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Saccharomyces cerevisiae LIF1: a function involved in DNA double-strand break repair related to mammalian XRCC4

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Saccharomyces cerevisiae **DNA ligase IV (LIG4) has been shown previously to be involved in non-homologous DNA end joining and meiosis. The homologous mammalian DNA ligase IV interacts with XRCC4, a protein implicated in V(D)J recombination and double-strand break repair. Here, we report the discovery of LIF1, a *S.cerevisiae* protein that strongly interacts with the C-terminal BRCT domain of yeast LIG4. LIG4 and LIF1 apparently occur as a heterodimer *in vivo*. LIF1 shares limited sequence homology with mammalian XRCC4. Disruption of the *LIF1* gene abolishes the capacity of cells to recircularize transformed linearized plasmids correctly by non-homologous DNA end joining. Loss of LIF1 is also associated with conditional hypersensitivity of cells to ionizing irradiation and with reduced sporulation efficiency. Thus, with respect to their phenotype, *lif1* strains are similar to the previously described *lig4* mutants. One function of LIF1 is the stabilization of the LIG4 enzyme. The finding of a XRCC4 homologue in *S.cerevisiae* now allows for mutational analyses of structure–function relationships in XRCC4-like proteins to define their role in DNA double-strand break repair.**

Keywords: BRCT domains/DNA ligase IV/DNA repair/illegitimate recombination/XRCC4

Introduction

Double-strand breaks in DNA constitute a significant threat to the stability of cellular genomes, and highly efficient repair pathways have evolved to counteract this form of DNA damage. In eukaryotes, two pathways for double-strand break repair (DSBR) can be distinguished, homologous recombination and non-homologous end joining (NHEJ). In mammalian cells, NHEJ seems to be the major pathway, whereas yeast preferentially employs homologous recombination for DSBR. Nevertheless, yeast cells possess the ability to perform NHEJ, but the full biological role of this pathway remains to be elucidated. Some of the components involved in NHEJ, like the Ku proteins and DNA ligase IV, are conserved between yeast and man, whereas others, e.g. DNA-dependent protein kinase (DNA-PK) or the XRCC4 protein, have not yet been detected in yeast. Thus, the latter may be either highly diverged or not present at all in yeast.

Human XRCC4 was identified by its ability to complement radiation sensitivity and a deficiency in V(D)J recombination of hamster XR-1 cells, which have been shown to lack both alleles of the *XRCC4* gene (Stamato *et al.*, 1983; Li *et al.*, 1995). The *XRCC4* gene encodes a polypeptide with a predicted molecular mass of 38 kDa which occurs as a phosphoprotein and migrates at ~55 kDa on SDS–PAGE (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997; Mizuta *et al.*, 1997). XRCC4 appears to be conserved in mammals, with human and mouse sequences sharing ~75% identity, but homologues have not been detected in other eukaryotes. Recently, XRCC4 was shown to interact with the C-terminal region of human DNA ligase IV and to stimulate double-strand break joining efficiency of this enzyme *in vitro* (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). On the basis of the XR-1 cellular phenotype, these results indicated that a complex involving DNA ligase IV and XRCC4 might be responsible for joining of double-strand breaks generated by DNA-damaging treatments or during V(D)J recombination in mammalian cells.

DNA ligase IV has been isolated as one of at least three distinct ATP-dependent DNA ligases in mammalian cells (Wei *et al*., 1995; Robins and Lindahl, 1996). These enzymes share highly conserved catalytic core domains but differ completely in their N- and C-terminal regions. These extra-catalytic domains appear to be required for interactions with other proteins and/or might target the DNA ligases to different DNA metabolic pathways. DNA ligase I is an essential replication factor; its main function is the joining of Okazaki fragments during lagging-strand DNA synthesis, but it may also play a role in some forms of DNA repair and recombination (Barnes *et al*., 1990; Waga *et al*., 1994; Petrini *et al*., 1995; Mackenney *et al*., 1997). DNA ligase III interacts strongly with the DNA repair protein XRCC1 and accounts for the final joining step in the major pathway of base excision repair (Kubota *et al.*, 1996; Cappelli *et al.*, 1997; Nash *et al.*, 1997). An alternatively spliced form of DNA ligase III is present in testis and may be active in meiotic recombination (Mackey *et al*., 1997), and DNA ligase II is another smaller form of DNA ligase III.

Direct evidence for an involvement of DNA ligase IV in DSBR became available with the recent identification of *Saccharomyces cerevisiae LIG4*, a gene encoding a homologue of mammalian DNA ligase IV. From investigations of the *lig4* mutant phenotype in yeast, we and others could show that unlike the yeast DNA ligase I homologue, CDC9, LIG4 is not essential for growth but is involved in non-homologous DSBR; in addition, it has a function in meiosis (Schär *et al.*, 1997; Teo and Jackson, 1997; Wilson *et al.*, 1997). Similarly to mammalian DNA ligase IV, the yeast enzyme has a characteristic C-terminal region consisting of two tandemly arrayed BRCT domains which have been implicated in protein–protein interactions

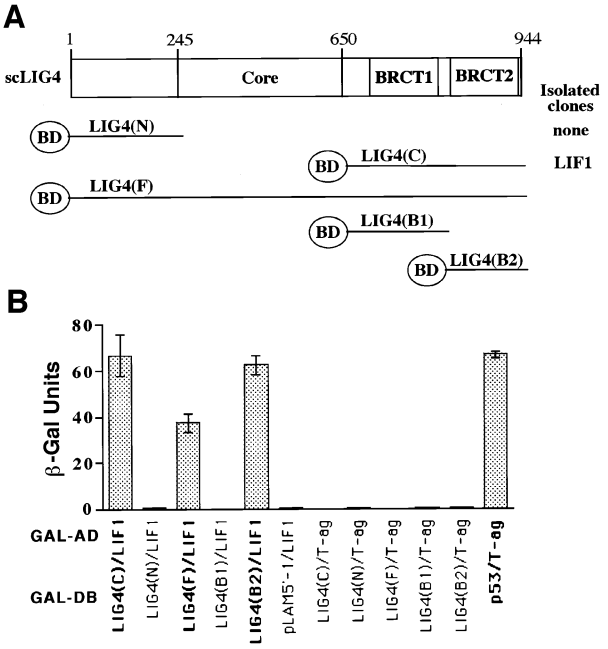

Fig. 1. Identification of LIF1 by yeast two-hybrid screening. (**A**) Bait constructs used in this study. Five DNA fragments representing parts of the *LIG4* ORF were fused to the GAL4 DNA-binding domain in the yeast two-hybrid vector pAS2-1. LIG4(N) and LIG4(C) were used for screening a *S.cerevisiae* cDNA library cloned in the GAL4 activation domain vector pACT-2. All other constructs were used for confirmation and characterization of identified interactions. *LIF1* cDNAs were isolated with the C-terminal LIG4(C) fragment of *LIG4*. (**B**) LIF1 interacts with the downstream BRCT domain of *LIG4* as determined by quantitative β-galactosidase assays. β-Gal activities shown are averages obtained with at least three independent clones analysed for each vector combination. GAL-AD, GAL4 activation domain; GAL-BD, GAL4 DNA-binding domain; T-ag, SV40 large T-antigen; p53, murine $p53_{(72–390)}$; pLAM5′-1, human lamin $C_{(66–230)}$.

(Callebaut and Mornon, 1997; Critchlow *et al*., 1997; Nash *et al*., 1997).

To dissect further the DNA ligase IV-dependent DSBR pathway and its biological role in *S.cerevisiae*, we set out to isolate and characterize proteins physically interacting with yeast LIG4. Here, we describe the functional analysis of a so far uncharacterized yeast gene, *LIF1* (ligase-interacting factor 1), the product of which specifically binds to the C-terminal BRCT domain of LIG4. This interaction involves a part of LIF1 which shows sequence homology with the mammalian XRCC4 protein. Genetic examination of the *lif1* mutant phenotype revealed a defect in non-homologous DSBR.

Results

Identification and isolation of LIF1

For two-hybrid screening, we constructed five bait vectors, each containing a part of LIG4 fused to the DNA-binding domain (BD) of the yeast GAL4 transcriptional activator (Figure 1A). The constructs LIG4(N) and LIG4(C) were used for initial screening of a *S.cerevisiae* cDNA library whereas the others were employed for subsequent confirmation and further characterization of identified interactions.

Ten positive clones were isolated from a screen of $>10^6$ *S.cerevisiae* cDNA clones with the LIG4(C) bait, the construct expressing both the BRCT domains of *LIG4*. Eight of them carried overlapping cDNA sequences from an open reading frame (ORF) on chromosome VII (YGL090w) which has coding potential for a 48.3 kDa protein. We designated this *S.cerevisiae* gene and its product LIF1. One of the *LIF1* clones carried the coding sequence for the first 260 amino acids of the protein [$pLIF1_{(1–260)}$] fused to the GAL4 activation domain (AD), whereas all others started in the region around codon 190 and extended over the entire C-terminal part of LIF1. In quantitative β-galactosidase assays, $pLIF1_{(1–260)}$ showed the strongest interaction with LIG4(C). Further two-hybrid-based characterization of this interaction with various LIG4 bait constructs revealed that the combination of $pLIF1_{(1–260)}$ and LIG4(C) produced β-galactosidase activities as strong as our positive control (Figure 1B). High levels of β-galactosidase activities were also induced with bait constructs expressing either the entire *LIG4* ORF [LIG4(F)] or the downstream BRCT domain of LIG4 [LIG4(B2)]. Other combinations, including a series of controls but also the LIG4(N) and LIG4(B1) constructs encoding the N-terminus and the upstream BRCT domain of the ligase, respectively, showed low to non-detectable β-galactosidase activities (Figure 1B). Further analyses revealed that a two-hybrid construct expressing residues 137–274 of LIF1 as a GAL4–AD fusion protein was able to induce *lacZ* expression in combination with LIG4(C), but not so with two constructs containing either residues 1–135 or 278–421 of LIF1 (data not shown). These data localize the LIG4-interacting domain in LIF1 within residues 137 and 274, a region which includes the common sequence encoded by all isolated *LIF1* cDNA clones. Finally, full-length LIF1 expressed as a GAL4–BD fusion protein induced β-galactosidase activity also with LIG4(C) subcloned in-frame with GAL4–AD (data not shown). No positive clones were obtained in cDNA library screening with the N-terminal domain of LIG4 [LIG4(N)].

LIF1 is a protein related to mammalian XRCC4

The nucleotide sequence of *LIF1* was confirmed by DNA sequencing and shown to be identical with ORF YGL090w in the *S.cerevisiae* genome database (accession No. Z72612). The *LIF1* gene encodes an acidic protein of 421 amino acids (pI = 4.86) with a calculated molecular mass of 48.3 kDa. Its predicted subcellular localization is nuclear (Horton and Kenta, 1996) and contains a cluster of putative nuclear localization signals between residues 263 and 285 (Figure 2A). Preliminary database searches with the LIF1 amino acid sequence revealed no significant homologies to other known proteins. However, a direct comparison with human XRCC4 showed that LIF1 shares 22% identity and 49% similarity with this human protein (Figure 2A). Interestingly, the highest degree of sequence conservation between the two proteins was found in the region encoded by the overlapping cDNA sequences of the *LIF1* clones isolated in the two-hybrid screen (Figure 2A). Thus, yeast LIF1 and human XRCC4 are most similar in the region which is involved in the interaction of LIF1 with the C-terminal BRCT domain of yeast LIG4.

LIF1 migrated anomalously slowly during SDS–PAGE (Figure 2B). Similar observations have been made for human XRCC4 (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997; Mizuta *et al.*, 1997). Here, an N-terminal His-

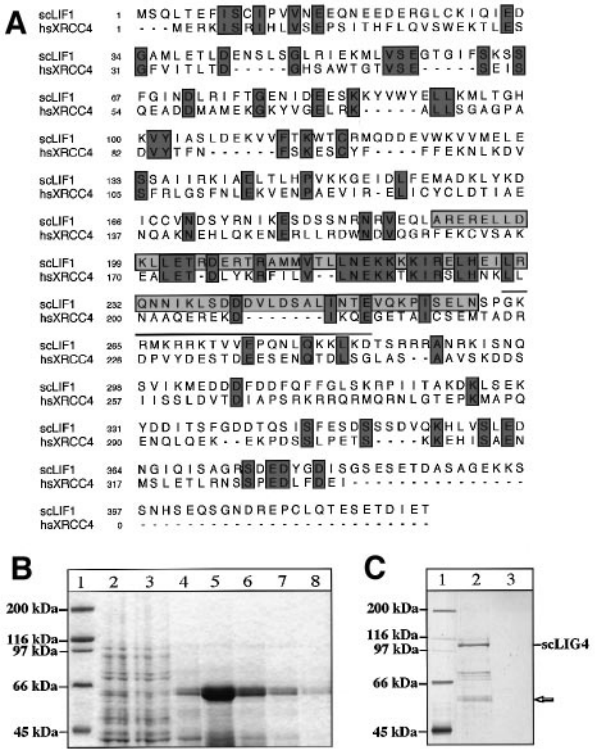

Fig. 2. Structural relationships between *S.cerevisiae* LIF1 and human XRCC4. (**A**) Amino acid sequence alignment of the yeast LIF1 protein (scLIF1, SGD YGL090W) and human XRCC4 (hsXRCC4, accession No. U40622). Identical residues are boxed and appear dark grey. The light grey shaded box between amino acids 190 and 260 of scLIF1 marks the overlapping region encoded by all cDNA clones isolated in the two-hybrid screen. The black line above residues 263–284 of scLIF1 indicates putative nuclear localization signals. (**B**) LIF1 polypeptide migrates anomalously slowly in SDS–PAGE. Recombinant His-tagged LIF1 was overexpressed in and purified from *E.coli*. The panel shows the protein content in relevant nickel column fractions by Coomassie staining after SDS–PAGE. Lane 1, molecular weight standard; lanes 2 and 3, loaded and unbound proteins (2.5 μl), respectively; lanes 4–8, consecutive LIF1-containing fractions (10 μl) eluted from the Ni-NTA column. (**C**) A protein similar in size to LIF1 co-purifies with His-tagged LIG4 expressed in a *lig4* mutant yeast strain. Shown are the relevant nickel column fractions in a silver-stained SDS–polyacrylamide gel. Lane 1, molecular weight standard; lane 2, 20 μl of a 500 mM imidazole eluate from a nickel column loaded with extract of *lig4* mutant cells carrying a *LIG4*-expressing plasmid (pPRS154); lane 3, 20 μl of the corresponding fraction from an extract of the *lig4* cells carrying the expression vector only (pYES2). Molecular weights are indicated on the left, His-LIG4 is indicated on the right side and the co-eluting band(s) at 60 kDa is marked by an arrow.

tagged form of the LIF1 protein (expected size, 50 kDa) overexpressed in *Escherichia coli* migrated at ~60 kDa (Figure 2B). A split band of LIF1 protein occurred, implicating two slightly different forms of the protein (Figure 2B). Moreover, a protein of similar size co-eluted with His-tagged LIG4 protein when the latter was overexpressed in and purified from a *lig4* mutant strain (pPRS154 in PRSY003,1) (Figure 2C); in addition, after initial nickel affinity chromatography, the protein co-eluted with LIG4 in an FPLC-Mono S step at 1 M NaCl, resisting washes with 600 mM NaCl (data not shown). A separate protein band at ~75 kDa (Figure 4C) was identified as a proteolytic fragment of LIG4 by ligase adenylation assays and Western blot analysis (data not shown). These results indicate that yeast LIG4 and LIF1 occur as a heterodimer. Human DNA ligase IV was also isolated initially as a heterodimer from HeLa cell nuclei (Robins and Lindahl, 1996), with its partner subsequently identified as XRCC4 (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997).

LIF1 is not essential for growth but mutants show defects in radiation resistance and sporulation efficiency

A heterozygous *LIF1* gene disruption was generated in a diploid *LIG4/lig4::kanMX* strain (PRSY030) by replacing the coding sequence for amino acids 13–383 of the *LIF1* ORF with an URA3 expression cassette employing a short homology PCR strategy. URA^+ transformants were examined for correct heterozygous gene replacement by PCR and genomic Southern blot analysis, and then sporulated to segregate haploid meiotic progeny. Thirty of 40 dissected tetrads produced four viable spores, with the uracil prototrophy and the geneticin resistance segregating 2:2 in 29 cases, suggesting a non-essential function for the *LIF1* gene and a viable phenotype for *lig4 lif1* double mutants. After genotyping haploid segregants, isogenic pairs of *lif1::URA3* mutants and *lig4::kanMX4 lif1::URA3* double mutants (PRSY031–PRSY034, Table II) were isolated and subjected to further phenotypic analyses.

lif1 single and *lig4 lif1* double mutants did not show any significant retardation on exponential growth or during cell cycle progression as established by parallel examination of growth rates and distribution patterns of unbudded, small budded and large budded cells in vegetative cultures (data not shown). They were neither temperature-sensitive (up to 38°C) nor cold-sensitive (down to 18°C). However, in complete medium (YPD), *lif1* and, to a lesser extent, also *lig4* mutants consistently grew to higher densities ($3–4\times10^8$ cells/ml) than isogenic wild-type cells ($2–2.5\times10^8$ cells/ml), and this effect was enhanced in the *lig4 lif1* double mutant which grew to the unusual density of $5–5.7\times10^8$ cells/ml under the same conditions. Examination of cell morphology in late exponential–early stationary phase populations (1×10^8 cells/ml) revealed an increased fraction of small budded cells in *lif1*, *lig4* and *lig4 lif1* cultures, suggesting that the mutant cells re-initiate S-phase more frequently than wild-type cells under limiting growth conditions. In late stationary phase populations (after 5–7 days incubation at 30°C), the majority (>80%) of cells were unbudded in the mutant as well as in the wild-type cultures.

The effect of *LIF1* disruption on cellular resistance to DNA damage induced by ionizing irradiation was investigated. Previous analyses of the *lig4* mutant phenotype by several groups had revealed an apparent discrepancy: whereas no significantly increased sensitivity to different DNA-damaging agents was reported consistently for *lig4* single mutants, increased hypersensitivity of *rad52* mutants to ionizing irradiation was observed for *rad52 lig4* double mutants in two studies (Teo and Jackson, 1997; Wilson *et al.*, 1997) but not in another (Schär *et al.*, 1997). We re-examined this issue for the *lig4* mutants and assessed the radiation sensitivity of *lif1* mutants under conditions that minimize the contribution of the *rad52* phenotype in the analysis, so that in the absence of homologous recombination a minor contribution to DSBR by a LIG4-dependent pathway might be uncovered. For this purpose, we irradiated haploid cells in late stationary

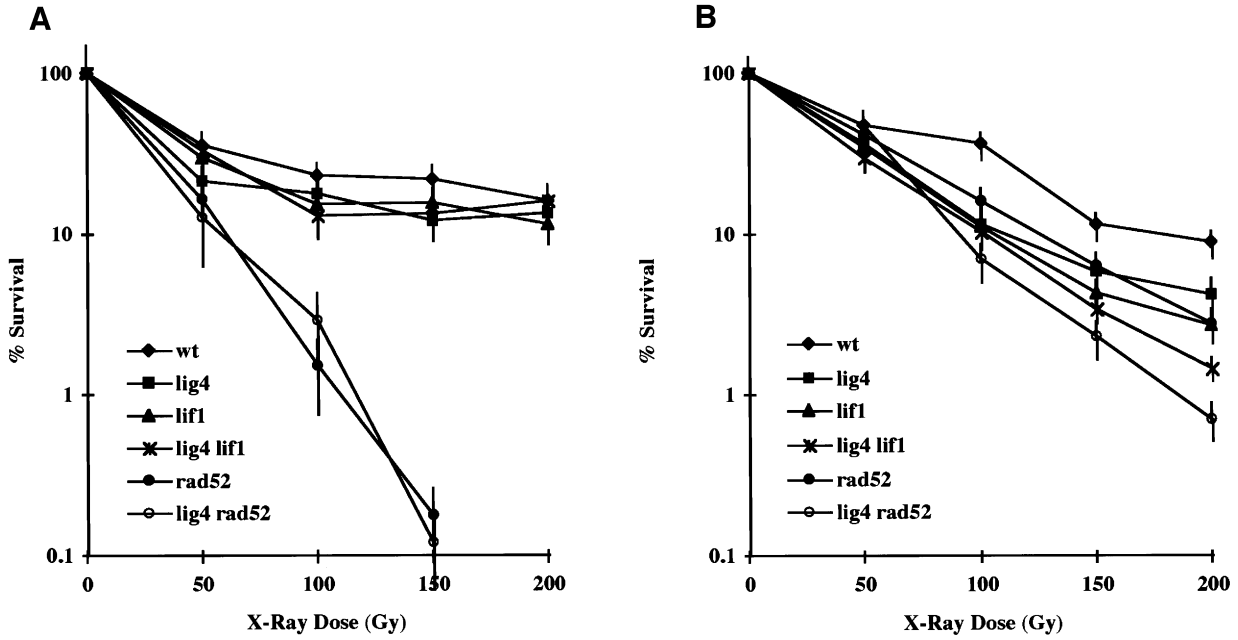

Fig. 3. Sensitivity of *lif1* mutant cells to ionizing radiation. (**A**) Exponentially growing cultures; (**B**) stationary phase cultures. Cells were exposed to X-rays as indicated at a dose rate of 10 Gy/min. Survival data are shown from at least three independent experiments. Plotted are averaged survival data with standard deviations from at least three independent experiments. 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 II and are referred to in the figure as: wt, FF18734; *lig4*, PRSY003,1; *lif1*, PRSY031,a; *lig4 lif1*, PRSY033,a; *rad52*, FF18743; *rad52 lig4*, PRSY005.

phase cultures, which are mainly in the G_0 phase of the cell cycle. Due to the lack of sister chromatids as homologous partners, such cells are expected to be limited in their capacity for DSBR by homologous recombination and, consequently, non-homologous DSBR pathways should be more important in cellular resistance against ionizing irradiation.

Employing different experimental strategies and varying irradiation dose rates from 2.5 to 10 Gy/min, we consistently obtained results as illustrated in Figure 3. With exponential and early stationary phase cells ($\leqslant 1\times10^8$ cells/ml, $\geqslant$50% budding), we confirmed our previous observations with *lig4* and *rad52 lig4* strains (Schär *et al.*, 1997) and found that the radiation sensitivity of *lif1* single and *lig4 lif1* double mutants was also only insignificantly different from that of *lig4* mutants and wild-type cells (Figure 3A). In late stationary phase cultures (5–7 days at 30°C, $>2\times10^8$ cells/ml), however, the situation changed. The radiation sensitivity of *lif1* (3.3-fold relative to wild-type), *lig4* (2.1-fold) and *lig4 lif1* (6.0-fold) mutant cells was enhanced and, as expected, the contribution of RAD52 to cellular resistance was much less pronounced (Figure 3B). At X-ray doses >100 Gy, the *lig4 lif1* double mutant strain was slightly more sensitive than either of the single mutants alone, implying that in the absence of LIF1 some residual LIG4-dependent repair can take place and vice versa. Most sensitive in the analysis was the *rad52 lig4* double mutant (12.4-fold relative to wild-type), and the comparison with the *rad52* (3.1-fold) and *lig4* (2.1-fold) single mutants suggests that, in resting cells, RAD52-dependent DSBR is in a synergistic relationship with the LIG4–LIF1-dependent pathway.

We also examined the effect of the *LIF1* disruption on the capacity of the cells to undergo meiotic differentiation. For this purpose, we sporulated isogenic diploid strains which were either homozygous *LIF1/LIF1* wild-type (FF18734×FF18984, Table II), homozygous *lif1/lif1* mutant (PRSY031×PRSY032) or heterozygous *LIF1/lif1* mutant (FF19734×PRSY032), and evaluated sporulation efficiency 24 and 48 h post-induction by counting the number of 4,6'-diamidino-2-phenylindole (DAPI)-staining bodies in at least 200 randomly selected meiocytes per time point. Similarly to *lig4/lig4* diploids (Schär *et al.*, 1997), the homozygous *lif1/lif1* strain sporulated very inefficiently (29% completed meioses after 48 h) as compared with the heterozygous *LIF1/lif1* mutant (62% after 48 h) or the homozygous wild-type (69% after 48 h); >95% of *lif1/lif1* mutant cells which failed to sporulate after 48 h showed a single DAPI-staining body, implying that they did not pass meiosis I. Spore viability in the sporulating fraction of homozygous *lig4* mutant cells was only marginally reduced (88%) in comparison with wild-type cells (96%), and segregation of heterozygous marker alleles was normal as evidenced in 42 dissected spore tetrads.

LIF1 is involved in non-homologous double-strand break joining

The physical interaction of LIF1 with LIG4 suggests an involvement of LIF1 in non-homologous repair of DNA double-strand breaks. To test this possibility, we performed plasmid rescue assays as described previously (Boulton and Jackson, 1996b; Schär *et al.*, 1997) and compared *lif1* mutants with isogenic *lig4* mutant and *lig4 lif1* double mutant yeast strains. Briefly, the yeast plasmid, pBTM116, was linearized by restriction enzyme digestion in a region without sequence homology to chromosomal DNA (Figure 4A); completion of digestion was verified by Southern blot hybridization. Competent *S.cerevisiae* cells were then transformed in parallel with limiting amounts

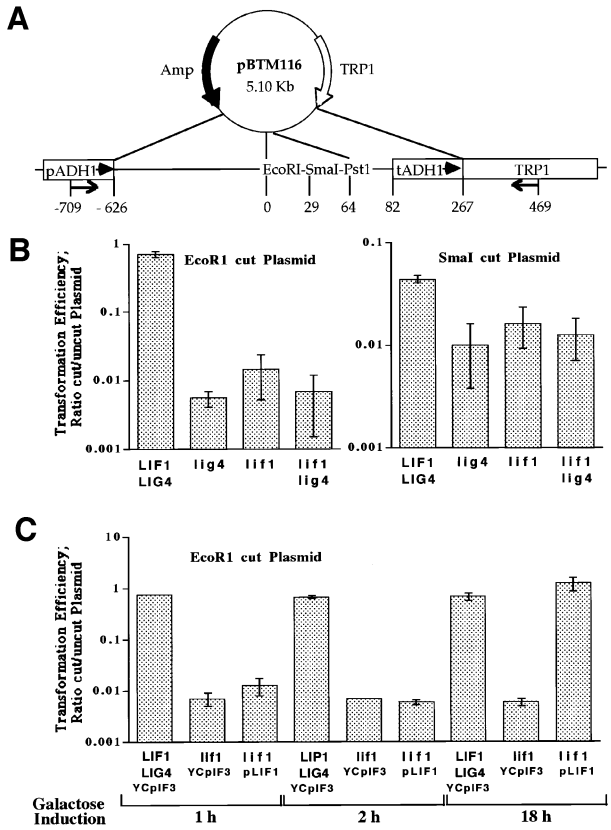

Fig. 4. *Saccharomyces cerevisiae lif1* mutant cells are deficient in recircularizing linearized plasmid DNA. Transformation assays were carried out using either supercoiled, *Eco*RI-digested or *Sma*I-digested pBTM116 plasmid (see Materials and methods). (**A**) Schematic map of the yeast replicative plasmid pBTM116, carrying Amp 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 which shares no homology with *S.cerevisiae* genomic sequences. This DNA is flanked by transcription control elements of yeast *ADH1* (pADH1, tADH1). Numbers below the map indicate the distance in base pairs of relevant plasmid elements from the *Eco*RI 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 panel) and *Sma*I-cut plasmid (right panel). (**C**) Graphic illustration of relative transformation efficiencies obtained with *Eco*RI-digested plasmid in yeast strains carrying either LIF1 in a galactose-inducible expression vector or the expression vector alone. Times of galactose-induced expression prior to transformation are indicated. Data are averages from at least three independent transformation experiments in which matching test and control strains were treated in parallel. Strains used were *LIF1 LIG4*, FF18734; *lig4*, PRSY003,1; *lif1*, PRSY031,a; *lif1 lig4*, PRSY033,a; LIF1 LIG4/YCpIF3, FF18734 carrying the expression vector only; *lif1*/YCpIF3, PRSY031,a carrying the expression vector only; *lif1*/pLIF1, PRSY031,a carrying a LIF1 expressing plasmid (pGEH014).

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 required for establishment of a TRP^+ phenotype depends on successful religation of the linear plasmids, the relative transformation efficiency obtained with linear versus circular plasmid DNA is a measure of the DSBR capacity of a yeast strain.

Transformation data with *Eco*RI- or *Sma*I-digested plasmid DNA are illustrated in Figure 4B. Wild-type cells were highly efficient in recircularizing linearized plasmids with 5′ overlapping cohesive ends. In contrast, *lif1* and, as previously shown, *lig4* mutant cells showed a dramatically, 100-fold reduced relative transformation efficiency with *Eco*RI-digested plasmid, whereas transformation with supercoiled plasmid was as efficient as in 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. Transformation efficiency was not reduced further in a *lif1 lig4* double mutant strain, indicating that LIF1 and LIG4 act in the same pathway for non-homologous double-strand break joining. Transformation experiments with *Sma*I-digested blunt-ended plasmid DNA produced essentially the same result, but the effects were less pronounced (Figure 4B); this was mainly because wild-type cells, but not the mutant cells, were transformed 40-fold less efficiently with blunt-ended plasmids than with cohesive-ended plasmids.

The deficiency in recircularizing *Eco*RI-cut plasmid was fully complemented in *lif1* mutant cells by expression of the *LIF1* ORF from yeast episomal vector pGEH014 under the control of a *GAL1* promoter (Figure 4C). However, complementation was only observed after the cells were grown for several hours under *GAL1*-inducing conditions (medium containing galactose and raffinose). No complementation was observed in parallel experiments with cells carrying the expression vector only (YCpIF3, Figure 4C). We have been unable to complement the reduced capacity for plasmid religation in *lif1* or *lig4* mutant strains with human DNA ligase IV or human XRCC4 protein overexpressed in these strains alone or in combination (data not shown).

To characterize the molecular events that led to the establishment of tryptophan prototrophy after transformation of cells with *Eco*RI- or *Sma*I-digested pBTM116, we analysed wild-type, *lif1* and *lig4* mutant transformants by PCR amplification of a plasmid segment spanning the putative *Eco*RI–*Sma*I junction, *Eco*RI or *Sma*I digestion and DNA sequencing of the fragments, and by plasmid stability assays and genomic Southern blot analysis. Table I shows that correct religation of the plasmid only represents a small fraction of events in the rare TRP^+ transformants of either *lif1* or *lig4* strains. Different inefficient, mostly homologous recombination-mediated backup pathways (gap repair, genomic integration, gene conversion) appeared to be responsible for the generation of the majority of TRP^+ transformants in these strains. Thus, the actual capacity of *lif1* and *lig4* mutant cells to religate the plasmid correctly after transformation is even lower than estimated from the comparison of the relative transformation efficiencies obtained with wild-type and mutant strains. Due to highly efficient plasmid religation by NHEJ in wild-type cells, wild-type TRP^+ transformants generated through these backup pathways which are dominant in *lif1* and *lig4* mutant cells were only detectable in experiments with *Sma*I-digested blunt-ended plasmid DNA, which appears to be a suboptimal substrate for the LIG4-dependent end-joining pathway (Table I).

LIF1 affects the stability of LIG4 protein

Since efficient complementation of the plasmid repair deficiency in *lif1* mutant cells required several hours of

Table I. Molecular events establishing TRP^+ prototrophs after transformation of yeast with linearized plasmid DNA

Fate of plasmid	Percentage of events detected in various strains					
	*Eco*RI-digested plasmid			*Sma*I-digested plasmid		
	WT	*lig4*	*lif1*	WT	*lig4*	*lif1*
Accurate religation	100	13	0	80	35	30
Inaccurate religation	0	7	4	10	5	5
Genomic integration at *ADH1* or gap repair by *ADH1* sequence	0	54	76	5	20	40
Unknown (TRP1 convertants?)	0	26	20	5	40	25

Thirty TRP^+ colonies were analysed for each strain transformed with *Eco*RI-digested plasmid and 20 for each strain transformed with *Sma*I-digested plasmid. Transformants were examined by PCR amplification, plasmid isolation, DNA sequencing, plasmid stability assays and Southern analysis of genomic DNA, as detailed in Materials and methods.

GAL1 promoter-driven *LIF1* expression from a yeast episomal vector (Figure 4C), it appeared that LIF1 might have a regulatory effect on the level of its partner, LIG4. Stabilizing functions of protein–protein interactions have been reported for a variety of heterodimeric factors including the Ku80 and Ku70 subunits of the Ku protein (Chen *et al.*, 1996).

Using purified yeast LIG4 enzyme and crude protein extracts prepared from wild-type and *lig4* mutant strains, we observed that a polyclonal antibody (TL14/15; Robins and Lindahl, 1996) directed against a peptide derived from the highly conserved core domain of human DNA ligase IV also recognizes the yeast homologue. Non-specific cross-reactions with other yeast proteins could be virtually eliminated by pre-incubation of the antiserum with *lig4* mutant extract (data not shown).

When Western blot analyses were performed on extracts from *lif1* mutant cells (PRSY031,a), no LIG4 protein was detectable (Figure 5A). As expected, the protein was not detected in extracts of *lig4* mutant (PRSY003,1) and *lig4 lif1* double mutant (PRSY033,a) cells, but it was readily observed in extracts of isogenic wild-type cells (FF18734), as was the 108 kDa His-tagged human DNA ligase IV overexpressed (pGEH007) in a *lig4* mutant strain which served as positive control (Figure 5A). After prolonged *GAL1*-induced expression of *LIF1* from an episomal plasmid, LIG4 protein was detected in the *lif1* mutant strain, whereas in extracts of the vector control cells it remained at undetectable levels (Figure 5B). Thus, the loss of LIG4 protein is a phenotype of the *lif1* mutants, and can be complemented by expression of LIF1 protein.

The absence of LIG4 protein in *lif1* mutant cells is not associated with down-regulation of transcription. Figure 5C shows the result of Northern blot analysis using *LIG4* and, as an internal control, 18S rRNA-specific probes. The relative amount of *LIG4* mRNA was not decreased in a *lif1* mutant strain in comparison with the wild-type strain, and it may even be slightly up-regulated in the mutant cells. These data establish that the regulatory effect of LIF1 on LIG4 is due to protein stabilization and not to differences in gene expression. In conclusion, one function of LIF1 is to stabilize DNA ligase IV in yeast. It seems likely that mammalian XRCC4 protein might have a similar stabilizing effect on DNA ligase IV, but this has not been investigated in XR-1 cells.

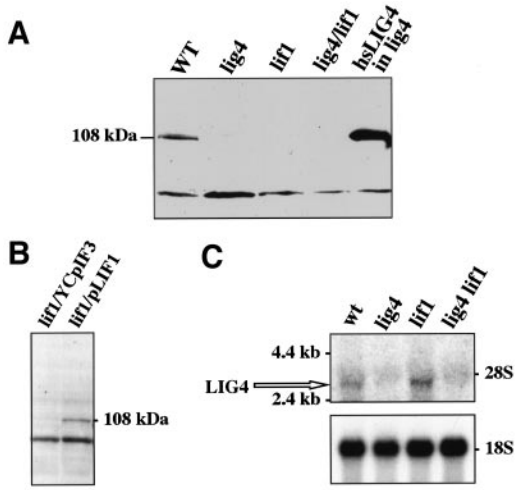

Fig. 5. LIG4 is destabilized in *lif1* mutant cells. (**A**) Western blot analysis of crude cell extracts using a polyclonal anti-human DNA ligase IV peptide antiserum for detection of yeast LIG4 protein (see Materials and methods). LIG4 protein is detectable in wild-type cells (lane 1) but not in *lig4* (lane 2), *lif1* (lane 3) or *lig4 lif1* (lane 4) mutant cells. Detection of human DNA ligase IV expressed in the *lig4* mutant is shown as positive control in lane 5. (**B**) Western blot analysis of *lif1* mutant cells either expressing the wild-type *LIF1* gene from an episomal plasmid (*lif1*/pLIF1) or carrying the expression vector only (*lif1*/YCpIF3). (**C**) Northern blot analysis of total RNA extracted from different strains (see Materials and methods). *LIG4* mRNA is detectable in wild-type and *lif1* mutant strains but not in *lig4* nor *lig4 lif1* mutants. The lower panel shows hybridization of the same blot with 18S rRNA used as an internal standard. Strains used were wt, FF18734; *lig4*, PRSY003,1; *lif1*, PRSY031,a; *lif1 lig4*, PRSY033,a; *lig4*/hsLIG4, PRSY003,1 carrying a hsLIG4-expressing plasmid (pGEH007).

LIF1 is not involved in telomere length maintenance

Several proteins that participate in NHEJ, including the yeast Ku homologues YKU70 and YKU80, the silencing factors SIR2, SIR3 and SIR4, and the RAD50, MRE11 and XRS2 functions, have been implicated in telomere length maintenance (Boulton and Jackson, 1996a, 1998; Porter *et al.*, 1996). In contrast, telomeres are maintained as in wild-type cells in yeast *lig4* mutants (Teo and Jackson, 1997; Boulton and Jackson, 1998). To address any involvement of LIF1, the protein partner of LIG4, we examined telomere lengths in *lif1* mutants and *lig4 lif1* double mutant cells by the same experimental approach

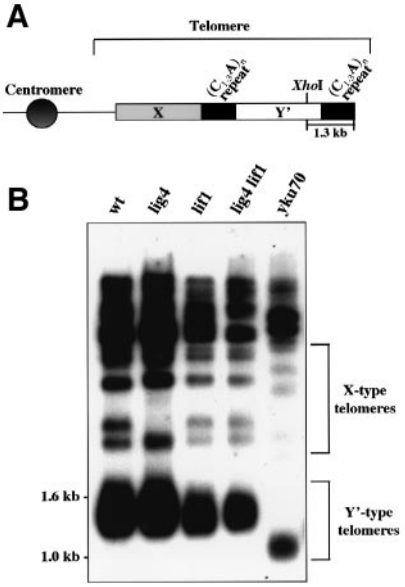

Fig. 6. LIF1 is not involved in telomeric maintenance. (**A**) Schematic illustration of telomeric regions in yeast. The relative location of the $C_{1-3}A$ repeats between X and Y' regions is indicated. *Xho*I cleavage generates telomeric fragments of ~1.3 kb in wild-type strains which include ~400 bp of $C_{1-3}A$ repeats. (**B**) Telomere length is not altered in *lig4*, *lif1* or *lig4 lif1* mutant strains as compared with wild-type strains. *Xho*I-digested genomic DNA of the strains indicated was analysed by hybridization with end-labelled poly$(GT)_{20}$ (see Materials and methods). Weaker signals in *lif1* and *lif1 lig4* strains are due to less DNA loaded onto the gel. A *yku70* strain was used as a control to confirm telomeric instability. Strains used were wt, FF18734; *lig4*, PRSY003,1; *lif1*, PRSY031,a; *lif1 lig4*, PRSY033,a; yku70, yku70α.

as employed in previous studies (Boulton and Jackson, 1998). Figure 6 shows that after culturing cells for >100 generations, telomere lengths were not affected in *lif1* mutants or *lig4 lif1* double mutants as compared with wild-type and *lig4* mutant strains, whereas telomere shortening was clearly visible in the *yku70* mutant. Although the *yku70* mutation is in a genetic background (W303) different from that of the other strains examined (A364A), it has been shown previously that *Xho*I digestion of genomic DNA and Southern hybridization with a poly$(GT)_{20}$ probe results in comparable telomeric restriction fragments in both strain backgrounds (Lustig and Petes, 1986; Porter *et al.*, 1996). Thus, the present data establish that LIF1, like LIG4, is not involved in telomere length maintenance and has no obvious role in protection of telomere ends from degradation.

Discussion

Human DNA ligase IV occurs as a heterodimer together with the XRCC4 protein. The phenotype of a hamster XRCC4-deficient cell line, XR-1, indicates that the complex plays a role in DSBR and V(D)J recombination. The identification and mutational analysis of a DNA ligase IV homologue in *S.cerevisiae* corroborated an involvement of the enzyme in DSBR independent of homologous recombination, and suggested the possible existence of a yeast counterpart to mammalian XRCC4. However, *S.cerevisiae* genome database searches failed to produce evidence for the presence of a gene encoding a related protein in yeast. Following a yeast two-hybrid-based screening strategy, we were able to identify *LIF1*, a previously uncharacterized yeast gene encoding a protein which physically interacts with the C-terminal BRCT domain of LIG4.

LIF1 is structurally and functionally related to XRCC4

The C-terminal BRCT domains of mammalian DNA ligase IV interact stably with XRCC4 (Critchlow *et al.*, 1997; Grawunder *et al.*, 1997). The N-terminal 204 amino acids of XRCC4 are sufficient to reverse the DSBR defect of XR-1 cells significantly (Leber *et al.*, 1998). These data suggest that the interacting domain is localized within this N-terminal region. By comparison, the overlapping cDNA sequences present in all *LIF1* two-hybrid clones isolated located the LIG4-interacting region within the LIF1 protein between residues 191 and 260. A linear alignment of the amino acid sequences of yeast LIF1 and human XRCC4 positions residue 204 of XRCC4 opposite residue 236 of LIF1 and reveals a region of highly conserved amino acids (residues 201–230 of LIF1 and 172–198 of XRCC4) which is present in the XRCC4 and LIF1 sequences required for association with DNA ligase IV and, therefore, may be a likely candidate for mediating this interaction (Figure 2A). Thus, LIF1 interacts with yeast DNA ligase IV in a similar way to mammalian XRCC4 with DNA ligase IV. The overall similarity between the two proteins is moderate (22% identity, 49% similarity) and explains why LIF1 has not been identified in previous database searches. However, identities of ~20% at the amino acid level are not unusual between mammalian and yeast homologues, and have also been seen in other factors involved in NHEJ, such as the yeast Ku homologues YKU70 and YKU80 in comparison with the human proteins (Beall *et al.*, 1994; Boulton and Jackson, 1996a,b; Milne *et al.*, 1996).

Mutant phenotypes and implications for the physiological roles of LIF1

The *LIF1* gene is not essential; *lif1* mutant haploid and diploid cells grow at normal rates, are respiratory-competent and show normal cell cycle progression during exponential growth. It may be concluded that LIF1 is not required for DNA replication or maintenance of mitochondria. However, *lif1*, *lig4* and *lig4 lif1* mutant strains consistently grew to 2- to 3-fold higher densities than isogenic wild-type strains under identical conditions, and this correlated with a higher proportion of small budded cells in early stationary phase cultures of the mutant strains. This phenotype may implicate a role for the LIG4–LIF1 complex in growth regulation and/or cell cycle arrest. Two other experimental observations indirectly support such a model: (i) BRCT motifs are present in LIG4, and such motifs have been identified in other proteins involved in regulation of cell cycle progression which show a phenotypic link to DNA repair, e.g. budding yeast RAD9 and fission yeast Rad4 (Callebaut and Mornon 1997); and (ii) homozygous *lif1* and *lig4* diploids sporulate inefficiently due to a defect prior to meiosis I, but spore viability in the sporulating fraction of cells is normal. A simple explanation of this phenotype would be that the *lig4* and *lif1* mutants fail to respond efficiently to the nutritional signals which force wild-

type cells in G_1 to enter meiosis. If so, the sporulation inefficiency of the mutants would reflect a regulatory deficiency in G_1 of the cell cycle rather than a meiotic problem. Interestingly, a DNA ligase IV homologue of *Candida albicans* was cloned by complementation of an *ime1* mutation in *S.cerevisiae*, and *IME1* is part of a regulatory pathway leading to induction of meiosis in diploid budding yeast (Andaluz *et al*., 1996).

Strains lacking functional LIG4–LIF1 complexes display slight and conditional sensitivity to ionizing radiation. Whereas exponentially growing mutant and wild-type cells are not significantly different from each other (Figure 3A), *lif1* and *lig4* mutant strains become hypersensitive to irradiation in stationary phase (Figure 3B), and this phenotype is enhanced in *lig4 lif1* double mutants. The conditional radiosensitivity of yeast *lig4*, *lif1* and *lig4 lif1* mutant cells has an interesting parallel in the XRCC4-defective hamster XR-1 cell line which displays a unique cell-cycle-dependent radiosensitivity, being hypersensitive in G_1 phase and early S phase but not in later stages of the cell cycle (Stamato *et al*., 1988). In stationary phase, the sensitivity of either a *lif1* or *lig4* mutant is similar to that of a *rad52* mutant deficient in homologous recombination. In these cultures, cells are mainly in G_0 of the cell cycle; as they are haploid, their ability to repair double-strand breaks by homologous recombination is minimized and NHEJ pathways may be required. The synergistically enhanced sensitivity of the *rad52 lig4* double mutant indicates that RAD52- and LIG4–LIF1-dependent pathways contribute equally to repair of X-ray-induced DNA damage in resting yeast cells. Similarly, the enhanced radiosensitivity of the *lig4 lif1* double mutant indicates that, to some degree, LIF1 and LIG4 are also able to act independently of each other in protecting cells against radiation damage.

The most striking phenotype of *lif1* and *lig4* mutant cells is observed in a transformation-based plasmid double-strand break rejoining assay. Whereas wild-type cells are highly efficient in precise recircularization of linearized plasmids after transformation, this capacity is nearly abolished in *lif1* or *lig4* mutants (Figure 4B, see also Schär *et al*., 1997). The finding that wild-type cells religated blunt ends less efficiently (Figure 4B) and less accurately (Table I) than cohesive ends, whereas *lif1* or *lig4* mutants show no such difference, suggests that the LIG4–LIF1-dependent DNA joining pathway prefers DNA termini with protruding single strands to blunt ends as substrate. *LIF1* and *LIG4* appear to be epistatic with respect to this phenotype and, thus, act in the same pathway for non-homologous plasmid religation (Figure 4B).

In addition to LIG4 and LIF1, an appreciable number of yeast factors have been identified which appear to be required for efficient non-homologous religation of linearized plasmids after transformation. These include the Ku proteins, YKU70 and YKU80 (Boulton and Jackson, 1996a,b), the silencing factors SIR2, SIR3 and SIR4, and the recombination proteins RAD50, MRE11 and XRS2 (Boulton and Jackson, 1998). LIG4 and LIF1 are the only proteins among these which show an impaired plasmid repair phenotype but do not seem to be involved in telomere length maintenance (Boulton and Jackson, 1998; Figure 6). Comparing the fidelity of plasmid repair in strains transformed with linearized plasmid DNA having protruding single-strand termini, accurately ligated products can be found in the rare transformants of *lig4*, *rad50*, *mre11* and *xrs2* strains but not in *yku70*, *sir2*, *sir3* and *sir4* mutants, nor in *lif1* mutants (Table I; Boulton and Jackson, 1998). Thus, with respect to telomere length maintenance, *lif1* mutants seem identical to *lig4* mutants, but with respect to accuracy of plasmid religation they appear to be more similar to *yku70*, *sir2*, *sir3* and *sir4* mutant strains. These data imply that LIF1 may fulfil a structural role in association with LIG4, possibly acting as a mediator between factors protecting DNA ends, and the ligase which needs to be recruited to rejoin DNA double-strand breaks. Similar speculations have been made for the possible role of XRCC4 in mammalian cells (Critchlow *et al*., 1997). Detailed genetic analysis in the yeast system should clarify the role of the LIF1 protein further.

Materials and methods

Genetic methods, assessment of sporulation efficiency and radiation sensitivity

Yeast complete medium (YPD), pre-sporulation medium and synthetic drop out media were prepared as described by Sherman *et al.* (1982). Media for selection of respiration-proficient cells and sporulation medium were those described by Bähler *et al.* (1994). Strains were propagated and sporulated at 30°C, unless otherwise indicated. Transformations were performed by a slight modification of the high-efficiency lithium acetate method (Gietz and Schiestl, 1991). Analysis of transformants was performed as described (Schär *et al*., 1997). Mitotic growth, sporulation efficiency and spore viability of different strains were also examined as described previously (Schär *et al*., 1997).

Radiation sensitivity of cells was assessed as follows: 20 ml of YPD cultures were inoculated with 3×10^6 cells/ml from stationary phase pre-cultures and grown to late log–early stationary phase (~10^8 cells/ml) or late stationary phase ($>2\times10^8$ cells/ml) at 30°C with vigorous shaking. After plating serial dilutions from 10^5 cells to 10^1 cells onto YPD agar plates, cells were exposed to different doses of X-rays at varying dose rates (2.5, 5 or 10 Gy/min) using a Seifert, Isovolt X-ray generator (300 kV, 9.0 mA, 0.5 mm Al-filter). Incubations were at 30°C for 2–3 days before counting surviving clones.

Yeast strains

The *S.cerevisiae* strains used in this study are listed in Table II. They are all isogenic derivatives of two closely related, congenic series represented by FF18734 and FF18984 in an A364A background (F.Fabre, personal commununication) and were obtained by transformation and crossing within the set as indicated in Table II. Appropriate genotypes were isolated from dissected spore tetrads using standard replica plating techniques. The *LIF1* gene disruption was generated by standard gene replacement techniques using a PCR-derived disruption construct consisting of the *URA3* gene flanked by 80 bp of homologous DNA sequences from the 5′ and 3′ ends of the *LIF1* ORF (primer sequence available on request). For transformation of PRSY002, 1 μg of gel-purified PCR fragment was used. URA^+ transformants were first selected on media lacking uracil and then genotyped by replica plating onto diagnostic media, by standard genomic PCR and by genomic Southern blot analysis. Verified transformants carrying the expected heterozygous replacement within the *LIF1* ORF with the *URA3* gene (PRSY030,n) were then sporulated, and haploid progeny with appropriate genotypes were identified in dissected spore tetrads.

Plasmids, DNA manipulations and sequence analyses

Different fragments of *LIG4* were PCR-amplified from *S.cerevisiae* genomic DNA isolated according to standard procedures (Ausubel, 1994). PCR amplifications were carried out using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). All constructs were verified by sequencing using an ABI 377 DNA sequencer (Perkin Elmer, Foster City, CA).

Restriction sites were included in the PCR primers for subsequent in-frame cloning into the GAL4-BD vector pAS2-1 (Clontech, Palo Alto, CA); pGEH009 contains the entire *LIG4* ORF [LIG4(F)] cloned

Table II. *Saccharomyces cerevisiae* strains used

Strain	Genotype	Source or reference
FF18734	α *leu2-3 trp1-289 ura3-52 his7-2 lys1-1*	F.Fabre[a]
FF18984	**a** *leu2-3 ura3-52 his7-1 lys2-1*	F.Fabre
FF18743	α *leu2-3 trp1-289 ura3-52 his7-2 lys1-1 rad52::URA3*	F.Fabre
PRSY001	Diploid, FF18734/FF18984	Schär *et al*. (1997)
PRSY002	PRSY001 but LIG4/lig4::kanMX4	Schär *et al*. (1997)
PRSY003,1	α *leu2-3 trp1-289 ura3-52 his7 lys1-1 lig4::kanMX4*	Schär *et al*. (1997)
PRSY005	α *leu2-3 trp1-289 ura3-52 his7-2 lys1-1 lig4::kanMX4 rad52::URA3*	Schär *et al*. (1997)
PRSY030	PRSY002 but *LIF1/lif1::URA3*	PRSY002 transformant
PRSY031,a	α *leu2-3 trp1-289 ura3-52 his7 lys1-1 lif1::URA3*	PRSY030 spore clone
PRSY032	**a** *leu2-3 ura3-52 his7 lys2-1 lif1::URA3*	PRSY030 spore clone
PRSY033,a	α *leu2-3 trp1-289 ura3-52 his7 lys1-1 lig4::kanMX4 lif1::URA3*	PRSY030 spore clone
PRSY034	**a** *leu2-3 ura3-52 his7 lys2-1 lig4::kanMX4 lif1::URA3*	PRSY030 spore clone
yku70α	α *ade2 his3 leu2 trp1 ura3 can1-100 yku70::URA3*	S.Jackson[b]

[a]Institut Curie, Paris, France.
[b]Wellcome/CRC Institute, Cambridge, UK.

into the *Nde*I sites of pAS2-1. The primers to generate the *LIG4* fragment were GH72200, 5′-GCATGCATCATATGATATCAGCACTAGATTCTATACC-3′ (restriction sites underlined), and GH72199, 5′-GCATGCATCATATGTCAGTAGTTGACTACGGGG-3′. pGEH010 is pAS2-1 containing the N-terminal 255 amino acids of *LIG4* [LIG4(N)] and the primers were: GH72200 (see above) and GH89052, 5′-GCATGCATCATATGTCAGGCGAATGCAAAGCCAAC-3′. pGEH011 is pAS2-1 containing the coding sequence for the C-terminal 313 amino acids of *LIG4* [LIG4(C)] and the primers were: GH72199 (see above) and GH89051, 5′-GCATGCATCATATGACAGATTGTTACACACTTAACG-3′. pGEH012 [LIG4(B1)] and pGEH013 [LIG4(B2)] are also pAS2-1 derived and contain the coding sequences for each of the C-terminal BRCT domains of *LIG4* in the *Nde*I–*Bam*HI restriction sites. Primers used were GH89051 and GH91938, 5′-CGGGATCCTCAATCACCCAAACAATCTACCC-3′ for LIG4(B1), and GH72199 and GH91937, 5′-GCATGCATCATATGCTGTCATCATTGTATAAATC-3′ for LIG4(B2). pGEH015 is pBluescript KS(+/–) containing the entire *LIF1* ORF which was PCR-amplified with primers GH91714, 5′-CGAATTCGA*ATG*TCCCAGCTGACGGAGTTC-3′ (start codon in italics) and GH91715, 5′-GCTCTAGAAGGC*CTA*TGTTTCTATATCCG-3′ (stop codon in italics).

pGEH014 was used for complementation experiments in *lif1* mutants and was constructed by subcloning the *LIF1* ORF from pGEH015 into the *Sal*I–*Xba*I sites of the galactose-inducible yeast expression vector YCpIF3 (Foreman and Davis, 1994). pGEH019 is *LIF1* subcloned into *Eco*RI–*Pst*I sites of pAS2-1 and was used to subclone *LIF1* in-frame with the His tag in expression vector pET-16b (Novagen, Madison, WI) (pGEH020). pGEH007 contains the entire ORF of human DNA ligase IV (106 kDa form) with seven N-terminal histidine residues in the *Eco*RI site of the galactose-inducible yeast expression vector pYES2 (Invitrogen, Leek, Netherlands) (primer sequence available on request). pPRS154 contains the entire His-LIG4 ORF in pYES2 and has been described (Schär *et al*. 1997). pBTM116 is an *E.coli*–yeast shuttle vector carrying 2 μ sequences for stable maintenance in yeast, 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, unpublished).

For analysis of telomeric length maintenance, 2 μg of genomic DNA derived from yeast cells that were grown for >100 generations since sporulation were digested with 30 U of *Xho*I, separated on 0.8% agarose gels, Southern blotted and hybridized with a poly$(GT)_{20}$ probe as described by Boulton and Jackson (1998).

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 web page; for yeast genome database searches we accessed MIPS and SGD through their web pages.

Two-hybrid screening

Two-hybrid screening (Fields and Song, 1989) was performed using the Matchmaker two-hybrid system from Clontech, largely following the instructions provided by the manufacturer. A *S.cerevisiae* cDNA library was converted to a two-hybrid library by subcloning into the *Xho*I sites of the activation domain vector pACT2 (Clontech) and was kindly provided by Dr N.Lowndes, ICRF Clare Hall Laboratories. Initial library screening was performed using pGEH010 and pGEH011. Filter assays and quantitative β-galactosidase assays were carried out according to standard protocols (Transy and Legrain, 1995; Bai and Elledge, 1997).

Bacterial expression and purification of LIF1

Recombinant His-tagged LIF1 protein was expressed in *E.coli* BL21(DE3) from pGEH020 under control of the T7 RNA polymerase promoter. Pre-cultures of 10 ml of LB broth + 50 μg/ml carbenicillin were inoculated with four freshly transformed BL21(DE3)/pGEH020 colonies, incubated overnight at 37°C with vigorous shaking, and used for inoculation of 1 l cultures (NZY-Broth + 0.2% casein acid hydrolysate and 50 μg/ml carbenicillin). Cultures were grown at 37°C to an OD_{600} of 0.5 and expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were then incubated for 6 h at 37°C, harvested by centrifugation and lysed overnight in 6 M guanidine–HCl, 0.1 M Na_2HPO_4, 0.01 M Tris–HCl; pH 8.0 (bacterial lysis buffer) with constant rotation. After centrifugation of the lysate (20 min, 15 000 *g*), soluble proteins in the supernatant were batch adsorbed to 1 ml of pre-equilibrated (with bacterial lysis buffer) SuperFlow Ni-NTA agarose (Qiagen, Hilden, Germany) at 4°C for 1 h. The Ni-NTA resin was then packed into a disposable column (Bio-Rad, Herts, UK) and washed with 30 ml of wash buffer (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris–HCl, 15 mM imidazole, pH 8.0). Finally, bound proteins were eluted with the same buffer containing 250 mM imidazole, and LIF1-containing fractions were identified by SDS–PAGE.

Expression in yeast and purification of LIG4 protein

Recombinant, His-tagged LIG4 protein was expressed in PRSY003,1 (*lig4*) from pPRS154 under control of the *GAL1* promoter. Pre-cultures of 100 ml of SD medium lacking uracil were inoculated with four freshly transformed PRSY003,1/pPRS154 colonies, incubated overnight at 30°C with vigorous shaking, and used for inoculation of 1 l of culture YP-medium containing 2% raffinose as carbon source. After 2 h at 30°C with vigorous shaking, galactose was added to a final concentration of 2% and cells were grown for a further 6 h and harvested by centrifugation. Cells were then suspended in 1 vol. of 2× yeast lysis buffer (40% glycerol, 200 mM Tris–HCl pH 8.0, 700 mM NaCl, 8 mM β-mercaptoethanol, 0.25% Tween-20) and lysed by vortexing in the presence of acid-washed glass beads. The lysis buffer was supplemented with 2× proteinase inhibitors to minimize degradation of the proteins [leupeptin (500 ng/ml), pepstatin A (2.7 μg/ml), phenylmethylsulfonyl fluoride (PMSF; 340 μg/ml) and benzamidine (660 μg/ml)]. Cell debris was removed by centrifugation (20 min, 4°C, 15 000 *g*), and soluble proteins in the supernatant were batch adsorbed to 1 ml of pre-equilibrated (with 1× yeast lysis buffer) SuperFlow Ni-NTA agarose (Qiagen, Hilden, Germany) at 4°C for 1 h. The Ni-NTA resin was packed into a disposable column (Bio-Rad) and washed with 2× 20 ml of wash buffer (1× yeast lysis buffer, without proteinase inhibitors) containing 50 and 70 mM imidazole. Proteins were then eluted in buffer containing 20% glycerol, 100 mM Tris–HCl pH 8.0, 350 mM NaCl, 5 mM dithiothreitol (DTT), 0.25% Tween-20 and 500 mM imidazole. LIG4 peak fractions were identified by SDS–PAGE after silver staining (SilverStain Plus,

Bio-Rad) and dialysed against 20% glycerol, 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 0.25% Tween-20.

Western blot analysis

Yeast cells were incubated in 50 ml of YPD medium and grown to late log-phase (16 h). Crude lysates were prepared as described above, and equal amounts of protein (20 μg/lane) separated by SDS–polyacrylamide gel (8%) electrophoresis. After transfer of the proteins to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using the Mini-PROTEAN blotting system (Bio-Rad), they were blocked in phosphate-buffered saline (PBS) containing 5% (w/v) non-fat dried milk, 0.1% Tween-20 for 16 h at 4°C, then incubated with an anti-human DNA ligase IV antibody which was raised against highly conserved parts of the core domain of human DNA ligase IV (TL14/15) (Robins and Lindahl, 1996) at a dilution of 1:200 in PBS containing 1% (w/v) non-fat dried milk, 0.1% Tween-20 for 1 h at room temperature. After several washes in this buffer, the membrane was incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase (Pierce, Rockford, IL). After additional wash steps, the membrane was incubated with ECL solution (Amersham, Bucks, UK) and exposed to X-ray film. To reduce unspecific background, the primary antibody was pre-incubated with 100 mg of protein extract from a *lig4* strain immobilized on a nitrocellulose membrane in PBS containing 1% (w/v) non-fat dried milk and 0.1% Tween-20 at 4°C overnight.

Northern blot analysis

Yeast cells were cultured in 50 ml of YPD overnight at 30°C, and 1.5 ml of late-log phase cells were harvested by centrifugation and lysed in TRIzol™ reagent (Gibco-BRL) by vortexing in the presence of acid-washed glass beads. Total RNA was then extracted, and equal amounts of RNA (10 μg/lane) were separated on a 1% denaturing agarose gel and transferred to a Hybond-N membrane according to the protocol provided by the manufacturer (Amersham, Bucks, UK). Hybridizations were carried out at 42°C (Sambrook *et al.*, 1989) and followed by two washes in 2× SSC, 0.05% SDS at room temperature and two washes in 2× SSC, 0.1% SDS at 50°C. The membrane was then exposed to X-ray film. The *LIG4*-specific probe was generated by *Nde*I digestion of pGEH009 and purification of the 2.8 kb *LIG4* fragment. ^{32}P-labelling was performed with the Multiprime DNA Labelling Kit (Amersham) and the probe was purified from unincorporated nucleotides on a Sephadex G-50 column (Pharmacia Biotech).

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