and mammals, it would not be surprising to find a functional Nej1-like counterpart in mammals, which would modulate the DNA ligase IV/XRCC4 activity [196]. In addition to Nej1, Wilson [161] has recently identified other NHEJ regulatory factors, Doa1, Mck1 and Fyv6, which play a role in growth-phase-dependent regulation of NHEJ. This suggests existence of a mechanism, by which NHEJ is activated in postdiauxic/stationary phase of growth. Therefore, it seems that the budding yeast possesses several regulatory factors, which can increase NHEJ efficiency under certain circumstances (mainly those that keep a cell with one copy of the genome), while under normal conditions, Yku70/80, Lig4/Lif1 and Rad50/Mre11/Xrs2 may provide all obligatory functions for simple religation NHEJ. Further investigations are therefore required for a complete understanding of the molecular mechanisms involved in different sub-pathways of NHEJ.

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