

A newly identified DNA ligase of *Saccharomyces cerevisiae* involved in *RAD52*-independent repair of DNA double-strand breaks

Primo Schär,¹ Gernot Herrmann, Graham Daly, and Tomas Lindahl²

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, UK

Eukaryotic DNA ligases are ATP-dependent DNA strand-joining enzymes that participate in DNA replication, repair, and recombination. Whereas mammalian cells contain several different DNA ligases, encoded by at least three distinct genes, only one DNA ligase has been detected previously in either budding yeast or fission yeast. Here, we describe a newly identified nonessential *Saccharomyces cerevisiae* gene that encodes a DNA ligase distinct from the CDC9 gene product. This DNA ligase shares significant amino acid sequence homology with human DNA ligase IV; accordingly, we designate the yeast gene *LIG4*. Recombinant *LIG4* protein forms a covalent enzyme-AMP complex and can join a DNA single-strand break in a DNA/RNA hybrid duplex, the preferred substrate in vitro. Disruption of the *LIG4* gene causes only marginally increased cellular sensitivity to several DNA damaging agents, and does not further sensitize *cdc9* or *rad52* mutant cells. In contrast, *lig4* mutant cells have a 1000-fold reduced capacity for correct recircularization of linearized plasmids by illegitimate end-joining after transformation. Moreover, homozygous *lig4* mutant diploids sporulate less efficiently than isogenic wild-type cells, and show retarded progression through meiotic prophase I. Spore viability is normal, but *lig4* mutants appear to produce a higher proportion of tetrads with only three viable spores. The mutant phenotypes are consistent with functions of *LIG4* in an illegitimate DNA end-joining pathway and ensuring efficient meiosis.

[Key Words: DNA repair; illegitimate recombination; meiosis; DNA ligase IV; CDC9]

Received April 15, 1997; revised version accepted June 13, 1997.

Mammalian cells contain several different nuclear DNA ligases. These enzymes share related catalytic domains but in addition have unique regions that appear to be required for interactions with other proteins, therefore conferring functional specificity. DNA ligase I is an essential replication factor that accounts for joining of Okazaki fragments during lagging-strand DNA synthesis (Barnes et al. 1990; Waga et al. 1994; Petrini et al. 1995; Mackenney et al. 1997) and may also be involved in some forms of DNA repair and recombination (Barnes et al. 1992; Prigent et al. 1994). DNA ligase III (Wei et al. 1995) interacts strongly with the DNA repair protein XRCC1 and appears to take part in base excision-repair (Thompson et al. 1990; Kubota et al. 1996). An alternatively spliced, testis-specific form of DNA ligase III has also been identified that could be active in meiotic recombination (Chen et al. 1995). DNA ligase II, a smaller DNA ligase present in nonproliferating cells, may be yet

another alternatively spliced form of DNA ligase III (Roberts et al. 1994). During our attempts to clone a cDNA for DNA ligase III, a separate open reading frame (ORF) was also identified that encodes a previously unrecognized enzyme, with an extended carboxy-terminal region not present in other ATP-dependent ligases, DNA ligase IV (Wei et al. 1995). Active DNA ligase IV was subsequently isolated from HeLa cell nuclei where it occurs as a heterodimer with an unidentified partner; the function of DNA ligase IV is presently unknown (Robins and Lindahl 1996).

The only mammalian DNA ligase for which a functional assignment can be made with some certainty is the replication factor DNA ligase I. For this reason, detection of yeast counterparts of DNA ligases III and/or IV, and functional investigations of mutants defective in these activities, would be of considerable interest. Unfortunately, only a single gene for DNA ligase has been discovered in either *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, *CDC9* versus *cdc17*⁺, and the sequences of their products suggest strongly that they are counterparts to mammalian DNA ligase I (Barker et al. 1985, 1987). Conditional-lethal *S. cerevisiae* *cdc9* or *S.*

¹Present address: Institute of Medical Radiobiology, University of Zürich, CH-8029 Zürich, Switzerland.

²Corresponding author.

E-MAIL lindahl@icrf.icnet.uk; FAX 44-0-171-269-3819.

pombe cdc17 mutants, when grown at a permissive temperature, accumulate Okazaki fragments during DNA replication, are hypersensitive to several DNA-damaging agents, exhibit enhanced mitotic intergenic recombination, and sporulate with reduced efficiency (Johnston and Nasmyth 1978; Sipiczki et al. 1990). Biochemical investigations also showed that the CDC9 gene product is a functional homolog of mammalian DNA ligase I, and suggested further the presence of a second DNA ligase activity in *S. cerevisiae* cells (Johnston and Nasmyth 1978; Tomkinson et al. 1992; Ramos et al. 1997). Yet, even when the complete sequence of the *S. cerevisiae* genome initially became available in 1996, no ORF encoding a homolog of either DNA ligase III or IV could be detected. When we performed a more detailed investigation, however, sequence motifs typical of DNA ligase IV were revealed and an ORF masked by sequencing errors could be identified. Here we describe this gene, and properties of defective yeast mutants. The most striking phenotype of a *S. cerevisiae* *LIG4* disruption is a greatly impaired ability to recircularize a transformed linearized plasmid by DNA double-strand break repair.

Results

The LIG4 gene and its product

The DNA sequence initially deposited in the yeast genome database described two adjacent ORFs (Sterky et al. 1996) close to the centromere on the left arm of chromosome XV, one of them (UND407) showing homology to the unique carboxy-terminal region of human DNA ligase IV (Wei et al. 1995), and the other (UNE452) to part of the catalytic domain of ATP-dependent DNA ligases. This information prompted us to re-examine the DNA sequence of the entire region covering the two putative ORFs. Sequence analysis of multiple, independently subcloned DNA fragments amplified from genomic *S. cerevisiae* DNA revealed one continuous ORF, which is present in the current *S. cerevisiae* genome database as ORF YOR005C. On chromosome XV, this ORF extends from an ATG translation start at nucleotide position 337343 (Crick strand) to an in-frame TGA translation stop 2833 bp downstream (position 334511) and has coding potential for a 109-kD protein of 944 amino acids. The deduced amino acid sequence of the *S. cerevisiae* *LIG4* protein (scLIG4) displays significant homology to human DNA ligase IV (hsLIG4, 25% identity score) and shares less, but still significant, sequence homology with CDC9 of *S. cerevisiae* (scCDC9, 21% identity), human DNA ligase I (hsLIG1, 21% identity) and human DNA ligase III (hsLIG3, 19% identity). Recently, a *Candida albicans* gene encoding a DNA ligase was identified and called *CDC9* (Andaluz et al. 1996). However, the protein encoded by this gene is only distantly related to *S. cerevisiae* CDC9 (20% identity) and shows greater homology to scLIG4 (33% identity). The presence of a characteristic extended carboxy-terminal region also strongly indicates that this caLIG is a homolog of scLIG4 and hsLIG4. These relationships are displayed in Figure 1A.

The predicted amino acid sequence of scLIG4, aligned with human DNA ligase I and IV and the *S. cerevisiae* CDC9 gene product, is shown in Fig. 1B. The ATG₃₃₇₃₄₃ is likely to be the translation start: (1) presence of multiple translation stops in all frames between this and the next in-frame upstream ATG; (2) translation starting at the next downstream ATG (residue 60) would remove conserved amino-terminal peptide sequence (23% identity to the amino terminus of the *C. albicans* LIG protein). The scLIG4 protein shares a common core domain with both human DNA ligases and scCDC9 that includes a number of highly conserved motifs characteristic of nucleotidyl transferases such as DNA ligases and mRNA capping enzymes (Shuman and Schwer 1995). Among them is the minimal active site consensus for all ATP-dependent ligases, K-DG-R (residues 282–287 of scLIG4) (Tomkinson et al. 1991), which together with the conserved regions around Glu-340, Phe-382, and Lys-464 of scLIG4 forms the basis of the nucleotide binding pocket (Subramanya et al. 1996). scLIG4 also shows six of 16 amino acid residues that define a highly conserved consensus peptide; three of them form the motif R-DK (residues 624–627 of scLIG4), which is found in all ATP-dependent DNA ligases. Outside the core domain, DNA ligases I and IV are different (Fig. 1B) and unlike DNA ligase I and scCDC9, human DNA ligase IV and the scLIG4 have long carboxy-terminal extensions. Although their similarity is not striking at the primary structure level, hydrophobic cluster analyses have revealed that of both these carboxy-terminal regions contain a tandem array of two “BRCT” sequence motifs, a feature identified in several DNA repair factors including *S. cerevisiae* RAD9 and REV1 and *S. pombe* Rad4 (Cut5), as well as in human BRCA1 (Callebaut and Moron 1997). Such motifs have been directly implicated in specific protein–protein interactions (Nash et al. 1997).

S. cerevisiae LIG4 encodes a protein with catalytic properties of a DNA ligase

The reaction mechanism of DNA ligases is well characterized. In the first step of the reaction, eukaryotic enzymes use ATP to form a covalent ligase–adenylate complex through the active site lysine residue (Lindahl and Barnes 1992). The activated AMP moiety of the DNA ligase–adenylate intermediate is then transferred to the 5′ phosphoryl end of a strand break in double-stranded DNA to form a DNA–AMP complex. Finally, the generation of a phosphodiester bond is catalyzed by unadenylated DNA ligase with concomitant release of the AMP residue. To establish such catalytic properties for the scLIG4 gene product, it was overexpressed in *Escherichia coli* and purified as an amino-terminal histidine-tagged version of the protein (His–LIG4). Bacterial overexpression of His–LIG4 in different systems produced mainly insoluble matter (>98%), but small amounts of soluble, active protein could be recovered after growth at low temperatures. The soluble lysate fractions of *E. coli* cells expressing His–LIG4 contained a number of polypeptides which bound tightly to a nickel–agarose affinity

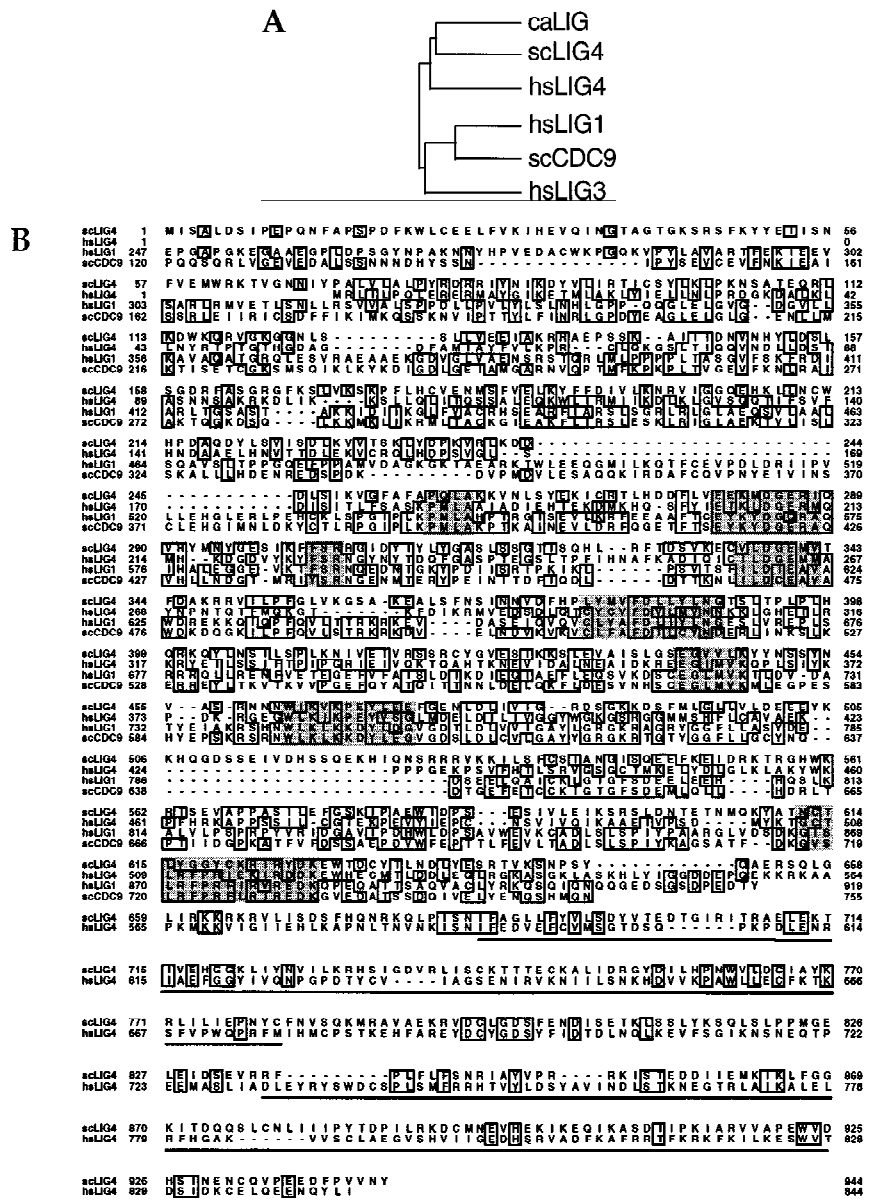


Figure 1. Relationship between the *S. cerevisiae* LIG4 gene product and those of other eukaryotic ATP-dependent DNA ligases. Sources of proteins compared are *caLIG* (*C. albicans* CDC9 EMBL accession no. X95001); *scLIG4* (*S. cerevisiae* LIG4, SGD, YOR005C); *hsLIG4* (*Homo sapiens* DNA ligase IV, EMBL accession no. X83441); *hsLIG1* (*Homo sapiens* DNA ligase I, GenBank accession no. M36067); *scCDC9* (*S. cerevisiae* CDC9, EMBL accession no. X03246); *hsLIG3* (*Homo sapiens* DNA ligase III, EMBL accession no. X84740). The amino acid sequences were aligned and the phylogenetic relationship calculated with the PILEUP and BESTFIT programs of the Genetic Computer Group (Devereux et al. 1984); gap weight, 5; gap length weight, 0.1. (A) Phylogenetic relationship of the scLIG4 protein to eukaryotic DNA ligase I, III, and IV type enzymes. (B) Amino acid sequence alignment of the yeast and human DNA ligase I and IV type enzymes. Identical residues are boxed. Shaded boxes denote positions of sequence motifs that are highly conserved between all ATP-dependent DNA ligases. The position of the adenylated lysine residue is residue 282 in scLIG4. Underscored are the carboxy-terminal regions that define the BRCT domains as identified by hydrophobic cluster analysis.

matrix, and were recovered specifically by elution with buffer containing 500 mM imidazole (Fig. 2A, lanes 3–5) after extensive washing (Fig. 2A, lanes 1,2). In parallel control fractionations of lysates from *E. coli* grown with the expression vector only, no detectable protein was recovered (Fig. 2A, lane 6). Two major His-LIG4 polypeptides of apparent masses of 108 and 75 kD were present in the imidazole eluate, the larger being close to the calculated molecular mass of the full-length His-LIG4 protein (111 kD), the lower most likely being a carboxy-terminal truncation of ~300 amino acids. On incubation with [α -³²P]ATP, both polypeptides formed a covalent enzyme-AMP complex as revealed by SDS-PAGE (Fig. 2A, lanes 9–11). No such activity was observed in corresponding vector control fractions (Fig. 2A, lane 12). Moreover, *E. coli* DNA ligase uses NAD instead of ATP

as a cofactor and would not be detected in these experiments, and assays with [³²P]NAD confirmed the absence of *E. coli* DNA ligase in the His-LIG4 imidazole eluate.

For further analysis of the second and third steps of the ligation reaction, we used the dialyzed peak fraction of the His-LIG4 imidazole eluate and the vector control material (Fig. 2A, lanes 4,10 and 6,12). The His-LIG4 produced a DNA-AMP intermediate with the hybrid substrate oligo (dT)₁₆ · poly(rA) (Fig. 2B, lane 3). No intermediates could be trapped with the same substrate lacking the 5' phosphate in the (dT)₁₆ component (Fig. 2B, lane 4), using an oligo (dT)₁₆ · poly(dA) substrate (Fig. 2B, lanes 5,6), or single-strand (dT)₁₆ (Fig. 2B, lane 7). Similarly, scLIG4 ligated (dT)₁₆ to multimers when annealed to a poly(rA) complementary strand (Fig. 2C, lane 3), but not detectably so with a poly(dA) strand (Fig. 2C,

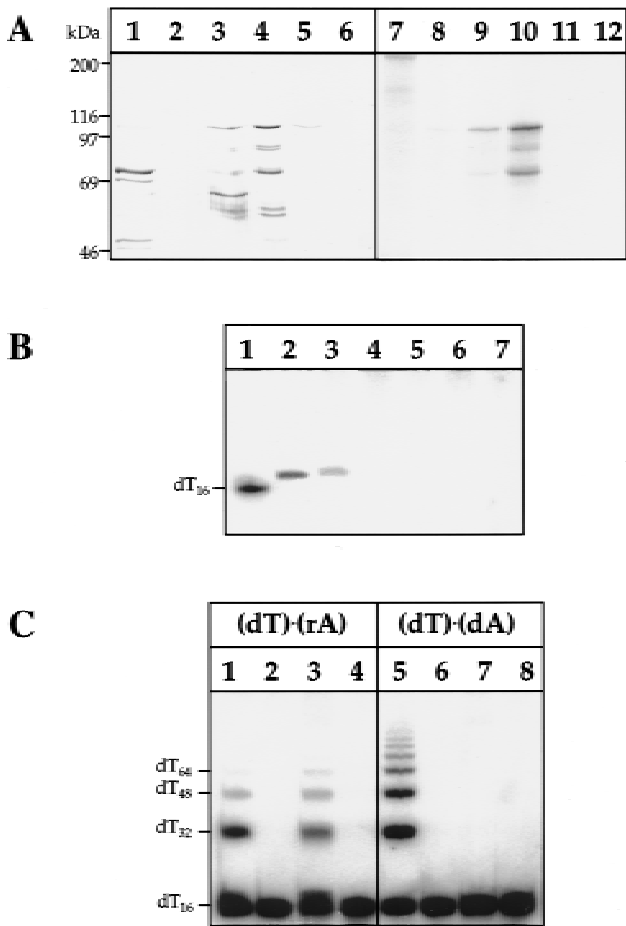


Figure 2. Recombinant scLIG4 protein shows the catalytic properties of an ATP-dependent DNA ligase. (A) Co-purification of histidine-tagged scLIG4 protein with enzyme-adenylate forming activity. The *left* panel (lanes 1–6) shows the protein content in relevant nickel column fractions (17 μ l aliquots), by silver-staining after SDS–polyacrylamide gel electrophoresis (7.5%). The *right* panel (lanes 7–12) shows the same fractions (4 μ l aliquots) assayed for enzyme–AMP complex formation as revealed after autoradiography. (Lanes 1, 7) First 80 mM imidazole wash; (lanes 2, 8) second 80 mM imidazole wash; (lanes 3, 9) first 500 mM imidazole elution; (lanes 4, 10) second 500 mM imidazole elution; (lanes 5, 11) third 500 mM imidazole elution; (lanes 6, 12) second 500 mM imidazole elution (corresponding to lanes 4 and 10) of a parallel vector control purification from *E. coli* BL21(DE3) cells with the pET16b vector alone. (B) Formation of DNA–adenylate intermediate. Reactions were carried out as described in Materials and Methods, and products were analyzed by denaturing polyacrylamide gel electrophoresis. (Lane 1) ³²P-labeled molecular mass standard (dT)₁₆; (lane 2), 1 mU of T4 DNA ligase incubated with oligo (dT)₁₆ · poly(rA); (lanes 3–7) 6 μ l of scLIG4 incubated with oligo (dT)₁₆ · poly(rA); [5'-OH] oligo (dT)₁₆ · poly(rA); oligo (dT)₁₆ · poly(dA); [5'-OH] oligo (dT)₁₆ · poly(dA); and oligo (dT)₁₆, respectively. (C) Ligation of polynucleotide substrates. Reactions were carried out as described in Materials and Methods. Lanes 1–4 show ligation assayed with [5-³²P] oligo (dT)₁₆ · poly(rA), lanes 5–8 with [5-³²P] oligo (dT)₁₆ · poly(dA). Enzymes were added as follows; (lanes 1, 5) T4 DNA ligase controls (1 mU and 0.1 mU, respectively); (lanes 2, 6) no enzyme; (lanes 3, 7) 6 μ l scLIG4; (lanes 4, 8) 6 μ l of corresponding vector control fractions.

lane 7). Phage T4 DNA ligase, used as a control, could ligate both substrates (Fig. 2C, lanes 1 and 5). The overexpressed scLIG4 enzyme did not join detectably *Eco*RI-cut plasmid DNA (data not shown); these data differ from *in vivo* results on efficient rejoining of linearized plasmid DNA by scLIG4 (see below, Fig. 4). Possibly, like human DNA ligase IV, scLIG4 occurs as a heterodimer *in vivo* (Robins and Lindahl 1996) so that only partial biochemical activity is observed with the monomer component.

LIG4 is not essential and mutants are only marginally hypersensitive to DNA damage

A heterozygous *LIG4* gene disruption was generated in a diploid strain (PRSY001) by replacing the *LIG4* ORF (amino acid residues 10–931) with a kanamycin resistance module employing the short homology PCR strategy described by Wach et al. (1994). Geneticin resistant transformants were examined for correct heterozygous gene replacement by PCR and Southern blot analysis (data not shown) and then sporulated to produce haploid meiotic progeny. Thirty of 42 dissected tetrads from two independent isolates (PRSY002, 1–2) produced four viable spores, with the Geneticin resistance segregating 2:2 in all cases, suggesting a nonessential function for the *LIG4* gene. The mutant phenotypes of haploid *lig4* segregants (PRSY003, 1, PSRY004, 2) were investigated further and the same strains were used for construction of *lig4 rad52* and *cdc9 lig4* double mutants by crossing (see Materials and Methods, Table 2, below).

lig4 single mutants did not show any effect on mitotic growth or on cell cycle progression as established by parallel examination of growth rates and distribution patterns of unbudded, small budded, premitotic, and postmitotic large budded cells in vegetative cultures (data not shown). They showed no temperature-sensitive phenotype up to 37°C nor any cold sensitivity. We also examined the effect of the *LIG4* disruption in exponentially growing haploid cells on resistance to DNA damage induced by exposure to ultraviolet light (UV), γ -ray irradiation or by incubation in the presence of methyl methanesulfonate (MMS). *lig4* mutant cells were slightly, but insignificantly more sensitive to DNA damage induced by high doses of UV light (>120 J/m²), ionizing radiation, or MMS than isogenic wild-type cells (Fig. 3). Also, *cdc9 lig4* double mutants were not significantly more sensitive to the DNA-damaging agents than *cdc9* or *lig4* single mutants, indicating an absence of synergism between the two DNA ligases (Fig. 3). Because MMS- and ionizing radiation-induced DNA damage may be repaired predominantly by homologous recombination in yeast, we investigated the possibility that repair defects associated with the lack of the *LIG4* function might be manifest only in genetic backgrounds where homologous recombination is nonfunctional. As expected, *rad52* single mutant cells were highly sensitive to MMS and ionizing radiation. Additional inactivation of *lig4* in *rad52* mutants did not result in further sensitization of the cells, however (Fig. 3B,C); in fact, *rad52* appears to be epistatic to *lig4*.

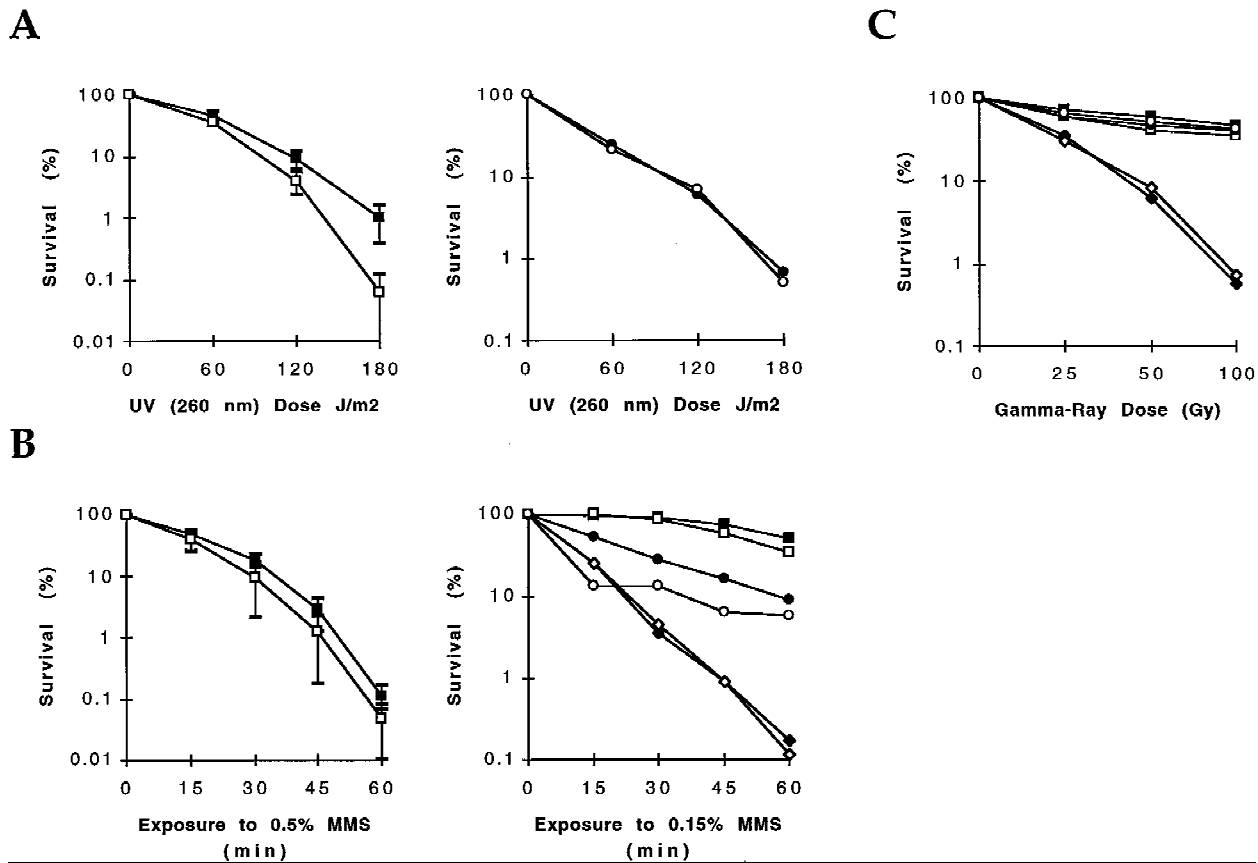


Figure 3. Sensitivity of *lig4* mutant cells to DNA damaging agents. Cells were exposed to UV, MMS, or ionizing radiation as indicated. Survival data are shown from at least three independent experiments. Standard deviations are shown where relevant for judgment of differences between strains. Matching test and control strains were always assayed in parallel as detailed in Materials and Methods. The strains used are listed with genotypes in Table 2 and are referred to in the figure as (wt) FF18734 and FF18984 (■); (*lig4*) PRSY003,1 and PRSY004,2 (□); (*rad52*) FF18743 (◆); (*lig4 rad52*) PRSY005, PRSY006 (◇); (*cdc9*) PRSY009 (●); and (*cdc9 lig4*) PRSY011 (○).

LIG4 is involved in nonhomologous double-strand break joining

To investigate whether *LIG4* might be involved in non-homologous joining of double-strand breaks in vivo, we performed plasmid rescue assays (Boulton and Jackson 1996b). Briefly, the yeast plasmid pBTM116 was digested with restriction enzymes in a region without sequence homology to chromosomal DNA (Fig. 4A), and completion of digestion was verified by Southern blotting (data not shown). Competent yeast cells were then transformed in parallel with limiting amounts of cut or uncut plasmid DNA, and the number of transformants was determined after selection for a plasmid-expressed genetic marker (*TRP1*). As plasmid replication and establishment of a *TRP*⁺ phenotype depends on successful religation of the cut plasmids, the transformation efficiency obtained with linear versus circular plasmid is a relative measure of the double-strand break repair capacity of a yeast strain.

Transformation data with *EcoRI*-digested plasmid

DNA are illustrated in Figure 4B. *LIG4* wild-type cells were highly efficient in recircularizing linearized plasmids with 5' overlapping cohesive ends. In contrast, *lig4* mutant cells showed a dramatic 300-fold reduced relative transformation efficiency with *EcoRI*-digested plasmid, whereas transformation with supercoiled plasmid was as efficient as in the *LIG4* wild-type cells. The same result was obtained in several different experiments and was independent of the overall transformation efficiency when limiting amounts of DNA were used. The deficiency in recircularizing *EcoRI*-cut plasmid was fully complemented in *lig4* mutant cells by expression of the *His-LIG4* fusion ORF carried on a yeast episomal vector (pPRS156) under the control of a *GAL1* promoter. No complementation was observed in parallel experiments with cells carrying the expression vector only (pYES2, Fig. 4B). Homologous recombination does not engage in joining of *EcoRI* ends in vivo, as *rad52* mutants did not show significantly reduced transformation efficiencies with linear plasmid. *rad52 lig4* double mutants, however, behaved as *lig4* single mutants and

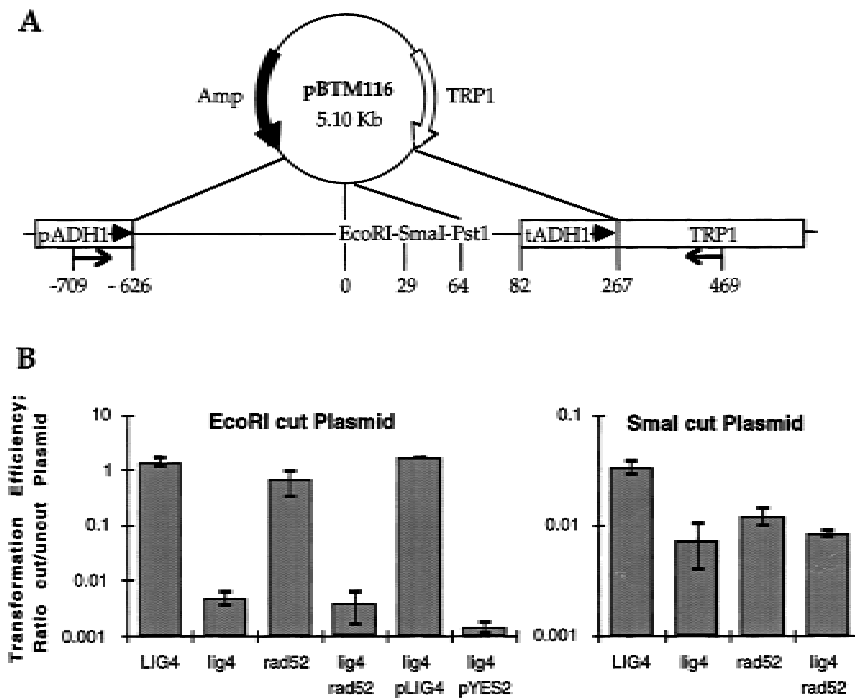


Figure 4. *S. cerevisiae* *lig4* mutant cells are deficient in recircularizing linearized plasmid DNA. Plasmid transformation assays were carried out as detailed in Materials and Methods using either supercoiled, *Eco*RI-digested, or *Sma*I-digested pBTM116 plasmid DNA for transformation. (A) Schematic map of the yeast replicative plasmid pBTM116, carrying Amp^r for selection in *E. coli* and the yeast *TRP1* gene as a selectable yeast marker. A multiple cloning sequence including the relevant *Eco*RI and *Sma*I sites is located within a stretch of DNA sequence which shares no homology with *S. cerevisiae* genomic sequences. This DNA is flanked by transcription control elements of yeast *ADHI* (pADHI, tADHI). Numbers below the map indicate the distance in base pairs of relevant plasmid elements from the *Eco*RI cut site, and the arrows mark the annealing sites of the PCR primers used for analysis of plasmid repair events. (B) Graphic illustration of relative transformation efficiencies (ratios cut/uncut plasmids) obtained with *Eco*RI- (left) and *Sma*I-cut plasmid. Data are averages from at least three independent transformation

experiments in which matching test and control strains were treated in parallel. Strains used LIG4, FF18734; *lig4*, PRSY003,1; *rad52*, FF18743; *lig4 rad52*; PRSY005; *lig4/pLIG4*, PRSY003,1 carrying a LIG4-expressing plasmid (pPRS156); *lig4/pYES2*, PRSY003,1 carrying the expression vector only.

joined *Eco*RI ends with a similarly reduced efficiency (Fig. 4B).

Transformation experiments with *Sma*I-digested blunt-ended plasmid DNA produced essentially similar results, but the effects were much less pronounced (Fig. 4B); this was mainly because LIG4 wild-type cells were reduced 42-fold in relative transformation efficiency with blunt-ended plasmids as compared with cohesive-ended plasmids. Interestingly, this difference depended on the LIG4 function as *lig4* mutants joined cohesive- and blunt-ended plasmid substrates with equal efficiency, but were still significantly (fivefold) less efficient in blunt-end joining than LIG4 wild-type cells (Fig. 4B). RAD52-dependent recombination events may contribute more to blunt-end than to cohesive-end ligation, as the difference between RAD52 wild-type and mutant cells increased slightly when rescue of *Sma*I-cut plasmid was assayed. Again, *rad52 lig4* double mutant cells produced relative transformation efficiencies with blunt-ended plasmids indistinguishable from those of the *lig4* single mutants.

To characterize the molecular nature of events that led to the establishment of tryptophan prototrophy after transformation of cells with *Eco*RI digested pBTM116, we analyzed 35 LIG4 wild-type and 48 *lig4* mutant transformants by PCR amplification of a plasmid segment spanning the putative *Eco*RI junction, *Eco*RI digestion, and DNA sequencing of the fragments, and by plasmid stability assays and Southern blotting (data not shown). All LIG4 wild-type transformants analyzed had acquired

an unstable TRP⁺ phenotype by correct religation of the plasmid. The situation in the *lig4* mutants was strikingly different and only 23% of the rare *lig4* mutant transformants analyzed showed a correct religation of the *Eco*RI-cut plasmid. In 4%, plasmid rejoining was associated with exonucleolytic degradation of DNA ends resulting in the loss of the *Eco*RI site and flanking nucleotides. In 61% of the mutant transformants, the entire ORF of the *ADHI* gene had been converted into the plasmid by homologous recombination through *ADHI* promoter and terminator sequences (Fig. 4A). Associated with this process was the loss of the heterologous sequences immediately flanking the *Eco*RI site on either side. Stability assays and Southern analysis of these transformants showed further that half of them (28% of all) contained a plasmid that circularized by homologous double-strand gap repair, whereas the other half (33% of all) carried an integrated copy of the gap-repaired plasmid at the *ADHI* locus (data not shown). Interestingly, these events appeared to be RAD52 dependent and were not detected in transformants of the *lig4 rad52* double mutant. The remaining 12% of the *lig4* mutant transformants analyzed represented more complex events. Some of them were stable but had no integrated plasmid sequence suggesting that they were TRP1 convertants.

LIG4-deficient diploids are delayed in meiosis I

DNA ligase functions are required not only in the veg-

etative state but also during meiotic differentiation of *S. cerevisiae* α/α diploids. To assess meiotic effects of a *lig4* disruption, we examined the sporulation capacity of homozygous and heterozygous *LIG4* wild-type and mutant diploids in meiotic time-course experiments, and viability of meiotic progeny in spore tetrads. Progression through meiosis was monitored by withdrawing aliquots of cells from sporulating populations at different times post-induction, and establishing distribution patterns of meiocytes containing 1, 2, 3, or 4 DAPI-staining bodies in at least 200 randomly counted cells per time point. There were significant differences between wild-type and mutant diploids before meiosis I (one DAPI body) and past meiosis II (four DAPI bodies), whereas the distribution of cells with two and three DAPI bodies was the same. Comparison of the cell class distribution showed that *lig4* mutant diploids are specifically delayed in processes before meiosis I, but once they have passed this stage they appear to be able to complete meiosis without difficulty. The meiosis I delay is best illustrated by comparing the accumulated fractions of cells past meiosis I (two, three, and four DAPI bodies) (Fig. 5). Moreover, *lig4/lig4* homozygotes sporulated less efficiently (53% completed meiosis after 72 hr) than isogenic homozygous *LIG4/LIG4* (87% after 72 hr), or heterozygous *LIG4/lig4* (81% after 72 hr) diploids (Fig. 5). Spore viability in the sporulating fraction of homozygous *lig4* mutant cells was normal (Table 1) and heterozygous marker alleles segregated 2:2. However, examination of the distribution of tetrads with 4, 3, 2, 1, and 0 viable spores in homozygous *LIG4* wild-type and mutant crosses revealed that *LIG4*-deficient diploids produced an increased proportion of tetrads with only three viable

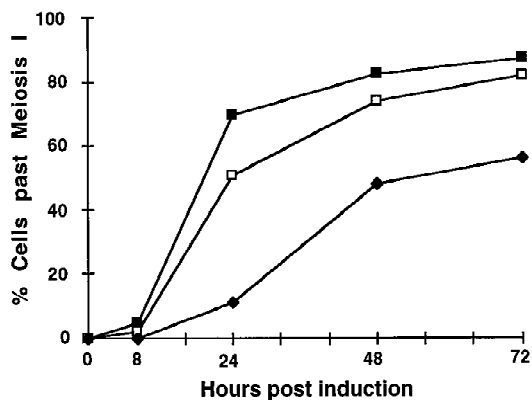


Figure 5. *LIG4*-deficient diploids are delayed in meiosis I. Fractions of cells in sporulating populations that have passed meiosis I, as evidenced by DAPI staining, are shown as a function of time postinduction. Analyses were performed as described in Materials and Methods and averages from two independent experiments are given. The values represent accumulated fractions of cells with two or more DAPI staining bodies in the sporulating populations. Strains are referred to as follows: (*LIG4/LIG4*) diploid FF18734/FF18984 (■); (*LIG4/lig4*) diploids FF18734/PRSY004,2 and PRSY003,1/FF18984 (□); (*lig4/lig4*) diploid PRSY003,1/PRSY004,2 (◆).

spores correlated with a decreased fraction of tetrads with four viable products (Table 1).

Discussion

A previously unrecognized *S. cerevisiae* gene encoding a protein whose predicted amino acid sequence shows a high degree of homology to ATP-dependent DNA ligases has been identified. We designate the gene *LIG4* because it forms a distinct group of DNA ligases together with human DNA ligase IV and a *C. albicans* DNA ligase, whereas it is related more distantly to CDC9, the yeast homolog of human DNA ligase I. *LIG4* encodes an enzyme catalyzing reactions characteristic of ATP-dependent DNA ligases, and disruption of the gene causes a phenotype that is consistent with a function of the gene product in nonhomologous joining of DNA double-strand breaks.

The *LIG4* protein is an ATP-dependent DNA ligase

The *LIG4* protein overexpressed in *E. coli* forms a covalent enzyme-AMP complex when incubated with ATP, a characteristic first step of the DNA ligation reaction of eukaryotic DNA ligases. Size determination of the radioactively labeled enzyme-AMP complex by SDS-PAGE showed that *LIG4* is a larger protein than the 87-kD CDC9 DNA ligase, and that *LIG4* migrated at the rate expected for the 109-kD protein predicted from the ORF shown in Figure 1B (111 kD for the His-tagged protein). The enzyme could also transfer the AMP residue to a DNA strand break and ligate the nick, as expected for a DNA ligase. However, *LIG4* only showed efficient activity in vitro when the complementary strand in the double helix was a polyribonucleotide rather than a polydeoxyribonucleotide; this most likely explains the failure of several research groups to detect a separate DNA ligase activity in extracts of *S. cerevisiae* *cdc9* mutant cells, when a standard substrate of nicked DNA was employed for enzyme assays. The unusual substrate specificity in vitro of purified monomeric *LIG4* may not be fully representative of the enzyme activity in vivo but does not appear to be a consequence of the amino-terminal His-tag, as the tagged enzyme expressed in *lig4* mutant yeast cells was able to complement their plasmid repair deficiency (Fig. 4B).

Role for *LIG4* in RAD52-independent double strand break repair and in progression through meiotic prophase I

The *LIG4* gene is not essential; *lig4* mutant haploid and diploid cells grow at normal rates, are respiratory-competent, and show normal cell cycle progression. Therefore, it may be concluded that this DNA ligase is not involved in DNA replication, nor in maintenance of mitochondria in *S. cerevisiae*. Surprisingly, *lig4* single mutants display only marginally increased sensitivity to UV, the alkylating agent MMS, or ionizing radiation. *S. cerevisiae* predominantly repairs UV damage by the

Table 1. Spore viability in tetrad analysis

Cross	Relevant genotype	n	Viable : dead spores in tetrads					Spore viability (%)
			4 : 0	3 : 1	2 : 2	1 : 3	0 : 4	
FF18734 × FF18984	<i>LIG4/LIG4</i>	98	87* 89%	6* 6.1%	4 4.1%	1 1.0%	0	96
PRSY003 × PRSY004	<i>lig4/lig4</i>	106	84* 79%	16* 15%	5 4.7%	1 0.9%	0	93

Spore tetrads from two independent diploids were analyzed. Full genotypes of strains are listed in Table 2. An asterisk (*) indicates tetrad classes with a significantly different distribution in wild-type and mutant diploids ($\chi^2 = 5.122$, 2×2 tables, $\chi^2 P_{0.05} = 3.84$).

nucleotide excision repair pathway represented by the *RAD3* epistasis group of genes, whereas MMS- and ionizing radiation-induced lesions are predominantly repaired by the homologous recombination pathway controlled by the *RAD52* epistasis group (for review, see Petes et al. 1991; Haber 1992a; Friedberg et al. 1995). The absence of significantly increased sensitivities in *lig4* mutant cells suggests that *LIG4* does not have an important role in either of these pathways, and the fact that no synergistic effect on cellular sensitivity was observed in a *cdc9 lig4* double mutant indicates that back-up compensation of a *LIG4* defect by the *CDC9* DNA ligase in these pathways does not occur. As *rad52* single and *lig4 rad52* double mutants are equally sensitive to ionizing radiation, there is no redundancy with the recombinational double-strand break repair pathway. In conclusion, *LIG4* does not appear to be active in excision-repair and mitotic homologous recombination processes in *S. cerevisiae*.

A strong phenotype was revealed when *lig4* mutant cells were challenged with a specific DNA substrate for ligation; cells lacking the *LIG4* function were dramatically impaired in their capacity to rejoin nonhomologous DNA ends of linearized replicative plasmids on transformation, and this repair pathway is *RAD52* independent. Nonhomologous, *RAD52*-independent double-strand break repair modes in *S. cerevisiae* have been observed previously, and it was shown that they require the functions of at least three genes, *RAD50*, *XRS2*, and *MRE11* (Schiestl and Petes 1991; Kramer et al. 1994; Mezard and Nicolas 1994; Schiestl et al. 1994; Moore and Haber 1996). At least two pathways appear to exist for nonhomologous recircularization of linearized plasmids on transformation; one of them accurately joins ends without the loss of nucleotide sequence and engages the yeast Ku70 and Ku80 homologs as accessory factors (illegitimate end-joining), whereas the other one results in the loss of variable amounts of terminal DNA sequence (illegitimate recombination) (Mezard and Nicolas 1994; Boulton and Jackson 1996a,b). Our analysis of the double-strand break repair events in the *LIG4* wild-type background showed that all transformants contained precisely religated plasmids independent of whether the linearized plasmids had cohesive- or blunt-ended termini or whether the host cells were *RAD52* wild-type or mutant. In contrast, only 23% of the scarce *lig4* mutant

transformants contained accurately religated plasmids, which might be accounted for by inefficient background ligation provided by the *CDC9* DNA ligase, and most (77%) were apparently channeled into alternative double-strand break repair pathways. In conclusion, our data are consistent with a critical role for the *LIG4* DNA ligase in a *RAD52*-independent illegitimate DNA end-joining pathway, which shows a strong preference for DNA ends with short homologies such as the single-stranded protruding sequences generated by some restriction enzymes.

Homozygous *lig4* mutants sporulate less efficiently than isogenic wild-type diploids. Reduced sporulation efficiency is a common phenotype of many genes encoding components of meiotic structures, or factors involved in cell division cycle control or DNA replication, repair, and homologous recombination; among them *CDC9*, the yeast DNA ligase I homolog and its fission yeast counterpart *cdc17+* (Simchen 1974; Sipiczki et al. 1990). The fact that *lig4* mutants are specifically impaired before meiosis I could suggest a function of *LIG4* in premeiotic DNA replication or meiotic prophase I. However, premeiotic DNA replication must have been completed accurately in the 42% of *lig4* diploids that produce asci with four viable haploid spores. Therefore, it seems more likely that *LIG4* functions in the subsequent meiotic prophase I, where homologous pairing of chromosomes is accompanied by initiation of high levels of homologous recombination by introduction of specific transient double-strand breaks and synaptonemal complex formation (for review, see Petes et al. 1991; Nicolas and Petes 1994; Roeder 1995). The formation and rejoining of specific double-strand breaks is essential for correct alignment of homologous chromosomes, without which proper segregation cannot occur, and it might engage more than one double-strand break repair pathway. It appears that *LIG4* is not essential for meiosis but beneficial to its efficiency and accuracy. A minor deficiency in chromosome segregation would be consistent with the *lig4* mutant phenotype; more detailed investigations of meiosis in *lig4* mutants will clarify this point.

Comparison with Ku of *S. cerevisiae* and mechanistic implications

There are interesting parallels and differences between

the mutant phenotypes of the *LIG4* gene and those of the recently identified *S. cerevisiae* homologs of the mammalian Ku70 and Ku80 proteins (Feldmann and Winacker 1993; Boulton and Jackson 1996a,b; Mages et al. 1996; Milne et al. 1996; Siede et al. 1996; Tsukamoto et al. 1996; Barnes and Rio 1997). *Ku* mutants display marked hypersensitivity to DNA damage in the absence of functional homologous recombination. In a plasmid repair assay, *Ku* mutants are only partly deficient in re-circularizing a plasmid with cohesive ends and rejoin a blunt-ended plasmid more efficiently. If both *LIG4* and *Ku* are involved in illegitimate DNA end-joining, one possible explanation of the apparent discrepancies in their mutant phenotypes could be that *Ku* proteins have a nonspecific DNA end protection role, whereas *LIG4* might recognize a much more limited range of DNA substrates; if such specific DNA end arrangements are only rarely generated in consequence to DNA-damaging treatment with UV, MMS, or ionizing radiation, it would be expected that the contribution of *LIG4* to repair of such damage is unimportant. Recently, Teo and Jackson (1997) showed that *yku70 lig4* double mutant strains are not further impaired in rejoining of DNA double-strand breaks compared with *lig4* or *yku70* single mutants, suggesting that both proteins may function together in some forms of nonhomologous recombination.

Several DNA metabolic processes are known to generate DNA structural intermediates that could be substrates for *LIG4*. One is mating type switching, a mitotic gene conversion process initiated by introduction of a specific transient double-strand break in the chromosome, which is then repaired by means of homologous recombination (for review, see Haber 1992b). Another possibility is meiotic recombination, which is associated with introduction of specific double-strand breaks at recombination hotspots (for review, see Nicolas and Petes 1994); the meiotic phenotypes described here could be consistent with an involvement of *LIG4* in repair of such double-strand breaks. Furthermore, a *cdc17* DNA ligase-defective mutant of *S. pombe* with impaired DNA replication, excision-repair, and mitotic recombination exhibits both unaffected meiotic recombination and mat-

ing type switching (Spiczki et al. 1990), suggesting that a separate DNA ligase is involved. A third possibility for a role of *LIG4* is in site-specific recombination, in particular RNA-mediated transposition of Ty elements. The preferential joining of DNA strands in a DNA/RNA hybrid double helix by the purified enzyme would be compatible with such a role. Retrotransposition is known to involve both the formation of DNA/RNA hybrid duplexes during the process of reverse transcription, and integration of cDNA into chromosomes mediated by precise cutting and rejoining of DNA double strands (Derr et al. 1991).

The present data on yeast *lig4* mutants suggest that mammalian DNA ligase IV may also be a nonessential DNA ligase involved in specific recombination processes. Construction of knockout mice deficient in this enzyme should elucidate further the physiological roles of the type IV group of DNA ligases, now identified both in yeast and in mammalian cells.

Materials and methods

Genetic methods

Yeast complete medium (YPD), pre-sporulation medium, and synthetic drop-out media were prepared as described (Sherman et al. 1982). Media for selection of respiration-proficient cells and sporulation medium have been described by Bähler et al. (1994). For selection of Geneticin resistance, 0.02% Geneticin G-418 (Gibco BRL) was employed in YPD. Strains were propagated and sporulated at 30°C except for *cdc9* mutants, which were grown at 25°C. Transformations were performed by a slight modification of the high-efficiency lithium acetate method (Gietz and Schiestl 1991).

Yeast strains

The *S. cerevisiae* strains used in this study are listed in Table 2. With the exception of L22-12C, they are all isogenic derivatives of two closely related, congeneric series represented by FF18734 and FF18984 in an A364A background (F. Fabre, pers. comm.) and were obtained by transformation and crossing within the set as indicated in Table 2. *cdc9* mutants (PRSY009, PRSY011) were obtained by crossing L22-12C with PRSY004,2 and made

Table 2. *S. cerevisiae* strains used

Strain	Genotype	Source
FF18734	α <i>leu2-3 trp1-289 ura3-52 his7-2 lys1-1</i>	F. Fabre (Institut Curie, Paris, France)
FF18743	α <i>lue2-3 trp1-289 ura3-52 his7-2 lys1-1 rad52::URA3</i>	F. Fabre
FF18984	a <i>leu2-3 ura3-52 his7-1 lys2-1</i>	F. Fabre
PRSY001	diploid, FF18743/FF18984	FF18743 \times FF18984
PRSY002,n	PRSY001 but <i>LIG4/lig4::kanMX4</i>	PRSY001 transformants
PRSY003,1	α <i>leu2-3 trp1-289 ura3-52 his7 lys1-1 lig4::kanMX4</i>	PRSY002,1 spore clone
PRSY004,2	a <i>leu2-3 ura3-52 his7 lys2-1 lig4::kanMX4</i>	PRSY002,2 spore clone
PRSY005	α <i>leu2-3 trp1-289 ura3-52 his7 lys1-1 lig4::kanMX4 rad52::URA3</i>	FF18743 \times PRSY004,2
PRSY006	a <i>leu2-3 ura3-52 his7 lys2-1 lig4::kanMX4 rad52::URA3</i>	FF18743 \times PRSY004,2
L22-12C	α <i>ade1/2 cdc9-1 his1 lys2</i>	L. Johnston (National Institute for Medical Research, London, UK)
PRSY009	α <i>leu2 his7-2 lys1-1 cdc9-1</i>	PRSY004,2 \times L22-12C (\times FF18734 > 87% FF18734)
PRSY011	α <i>leu2 his7-2 lys1-1 cdc9-1 lig4::kanMX4</i>	PRSY004,2 \times L22-12C (\times FF18734 > 87% FF18734)

congenic by backcrossing three times with FF18734. Appropriate genotypes were isolated from dissected spore tetrads using standard replica-plating techniques. The *LIG4* gene disruption was generated by standard gene replacement techniques using a PCR-derived disruption construct consisting of the kanMX4 module (Wach et al. 1994) flanked by 60 bp of homologous DNA sequences from the 5' and 3' ends of the *LIG4* ORF. This was gel-purified and 1 µg was used for transformation of PRSY001. Geneticin-resistant transformants were genotyped by replica-plating onto diagnostic media, by standard genomic PCR, and by Southern blot analysis. Verified transformants carrying the expected heterozygous replacement of the *LIG4* ORF with the kanMX4 cassette (PRSY002,n) were then sporulated and haploid progeny with appropriate genotypes identified in dissected spore tetrads using standard genetic and PCR genotyping techniques.

Plasmids

pPRS150 is pGEM-5Zf(+) (Promega) carrying the original PCR-amplified *LIG4* ORF (as described below). pPRS154 was used for expression of recombinant scLIG4 protein in *E. coli* with an amino-terminal histidine tag and was constructed by subcloning the entire *LIG4* ORF (the 2.8-kb *NdeI* fragment of pPRS150) into the *NdeI* site of pET16b (Novagen). pPRS156 was used for complementation of the plasmid repair phenotype in *lig4* mutants and was constructed by subcloning the *NcoI*-*BamHI* fragment of pPRS154, containing the entire His-LIG4 fusion ORF, into the *HindIII*-*BamHI* sites of the yeast expression vector pYES2 (Invitrogen), using an appropriate synthetic *HindIII*-*NcoI* adapter. Plasmid pFA6a-kanMX4 (Wach et al. 1994) was provided by Dr. W.D. Heyer (University of Bern, Switzerland) and was used as template for PCR-amplification of the *LIG4* disruption construct. pBTM116 was provided by Dr. S. Jackson (Wellcome/Cancer Research Campaign Institute, Cambridge, UK) and was used for plasmid rescue assays; it is an *E. coli*-yeast shuttle vector carrying 2µ sequences for stable maintenance, the *TRP1* gene as a selectable marker, and the promoter and terminator sequences of the *ADH1* gene flanking a multiple cloning sequence (P. Bartel and S. Fields, unpubl.).

PCR cloning and sequence analyses

The entire *LIG4* ORF was amplified by PCR of genomic DNA isolated from FF18743. The primers were designed (sense primer 5'-GCATGCATCATATGATATCAGCACTAGATTCTATACC-3'; antisense primer, 5'-GCATGCATCATATGTCAGTAGTTGACTACGGGG-3') to anneal across the *LIG4* translation start and stop (underscored), respectively, and *NdeI* restriction sites were added (italic) to facilitate subcloning of the PCR products. PCR products were then purified (QIAquick PCR Purification Kit, Qiagen), *NdeI*-digested, and subcloned into pGEM-5Zf(+) (Promega) after agarose gel purification. The resultant construct (pPRS150) was verified by automated DNA sequencing using an ABI 377 DNA Sequencer. DNA and protein sequence analyses were performed with the Genetics Computer Group program package, Version 8, 1994 (Devereux et al. 1994). For general database searches and comparisons, we used the BLAST, FASTA, and ENTREZ services provided at NCBI's world wide web page; for yeast genome database searches we accessed MIPS and SGD through their web pages.

Bacterial expression and purification of scLIG4

Recombinant, histidine-tagged scLIG4 protein was expressed in *E. coli* BL21(DE3) from pPRS154 under control of the T7 RNA

polymerase promoter. Precultures of 120-ml Luria broth containing 100 µg/ml ampicillin were inoculated with six freshly transformed BL21(DE3)/pPRS154 colonies, incubated overnight at 30°C with vigorous shaking, and used for inoculation of 6 liters of culture. Cultures were grown at 30°C to an OD₆₀₀ of 0.7–0.8, then shifted to 16°C and incubated further for 24 hr. Cells were harvested by centrifugation and stored as pellets at –80°C. Frozen cell pellets were thawed on ice, resuspended in three volumes of lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 0.25% Tween 20, 5 mM β-mercaptoethanol, 1 mM imidazole, 1 mM phenylmethanesulfonyl fluoride, at pH 8.0) and sonicated on ice for five cycles of 30 sec with intermittent chilling. After centrifugation of the cell lysate (20 min, 4°C, 15,000g), soluble proteins in the supernatant were batch adsorbed to 1 ml of pre-equilibrated (lysis buffer) Ni-NTA agarose (Qiagen) at 4°C for 1 hr. The Ni-NTA resin was packed into a disposable column (BioRad) and washed twice with 15 volumes of lysis buffer supplemented with 80 mM imidazole. Finally, bound proteins were eluted with lysis buffer containing 500 mM imidazole. LIG4 peak fractions were identified by SDS-polyacrylamide gel electrophoresis and dialyzed against 50 mM Tris-HCl, 60 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 10% glycerol (pH 7.5).

Enzyme assays

Ligase-AMP formation scLIG4 protein fractions (4 µl) or T4 DNA ligase were adenylated in reaction mixtures (20 µl) containing 60 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 µg/ml of bovine serum albumin, 5 mM dithiothreitol, and 1 µCi of [α -³²P]ATP (3000 Ci/mmol; Amersham) at 20°C for 15 min, and then analyzed by electrophoresis in SDS-7.5% polyacrylamide gels followed by autoradiography or phosphorimaging.

DNA-AMP formation Reaction mixtures (15 µl) as above were supplemented with 0.5 µg nonradio-labeled substrate DNA and scLIG4 protein fractions (6 µl) or T4 DNA ligase (Fig. 2B). Reactions were stopped by adding three volumes of 90% formamide-dyes, and heating to 95°C for 5 min. The reaction products were separated in 20% denaturing polyacrylamide gels and identified by autoradiography or phosphorimaging.

Ligation DNA ligation was assayed with the double-stranded polymer substrates [5'-³²P]oligo(dT)₁₆:poly(dA) and [5'-³²P]oligo(dT)₁₆:poly(rA). The substrates were made as described (Prigent et al. 1994). Samples (6 µl) of scLIG4 fractions were incubated in reaction mixtures (15 µl) containing 60 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 µg/ml bovine serum albumin, 5 mM dithiothreitol, 1 mM ATP, and 0.1 µg of polynucleotide substrate (20,000–50,000 cpm) at 25°C. After 3 hr incubation, the reactions were stopped by adding 90% formamide-dyes, heating at 95°C for 5 min and immediately chilling on ice. ³²P-labeled oligo(dT)₁₆ multimers were resolved by electrophoresis through denaturing 15% polyacrylamide gels and detected by autoradiography or phosphorimaging.

Mitotic growth and sensitivity to DNA-damaging agents

Mitotic growth was examined in YPD cultures by inoculating 10⁵ cells/ml from stationary phase cultures and incubating at 30°C with shaking. At indicated times, samples were removed from the cultures, and titers of viable cells were determined by plating onto YPD agar plates and incubation at 30°C for 3 days. The distribution of cells in G₁, S phase, G₂, and mitosis was assessed microscopically by cell morphology (budding state of

cells) and by staining of nuclear DNA with DAPI (4',6'-diamidino-2-phenylindol).

UV sensitivity YPD cultures (20 ml) were inoculated with 3×10^6 cells/ml from stationary phase precultures and grown to mid-log phase (10^7 cells/ml) at 30°C with shaking. After plating appropriate dilutions onto YPD agar plates (200–400 cells/plate), cells were exposed to UV light (254 nm UV Lamp XX-1552, Ultra Violet Products Ltd., $3 \text{ Jm}^{-2}\text{sec}^{-1}$) for varying times. To avoid photoreactivation, plates were incubated in the dark immediately after irradiation. Incubations were at 30°C for 3–4 days, except for *cdc9* mutants, which were either incubated at 25°C only, or for 1 hr at 37°C immediately after irradiation and then at 25°C for 3–4 days.

MMS sensitivity Mid-log-phase cultures were grown as described above. Cells were collected by centrifugation at room temperature and washed in 70 mM sodium phosphate buffer (pH 6.5), at 30°C. They were resuspended at 10^7 cells/ml in the same buffer containing 2% glucose. MMS was added to the desired final concentration, and the cells were incubated at 30°C, or 25°C in experiments involving *cdc9* mutants. Samples were removed at intervals as indicated, diluted 10^4 -fold in H₂O and plated onto YPD agar plates 1 hr after dilution. Surviving clones were counted after 3–4 days incubation at 30°C. *cdc9* mutants were either kept at 25°C throughout the procedure or incubated for 1 hr at 37°C after dilution with prewarmed H₂O.

Sensitivity to γ -irradiation Mid-log phase cells were grown and plated onto YPD agar plates as described above. Cells were exposed to different doses of γ -irradiation (¹³⁷Cs γ -beam, IBL437C irradiator, CIS Bio International) at a rate of 2.82 Gy/min. Three plates were irradiated at each dose and incubated at 30°C for 3–4 days before counting surviving clones.

Plasmid rescue assays

pBTM116 DNA was digested to completion with either *EcoRI* or *SmaI* restriction enzymes (New England Biolabs) as verified by agarose gel electrophoresis and Southern blotting. Restriction enzymes were inactivated by incubation at 65°C for 20 min, and plasmid DNA was purified by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation. Samples of undigested plasmid were subjected to the same procedure except that restriction enzymes were omitted. Equal amounts (160 ng) of undigested and digested plasmid DNA were used for parallel transformation of competent yeast cells. Serial dilutions were plated on medium lacking tryptophan and TRP⁺ transformants were counted after 4 days of incubation at 30°C. Plasmids were analyzed in individual transformants by standard colony PCR amplification (Jesnowski et al. 1995) of a 1170-bp segment spanning the putative *EcoRI* junction, and PCR products were sequenced as described above. The same transformants were assayed for plasmid stability. Selective precultures were grown in medium lacking tryptophan. Ten milliliter (nonselective) YPD cultures were inoculated with 1% of precultures and grown for another 24 hr at 30°C. Approximately 200 cells were then plated onto YPD-agar and grown for 3 days at 30°C. After replica-plating the colonies onto selective media and incubating for another 3 days at 30°C, the stability of tryptophan prototrophy was analyzed. For Southern blot analysis, cells from the selective preculture were pelleted and total cellular DNA was prepared. DNA (5 μ g), either undigested or digested with *EcoRI* or *PstI* was separated by agarose gel electrophoresis and analyzed by standard Southern blot hybridization.

Meiotic time course analysis and spore viability

Diploids were constructed freshly for each sporulation experiment by mating haploid parental strains of the *lys1-1* and *lys2-2* series, respectively, in 1-ml YPD liquid cultures followed by selection for LYS⁺ complementation on agar plates lacking lysine. Before sporulation, respiration-efficient diploids were selected in glycerol medium. Sporulation was then performed in liquid cultures at 30°C essentially as described by Padmore et al. (1991) starting from freshly isolated single colonies on YPD plates. Samples were withdrawn at different intervals, and cells fixed in 70% ethanol for DAPI staining of nuclear DNA. Examination of meiotic progression was done by bright-field and fluorescence microscopy. In parallel, spore viability was analyzed in tetrads dissected on YPD agar plates after incubation for 4–5 days at 30°C.

Acknowledgments

We thank Deborah Barnes and Christine Mezard for helpful discussions, Steve Jackson for providing plasmid pBTM116, and Francis Fabre, Wolf-Dietrich Heyer, Noel Lowndes, and Alain Nicolas for yeast strains and technical advice. P.S. was supported by an EMBO long-term fellowship and G.H. by grant He 2675/1-1 from the Deutsche Forschungsgemeinschaft.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Andaluz, E., G. Larriba, and R. Calderone. 1996. A *Candida albicans* gene encoding a DNA ligase. *Yeast* **12**: 893–898.
- Bähler, J., G. Hagens, G. Holzinger, H. Schertan, and W.-D. Heyer. 1994. *Saccharomyces cerevisiae* cells lacking the homologous pairing protein p175SEP1 arrest at pachytene during meiotic prophase. *Chromosoma* **103**: 129–141.
- Barker, D.G., J.H. White, and L.H. Johnston. 1985. The nucleotide sequence of the DNA ligase gene (CDC9) from *Saccharomyces cerevisiae*: A gene which is cell-cycle regulated and induced in response to DNA damage. *Nucleic Acids Res.* **13**: 8323–8337.
- . 1987. Molecular characterisation of the DNA ligase gene, CDC17, from the fission yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **162**: 659–667.
- Barnes, D.E., L.H. Johnston, K. Kodama, A.E. Tomkinson, D.D. Lasko, and T. Lindahl. 1990. Human DNA ligase I cDNA: Cloning and functional expression in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **87**: 6679–6683.
- Barnes, D.E., A.E. Tomkinson, A.R. Lehmann, A.D.B. Webster, and T. Lindahl. 1992. Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular hypersensitivity to DNA-damaging agents. *Cell* **69**: 495–503.
- Barnes, G. and D. Rio. 1997. DNA double strand break sensitivity, DNA replication, and cell cycle arrest phenotypes of Ku-deficient *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **94**: 867–872.
- Boulton, S.J. and S.P. Jackson. 1996a. Identification of a *Saccharomyces cerevisiae* Ku80 homologue; Roles in DNA double strand break rejoining and in telomeric maintenance. *Nucleic Acids Res.* **24**: 4639–4648.
- . 1996b. *Saccharomyces cerevisiae* Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. *EMBO J.*

- 15: 5093–5103.
- Callebaut, I. and J.P. Mornon. 1997. From BRCA1 to RAP1: A widespread BRCT module closely associated with DNA repair. *FEBS Lett.* **400**: 25–30.
- Chen, J., A.E. Tomkinson, W. Ramos, Z.B. Mackey, S. Danehower, C.A. Walter, R.A. Schultz, J.M. Besterman, and I. Husain. 1995. Mammalian DNA ligase III: Molecular cloning, chromosomal localization, and expression in spermatocytes undergoing meiotic recombination. *Mol. Cell. Biol.* **15**: 5412–5422.
- Derr, L.K., J.N. Strathern, and D.J. Garfinkel. 1991. RNA-mediated recombination in *S. cerevisiae*. *Cell* **67**: 355–364.
- Devereux, J., P. Haerberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **16**: 7025–7042.
- Feldmann, H. and E.L. Winnacker. 1993. A putative homologue of the human autoantigen Ku from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **268**: 12895–12900.
- Friedberg, E.C., G.C. Walker, and W. Siede. 1995. DNA repair and mutagenesis. pp. 662–669. American Society for Microbiology, Washington, D.C.
- Gietz, R.D. and R.H. Schiestl. 1991. Applications of high efficiency lithium acetate transformation of intact yeast cells using single-stranded nucleic acids as carrier. *Yeast* **7**: 253–263.
- Haber, J.E. 1992a. Exploring the pathways of homologous recombination. *Curr. Opin. Cell Biol.* **4**: 401–412.
- . 1992b. Mating-type gene switching in *Saccharomyces cerevisiae*. *Trends Genet.* **8**: 446–452.
- Jesnowski, R., J. Naehring, and K. Wolf. 1995. A rapid and reliable method for PCR-based amplification of chromosomal and mitochondrial DNA from intact yeast cells. *Curr. Genet.* **27**: 318–319.
- Johnston, L.H. and K.A. Nasmyth. 1978. *Saccharomyces cerevisiae* cell cycle mutant *cdc9* is defective in DNA ligase. *Nature* **274**: 891–893.
- Kramer, K.M., J.A. Brock, K. Bloom, K. Moore, and J.E. Haber. 1994. Two different types of double strand breaks in *Saccharomyces cerevisiae* are repaired by similar RAD52 independent, nonhomologous recombination events. *Mol. Cell. Biol.* **14**: 1293–1301.
- Kubota, Y., R.A. Nash, A. Klungland, P. Schär, D.E. Barnes, and T. Lindahl. 1996. Reconstitution of DNA base excision-repair with purified human proteins: Interaction between DNA polymerase β and the XRCC1 protein. *EMBO J.* **15**: 6662–6670.
- Lindahl, T. and D.E. Barnes. 1992. Mammalian DNA ligases. *Annu. Rev. Biochem.* **61**: 251–281.
- Mackenney, V.J., D.E. Barnes, and T. Lindahl. 1997. Specific function of DNA ligase I in SV40 DNA replication by human cell-free extracts is mediated by the amino terminal non-catalytic domain. *J. Biol. Chem.* **272**: 11550–11556.
- Mages, G.J., H.M. Feldmann, and E.L. Winnacker. 1996. Involvement of *Saccharomyces cerevisiae* *HDF1* gene in DNA double-strand break repair and recombination. *J. Biol. Chem.* **271**: 7910–7915.
- Mezard, C. and A. Nicolas. 1994. Homologous, homeologous, and illegitimate repair of double-strand breaks during transformation of a wild-type strain and a *rad52* mutant strain of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**: 1278–1292.
- Milne, G.T., S. Jin, K.B. Shannon, and D.T. Weaver. 1996. Mutations in two Ku homologs define a DNA end-joining repair pathway in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**: 4189–4198.
- Moore, J.K. and J.E. Haber. 1996. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16**: 2164–2173.
- Nash, R.A., K.W. Caldecott, D.E. Barnes, and T. Lindahl. 1997. XRCC1 protein interacts with one of two distinct forms of DNA ligase III. *Biochemistry* **36**: 5207–5211.
- Nicolas, A. and T.D. Petes. 1994. Polarity of meiotic gene conversion in fungi: Contrasting views. *Experientia* **50**: 242–252.
- Padmore, R., L. Cao, and N. Kleckner. 1991. Temporal comparison of recombination and synaptonemal complex formation during meiosis in *S. cerevisiae*. *Cell* **66**: 1239–1256.
- Petes, T.D., R.E. Malone, and L.S. Symington. 1991. Recombination in yeast. In *The molecular and cellular biology of the yeast Saccharomyces: Genome dynamics, protein synthesis and energetics* (ed. J.R. Broach, E. Jones, and J. Pringle), Vol. 1, pp. 407–521. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Petrini, J.H.J., Y. Xiao, and D.T. Weaver. 1995. DNA ligase I mediates essential functions in mammalian cells. *Mol. Cell. Biol.* **15**: 4303–4308.
- Prigent, C., M.S. Satoh, G. Daly, D.E. Barnes, and T. Lindahl. 1994. Aberrant DNA repair and DNA replication due to an inherited enzymatic defect in human DNA ligase I. *Mol. Cell. Biol.* **14**: 310–317.
- Ramos, W., N. Tappe, J. Talamantez, E.C. Friedberg, and A.E. Tomkinson. 1997. Two distinct DNA ligase activities in mitotic extracts of the yeast *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **25**: 1485–1492.
- Roberts, E., R.A. Nash, P. Robins, and T. Lindahl. 1994. Different active sites of mammalian DNA ligases I and II. *J. Biol. Chem.* **269**: 3789–3792.
- Robins, P. and T. Lindahl. 1996. DNA ligase IV from HeLa cell nuclei. *J. Biol. Chem.* **271**: 24257–24266.
- Roeder, G.S. 1995. Sex and the single cell: Meiosis in yeast. *Proc. Natl. Acad. Sci.* **92**: 10450–10456.
- Schiestl, R.H. and T.D. Petes. 1991. Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci.* **88**: 7585–7589.
- Schiestl, R.H., J. Zhu, and T.D. Petes. 1994. Effect of mutations in genes affecting homologous recombination on restriction enzyme-mediated and illegitimate recombination in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **14**: 4493–4500.
- Sherman, F., G.K. Fink, and J.B. Hicks. 1982. *Methods in yeast genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Shuman, S. and B. Schwer. 1995. RNA capping enzyme and DNA ligase: A superfamily of covalent nucleotide transferases. *Mol. Microbiol.* **17**: 405–410.
- Siede, W., A.A. Friedl, I. Dianova, F. Eckardt-Schupp, and E.C. Friedberg. 1996. The *Saccharomyces cerevisiae* Ku autoantigen homologue affects radiosensitivity only in the absence of homologous recombination. *Genetics* **142**: 91–102.
- Simchen, G. 1974. Are mitotic functions required in meiosis? *Genetics* **76**: 745–753.
- Sipiczki, M., A.M. Grossenbacher-Grunder, and Z. Bodi. 1990. Recombination and mating-type switching in a ligase-deficient mutant of *Schizosaccharomyces pombe*. *Mol. & Gen. Genet.* **220**: 307–313.
- Sterky, F., A. Holmberg, B. Pettersson, and M. Uhlén. 1996. The sequence of a 30 kb fragment on the left arm of chromosome XV from *Saccharomyces cerevisiae* reveals 15 open reading frames, five of which correspond to previously identified genes. *Yeast* **12**: 1091–1095.
- Subramanya, H.S., A.J. Doherty, S.R. Ashford, and D.B. Wigley. 1996. Crystal structure of an ATP-dependent DNA ligase from bacteriophage T7. *Cell* **85**: 607–615.

- Teo, S.-H. and S.P. Jackson. 1997. Identification of *Saccharomyces cerevisiae* DNA ligase IV: Involvement in DNA double-strand-break repair. *EMBO J.* (in press).
- Thompson, L.H., K.W. Brookman, N.J. Jones, S.A. Allen, and A.V. Carrano. 1990. Molecular cloning of the human *XRCC1* gene, which corrects defective DNA strand break repair and sister chromatid exchange. *Mol. Cell. Biol.* **10**: 6160-6171.
- Tomkinson, A.E., N.F. Totty, M. Ginsburg, and T. Lindahl. 1991. Location of the active site for enzyme-adenylate formation in DNA ligases. *Proc. Natl. Acad. Sci.* **88**: 400-404.
- Tomkinson, A.E., N.J. Tappe, and E.C. Friedberg. 1992. DNA ligase I from *Saccharomyces cerevisiae*: Physical and biochemical characterization of the *CDC9* gene product. *Biochemistry* **31**: 11762-11771.
- Tsukamoto, Y., J.-i. Kato, and H. Ikeda. 1996. Hdf1, a yeast Ku-protein homologue, is involved in illegitimate recombination, but not in homologous recombination. *Nucleic Acids Res.* **24**: 2067-2072.
- Wach, A., A. Brachat, R. Pöhlmann, and P. Philippsen. 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793-1808.
- Waga, S., G. Bauer, and B. Stillman. 1994. Reconstitution of complete SV40 DNA replication with purified replication factors. *J. Biol. Chem.* **269**: 10923-10934.
- Wei, Y.-F., P. Robins, K. Carter, K. Caldecott, D.J.C. Pappin, G.-L. Yu, R.-P. Wang, B.K. Shell, R.A. Nash, P. Schär, D.E. Barnes, W.A. Haseltine, and T. Lindahl. 1995. Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination. *Mol. Cell. Biol.* **15**: 3206-3216.