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Biochemical and genetic characterization of the DNA ligase encoded by *Saccharomyces cerevisiae* open reading frame YOR005c, a homolog of mammalian DNA ligase IV

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ABSTRACT

Here we demonstrate that the *Saccharomyces cerevisiae* DNA ligase activity, which we previously designated DNA ligase II, is encoded by the genomic DNA sequence YOR005c. Based on its homology with mammalian *LIG4*, this yeast gene has been named *DNL4* and the enzyme activity renamed Dnl4. In agreement with others, we find that *DNL4* is not required for vegetative growth but is involved in the repair of DNA double-strand breaks by non-homologous end joining. In contrast to a previous report, we find that a *dnl4* null mutation has no effect on sporulation efficiency, indicating that Dnl4 is not required for proper meiotic chromosome behavior or subsequent ascosporogenesis in yeast. Disruption of the *DNL4* gene in one strain, M1-2B, results in temperature-sensitive vegetative growth. At the restrictive temperature, mutant cells progressively lose viability and accumulate small, nucleated and non-dividing daughter cells which remain attached to the mother cell. This novel temperature-sensitive phenotype is complemented by retransformation with a plasmid-borne *DNL4* gene. Thus, we conclude that the abnormal growth of the *dnl4* mutant strain is a synthetic phenotype resulting from Dnl4 deficiency in combination with undetermined genetic factors in the M1-2B strain background.

INTRODUCTION

Three mammalian genes encoding DNA ligases, *LIG1*, *LIG3* and *LIG4*, have been identified (reviewed in 1). The enzymes encoded by the *LIG1* and *LIG3* genes were characterized prior to cloning of the genes (2–6). In contrast, the *LIG4* gene was isolated before DNA ligase IV activity had been detected (6,7). Although these enzymes are responsible for the DNA joining steps in the pathways of DNA replication, DNA repair and genetic recombination that occur in mammalian cells, the cellular functions of each of the DNA ligases have not been definitively established. There is substantial experimental evidence demonstrating that DNA ligase I is the enzyme that joins Okazaki fragments during DNA replication (8–11). However, the sensitivity of both DNA ligase I- (8,12) and DNA ligase III-deficient cell lines (13,14) to alkylating agents indicates that the DNA ligases may play distinct roles in the cellular responses to DNA damage.

The *Saccharomyces cerevisiae CDC9* gene, which encodes a DNA ligase, was initially identified in a screen for conditional lethal cell division cycle mutants (15). Subsequently, it was demonstrated that *cdc9* mutants exhibit hypersensitivity to a wide range of DNA damaging agents and hyper-recombination (16–19). Based on amino acid sequence homology, the polypeptide encoded by the *CDC9* gene is most similar to human DNA ligase I, suggesting that these enzymes may be functionally equivalent (1,5). In agreement with this notion, mammalian DNA ligase I and Cdc9 DNA ligase have the same polynucleotide substrate specificity (20) and cDNAs encoding human DNA ligase I complement the conditional lethal phenotype of an *S.cerevisiae cdc9* mutant (5).

Until recently it had been assumed that Cdc9 DNA ligase was the only species of DNA ligase in *S.cerevisiae*. However, the partial purification and characterization of a yeast DNA ligase activity, DNA ligase II, which is biochemically and immunologically distinct from Cdc9 DNA ligase (21) and the identification of an open reading frame, YOR005c, in the yeast genome that contains motifs which are conserved in ATP-dependent DNA ligases suggests that yeast cells also contain more than one species of DNA ligase (21–24). Here, we demonstrate that DNA ligase II is encoded by YOR005c. Given the homology between the amino acid sequences of YOR005c and mammalian DNA ligase IV (21–24), the open reading frame will be referred to as *DNL4* and the enzyme activity will be renamed Dnl4.

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MATERIALS AND METHODS

Expression plasmid constructs

The YOR005c (*DNL4*) ORF was amplified by the PCR (25) from yeast genomic DNA, using Pfu polymerase and primers [sense, d(CGGGATCC**ATG**ATATCAGCACTAGATTCTATA); antisense, d(AACTGCAG**TTA**AATGCTGTTCAGATATTGCTT)] that anneal across the YOR005c ORF translation start and stop codons (bold), respectively. The underlined *Bam*HI and *Pst*I restriction sites were added to facilitate subcloning of the PCR product. After purification using a Qiaquick PCR Purification Kit (Qiagen), the PCR product was digested with *Bam*HI and *Pst*I and subcloned into pBluescript IIKS (Stratagene). The nucleotide sequences at the ends of the PCR fragment were confirmed by double-strand DNA sequencing prior to subcloning in-frame into the *Escherichia coli* expression vectors pGSTag (26) and pQE30 (Qiagen), to produce GST and poly(histidine) fusion proteins, respectively. In addition, the YOR005c (*DNL4*) ORF was subcloned into the yeast expression vector pTB326 to produce the plasmid pWR-YOR005c, in which the ORF is expressed from the ADH promoter.

In vitro transcription and translation

Coupled *in vitro* transcription and translation reactions were performed with the TNT Coupled Reticulocyte Lysate System (Promega) using [^{35}S]methionine as recommended by the manufacturer. Labeled polypeptides were partially purified by ammonium sulfate precipitation as described (27).

Bacterial and yeast strains

The temperature-sensitive, ligase-defective *E.coli* strain AK76 *lig* ts7 was provided by Francis Barany (Cornell University Medical College). The congenic *S.cerevisiae* strains, M1-2B and L94-4D (Table 1), were supplied by Lee Johnston (National Institute of Medical Research, UK).

Construction and verification of *dnl4* mutant strains

The *DNL4* gene (YOR005c) was inactivated in several different yeast strains (Table 1) using the one-step gene disruption technique (28). Sense [d(GCCAAGCTTCGGTGGTCGAGTGTTCTT)] and antisense [d(CGGAAGCTTTATCGTGATGAGCAAGAAGGGA)] primers, which anneal 1 kb upstream of the YOR005c ORF translation start codon and 1 kb downstream of the stop codon, respectively, were used to amplify YOR005c (*DNL4* gene) and flanking regions of DNA from the genome of the *S.cerevisiae* strain M1-2B by PCR. The underlined *Hin*dIII restriction sites were added to facilitate subcloning of the PCR product into the *Hin*dIII site of pBluescript IIKS. The *S.cerevisiae URA3* gene was amplified from the plasmid pYES2 (Invitrogen) by PCR with the following primers: sense, d(ACCTCCGGACTCATCTTTGACAGCTTATCATCG) (underlined *Bsp*EI site); antisense, primer d(TCGCAGCTGCCCGGCGTCAATACGGGATAAT) (underlined *Pvu*II site). To construct a *dnl4*::*URA3* null allele, the 600 bp *Bsp*EI–*Eco*47III fragment of the YOR005c ORF, which encodes the putative adenylation site of the DNA ligase (1), was replaced by blunt end ligation of the 1.1 kb *Bsp*E1–*Pvu*II PCR fragment that encodes the *URA3* gene. The resultant plasmid, pdnl4::URA3, was digested with *Hin*dIII to release the *dnl4*::*URA3* null allele with flanking DNA sequences to direct targeted gene replacement at the *DNL4* locus by homologous recombination during yeast transformation. The genomic structure of URA3$^+$ colonies that grew on drop-out medium lacking uracil was analyzed by Southern blotting (29) and/or PCR to identify strains in which the *DNL4* gene had been replaced by the *dnl4*::*URA3* null allele. Tetrad analysis of the transformants in two related strain backgrounds, 'SideXΔ' and 'SideYΔ' (Table 1), demonstrated single gene segregation for the disrupted *dnl4*::*URA3* null allele. In addition, the observed genetic segregation of the *dnl4*::*URA3* null allele in both genetic backgrounds was confirmed physically by Southern blotting analysis of the tetrad products. The identical phenotypic and genetic behavior of the *dnl4*::*URA3* null allele in the multiply backcrossed hybrid, CG2706, compared with the primary disruptant hybrid, CG2596, demonstrates the single gene inheritance of the *dnl4*::*URA3* null allele and the absence of phenotypic or sporulation modifiers in these transformed strains.

Table 1. *Saccharomyces cerevisiae* strains and relevant genotypes

M1-2B	MATα *ura3 trp1*
WR-1	M1-2B *dnl4*::*URA3*
WR-2	M1-2B *dnl4*::*URA3*
WR-3	M1-2B *dnl4*::*URA3*
L94-4D	MATα *cdc9-7 ura3 trp1*
WR-4	L94-4D *dnl4*::*URA3*
CG2582	MAT**a** *Side X*Δ
CG2568	MATα *dnl4*::*URA3 Side X*Δ
CG2573	**a**/α HO SideXΔ/SideXΔ *dnl4*::*URA3*/*dnl4*::*URA3*
CG2579	**a**/α HO SideYΔ/SideYΔ *dnl4*::*URA3*/*dnl4*::*URA3*
CG2583	**a**/α HO SideXΔ/SideXΔ *DNL4*/*DNL4*
CG2584	**a**/α HO SideYΔ/SideYΔ *DNL4*/*DNL4*
CG2595	**a**/α ho SideXΔ/SideYΔ *dnl4*::*URA3*/*dnl4*::*URA3*
CG2596	**a**/α ho SideXΔ/SideYΔ *dnl4*::*URA3*/*dnl4*::*URA3*
CG2597	**a**/α ho SideXΔ/SideYΔ *dnl4*::*URA3*/*DNL4*
CG2706	**a**/α ho SideXΔ/SideYΔ *dnl4*::*URA3*/*dnl4*::*URA3*, multiply backcrossed

Partial purification and assay of Cdc9 and Dnl4 from yeast

Yeast cells from a 1 l culture were harvested and resuspended in 50 mM Tris–HCl (pH 7.5), 0.75 M NaCl, 10 mM 2-mercaptoethanol, 1 mM KPO_4 and a cocktail of protease inhibitors (20). A clarified lysate, which was prepared as described previously (20), was applied to a hydroxyapatite column. Bound proteins were eluted stepwise with 50, 200 and 400 mM KPO_4 (pH 7.5) buffers, each containing 0.5 mM DTT and protease inhibitors. Protein in the eluates was quantitated by the method of Bradford (30). Fractions in each of the eluates that contained significant levels of protein were pooled and dialyzed against 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 50% glycerol and protease inhibitors prior to storage at –20°C. Assays to detect formation of labeled enzyme–adenylate complexes were carried out as described previously (21). After separation by

SDS–PAGE (31), labeled polypeptides were detected by autoradiography.

Analysis of cell viability and morphology

Overnight cultures, which were grown in the appropriate drop-out medium at 30°C, were diluted 1:50 in the same medium and then incubated at 37°C. At the indicated time intervals, aliquots (100 µl) were removed to determine the cell cycle distribution and viability. For the cell cycle distribution analysis, at least 1000 cells were classified as single cells, single cells with small buds, single cells with large buds or single cells with multiple small buds after visualization by light microscopy (400× magnification). To determine cell viability, cells in aliquots from the liquid cultures were counted under the light microscope (cells with one or more buds were counted as a single cell), diluted and then plated on the appropriate drop-out agar plates. After incubation at 30°C for 4–5 days, colonies were counted.

Sporulation and ascospore analysis

Frozen stocks of diploid strains were streaked directly onto a YPAD plate and incubated at 30°C for 2 days to form single colonies. Four independent colonies of each strain were patched onto a YPAD plate, incubated overnight at 30°C, replica plated to supplemented sporulation medium and incubated for 5 days at 30°C to allow completion of sporulation. Sporulation data obtained from the four independent colonies of each strain were examined for quantitative variation and pooled for subsequent analysis. Sporulated strains were examined by phase contrast light microscopy (320×) to assay the efficiency of sporulation, as calculated by the percent of the total population which formed phase bright, morphologically well-defined asci. To assay meiotic progression, the quality of sporulation (*r*) was calculated as the ratio of one and two spore asci (diagnostic for incomplete meiotic progression) to three and four spore asci (diagnostic for successful meiotic progression).

Viability of sporulation products was determined by micromanipulation and tetrad dissection on a YPAD plate followed by incubation for 3 days at 30°C to allow spore clone colony formation. For tetrad dissection, a loop of sporulated cells was incubated in 4% glusulase (NEN) for 4–5 min at 30°C to digest the ascal sac and to release the tetrad for micromanipulation on a Zeiss Tetrad Dissection Microscope (160×). Only complete four spore asci were chosen for dissection and viability analysis. To assay proper meiotic chromosome recombination and segregation, the viability pattern of dissected tetrads was determined and compared with the calculated wild-type pattern assuming random spore death.

RESULTS

The polypeptide encoded by YOR005c is a DNA ligase

ATP-dependent DNA ligases interact with ATP to form a covalent enzyme–AMP complex (1) in which the AMP moiety is linked to a lysine residue within a motif, KXDGXR, that is diagnostic for ATP-dependent DNA ligases (1). Searches of the *S.cerevisiae* genome with this motif identified the *CDC9* gene, as expected, and also a second open reading frame, YOR005c. The 944 amino acid polypeptide encoded by YOR005c contains other regions that exhibit homology with amino acid sequences conserved in the catalytic domain of eukaryotic DNA ligases and is most similar to human DNA ligase IV. Notably, the amino acid homology between the polypeptides encoded by YOR005c and the human *LIG4* gene is not restricted to the catalytic domain and in fact extends over the entire linear amino acid sequences (data not shown).

Although the *E.coli* DNA ligase utilizes NAD^+ as a co-factor (1), expression of ATP-dependent DNA ligases such as human DNA ligase I (32) and human DNA ligase III (Fig. 1) complements the conditional lethal phenotype of an *E.coli lig* mutant. In similar experiments, expression of YOR005c as a glutathione *S*-transferase (GST) (Fig. 1) and a poly(histidine) fusion protein (data not shown) enabled *E.coli* AK67 *lig* 7ts cells to grow at 42°C (Fig. 1). In contrast, *E.coli* AK67 *lig* 7ts cells harboring the empty vectors, either pGST-Tag (Fig. 1) or pQE30 (data not shown), failed to grow at the non-permissive temperature. This functional complementation of the conditional lethal phenotype of an *E.coli lig* mutant provides strong evidence that the polypeptide encoded by YOR005c has DNA ligase activity.

The open reading frame, YOR005c, encodes the activity previously designated as *S.cerevisiae* DNA ligase II

In a previous study we described the partial purification and characterization of a 90 kDa DNA ligase that we designated DNA ligase II, since it had different biochemical properties than Cdc9 DNA ligase and was not recognized by a Cdc9 antiserum (21). Although the predicted molecular weight of the polypeptide encoded by YOR005c is 104 000, a major labeled 90 kDa polypeptide was produced after *in vitro* transcription and translation of several independent isolates of YOR005c that were subcloned after PCR amplification from yeast genomic DNA. The electrophoretic mobility of the polypeptide labeled by *in vitro* translation of one such recombinant plasmid is shown in Figure 2A. The discrepancy between the calculated molecular weight and the observed molecular mass may be due to aberrant electrophoretic mobility of the polypeptide or translation from an internal methionine. Interestingly, the electrophoretic mobility of the *in vitro* translated YOR005c polypeptide is very similar to that of DNA ligase II isolated from *S.cerevisiae* (21).

To investigate the relationship between DNA ligase II and the DNA ligase encoded by YOR005c, we disrupted YOR005c in the haploid yeast strain M1-2B by homologous recombination. After fractionation of cell lysates by hydroxyapatite chromatography, we measured the levels of DNA ligase using an assay which monitors the formation of a labeled covalent enzyme–adenylate complex. In agreement with our previous study (21), the M1-2B extract contained two species of DNA ligase that could be resolved by hydroxyapatite chromatography (Fig. 2B, lanes 1 and 2). Cdc9 DNA ligase, which is eluted from the hydroxyapatite column by 200 mM KPO_4, was present at similar levels in the extracts from the M1-2B and M1-2B ΔYOR005c (WR-1) strains (Fig. 2B, compare lanes 1 and 3). In contrast, DNA ligase II was not detectable in the WR-1 extract after hydroxyapatite chromatography (Fig. 2B, lane 4), suggesting that YOR005c encodes DNA ligase II. To confirm this, WR-1 was transformed with a multicopy plasmid, pWR-YOR00Rc, that expresses YOR005c from an ADH promoter. As expected, DNA ligase II activity was restored to levels higher than those present in wild-type cells (Fig. 2B, compare lanes 2 and 6) whereas the level of Cdc9 DNA ligase was not significantly changed (Fig. 2B, compare lanes 1,

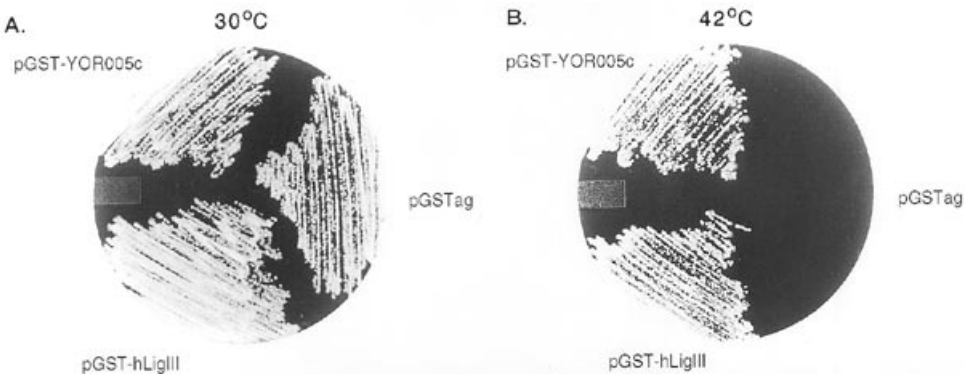

Figure 1. Effect of expression of the putative DNA ligase encoded by YOR005c (*DNL4*) on the temperature-sensitive phenotype of an *E.coli lig* mutant. The temperature-sensitive, ligase-defective *E.coli* strain AK76 *lig* ts7 transformed with the empty vector (pGSTag) and plasmids encoding GST–YOR005c (pGST-YOR005c) and GST–human DNA ligase IIIβ (pGST-hLigIII). Overnight cultures were streaked out on LB-AMP plates containing 2 mM IPTG. (**A**) Incubation overnight at 30°C. (**B**) Incubation at 30°C for 2 h and then at 42°C overnight.

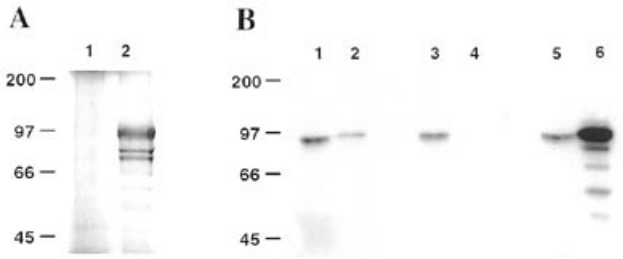

Figure 2. Size of the polypeptide encoded by YOR005c: effects of disruption and overexpression of YOR005c (*DNL4*) on yeast DNA ligase activities. (**A**) Coupled *in vitro* transcription and translation reactions were carried out with 2 μg of plasmid as described in Materials and Methods. Lane 1, pBluescript; lane 2, pBluescript-YOR005c (*DNL4*). (**B**) Yeast cell extracts were fractionated by hydroxyapatite chromatography as described in Materials and Methods. Proteins (10 μg) in the 200 and 400 mM KPO_4 eluates from the hydroxyapatite column were assayed for the ability to form labeled polypeptide–AMP complexes. Lane 1, M1-2B 200 mM KPO_4 eluate; lane 2, M1-2B 400 mM KPO_4 eluate; lane 3, WR-1 *dnl4*::*URA3* 200 mM KPO_4 eluate; lane 4, WR-1 *dnl4*::*URA3* 400 mM KPO_4 eluate; lane 5, WR-1 *dnl4*::*URA3* pWR-YOR005c (*DNL4*) 200 mM KPO_4 eluate; lane 6, WR-1 *dnl4*::*URA3* pWR-YOR005c (*DNL4*) 400 mM KPO_4 eluate. After denaturing gel electrophoresis, labeled polypeptides were detected in the dried gels by autoradiography. The positions of ^{14}C-labeled molecular mass standards (Amersham) are indicated on the left.

3 and 5). In conclusion, our results demonstrate that YOR005c encodes the activity that was previously designated yeast DNA ligase II. Since the DNA ligase encoded by YOR005c is homologous with human DNA ligase IV, we will refer to YOR005c as the *DNL4* gene and the gene product as Dnl4.

Inactivation of the *DNL4* gene does not confer sensitivity to DNA damaging agents

The viability of the WR-1 *dnl4*::*URA3* strain described above demonstrates that *DNL4* is not an essential gene. Furthermore, a *dnl4*::*URA3 cdc9-7* strain is viable even though extracts from the *cdc9-7* strain grown at the permissive temperature have no detectable DNA ligase activity (5). Inactivation of the *DNL4* gene in either a *CDC9* or *cdc9* genetic background does not increase sensitivity to killing by UV radiation or methylmethane sulfate (data not shown), indicating that the *DNL4* gene is not required for the repair of DNA damage that is recognized by the major DNA repair pathways operating in yeast.

In *S.cerevisiae*, the repair of DNA double-strand breaks occurs predominantly by a homologous recombination pathway that involves products of genes in the *RAD52* epistasis group. A pathway for the repair of DNA double-strand breaks by a non-homologous end joining (NHEJ) mechanism does exist in yeast cells and can be measured by recircularization of linearized plasmid molecules (33–35). Disruption of the *DNL4* gene reduced the transformation efficiency with linearized plasmid DNA compared with an isogenic wild-type strain 15- to 20-fold (data not shown), in agreement with previous reports (22–24). This deficiency presumably reflects an inability to recircularize the linear plasmid molecules by a NHEJ mechanism and implies that the product of the *DNL4* gene is required for the NHEJ pathway in yeast.

Inactivation of *DNL4* causes temperature-sensitive growth

During the experiments described above it became apparent that the WR-1 strain grew more slowly in liquid media compared with its isogenic parental strain. Results similar to those shown in Figure 3A were obtained with several independent *dnl4*::*URA3* isolates (Table 1) in the M1-2B genetic background. However, inactivation of the *DNL4* gene in two other genetic backgrounds, for example CG2568 (Table 1), did not result in abnormal growth. Further analysis of the WR-1 *dnl4*::*URA3* growth defect revealed that this strain, unlike its parental strain, was unable to grow at 37°C (Fig. 3B). Importantly, transformation of the WR-1 *dnl4*::*URA3* strain with a plasmid expressing the *DNL4* gene restored the ability of this strain to grow at 37°C (Fig. 3B), confirming the dependence of cell proliferation at 37°C upon *DNL4* gene function in this strain background. Furthermore, tetrad analysis of crosses between WR-1 and SideYΔ strains, either $DNL4^+$ or *dnl4*::*URA3* (not temperature-sensitive for growth) demonstrated multigenic inheritance and *dnl4*::*URA3*-dependence for segregation of the temperature-sensitive phenotype.

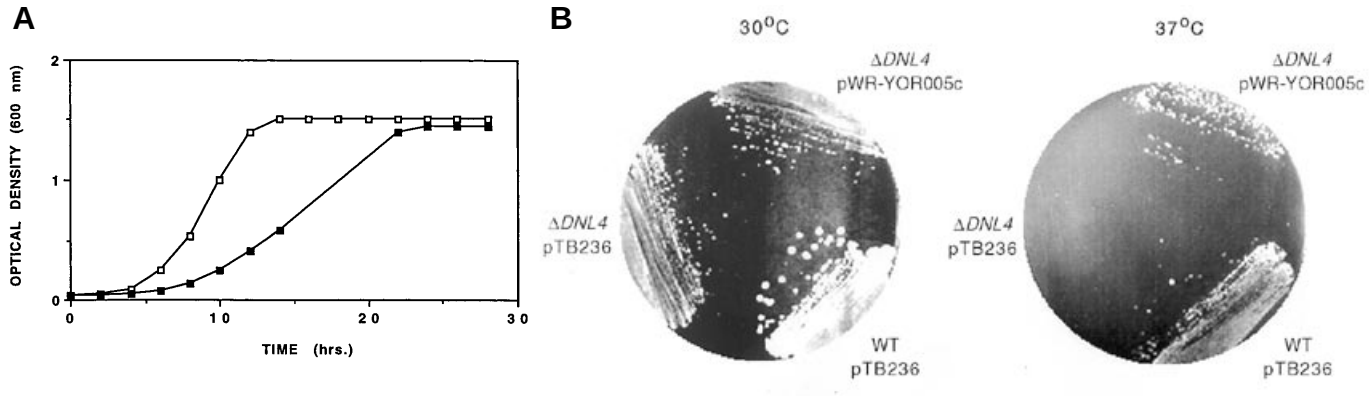

Figure 3. Effects of inactivation of the *DNL4* gene on cell growth. (**A**) Overnight cultures of WR-1 *dnl4*::*URA3* (filled-in squares) and its parental strain M1-2B (open squares) were diluted to 5×10^5 cells/ml with the appropriate selective medium and then incubated at 30°C. Proliferation was monitored by measuring absorbance at 600 nm. (**B**) Overnight cultures of WR-1 *dnl4*::*URA3* (Δ*DNL4*) and its parental strain M1-2B (WT) containing either the empty vector (pTB326) or the recombinant plasmid (pWR-YOR005c), in which YOR005c (*DNL4*) is expressed from an ADH promoter, were streaked onto drop-out plates lacking tryptophan. The plates were incubated for 4–5 days at 30 or 37°C as indicated.

Table 2. Viability and cell morphology of the *dnl4*::*URA3* strain at the non-permissive temperature

		Cell morphology			
		Cells with large buds (%)	Single cells (%)	Cells with small buds (%)	Cells with multiple buds (%)
M1-2B					
30°C	8 h	41	29	30	0
37°C	2 h	40	39	21	0
	8 h	45	33	22	0
	24 h	15	60	25	<1
M1-2B *dnl4*::*URA3*					
30°C	8 h	35	34	30	1
37°C	2 h	45	36	18	1
	8 h	35	40	20	5
	24 h	5	45	25	25
M1-2B *dnl4*::*URA3* pWR-YOR005c					
30°C	8 h	40	30	30	0
37°C	2 h	40	39	20	1
	8 h	38	37	24	1
	24 h	15	62	22	1

To further examine the temperature-sensitive growth phenotype, WR-1 cells growing exponentially in liquid medium at 30°C were transferred to 37°C and, at various time intervals, plated on solid medium. The viability of the WR-1 *dnl4*::*URA3* cells decreased progressively with time. After 24 h at 37°C, only 34% of the cells were able to form colonies. In contrast, 90% of the cells from the isogenic parental strain were able to form colonies after 24 h at 37°C. Introduction of a plasmid expressing the *DNL4* gene from a constitutively active ADH promoter markedly improved the viability of the *dnl4*::*URA3* strain to 65% after 24 h at 37°C (data not shown).

Next we examined the morphology of the yeast cells before and after the temperature shift by phase contrast microscopy. At 30°C, the WR-1 *dnl4*::*URA3* strain exhibited a small reduction in the percentage of cells with large buds and a small increase in the percentage of single cells compared with the isogenic parental strain (Table 2). After 24 h at 37°C, the cell cycle distribution in cultures of the M1-2B and WR-1 *dnl4*::*URA3* strains were dramatically different (Table 2). Notably, 25% of the *dnl4*::*URA3* cell population were large cells that appeared to have more than one bud. Examples of this abnormal cell morphology are shown in Figure 4A. This type of abnormal morphology was observed at a low frequency (1%) in the *dnl4*::*URA3* strain grown at 30°C but was not observed in the isogenic parental strain at this temperature (Table 2). Furthermore, transformation of the *dnl4*::*URA3* strain with a plasmid expressing the *DNL4* gene almost completely restored the morphology and cell cycle distribution of this strain to that of the isogenic parental strain, confirming the *DNL4* dependence of this phenotype (Table 2).

The DNA content of the *dnl4*::*URA3* cells that had been incubated for 24 h at 37°C was examined by DAPI staining. The majority of the putative buds on multiply budded cells contained DNA (Fig. 4B). This raised the question as to whether these structures were authentic buds. To test this, the cell population

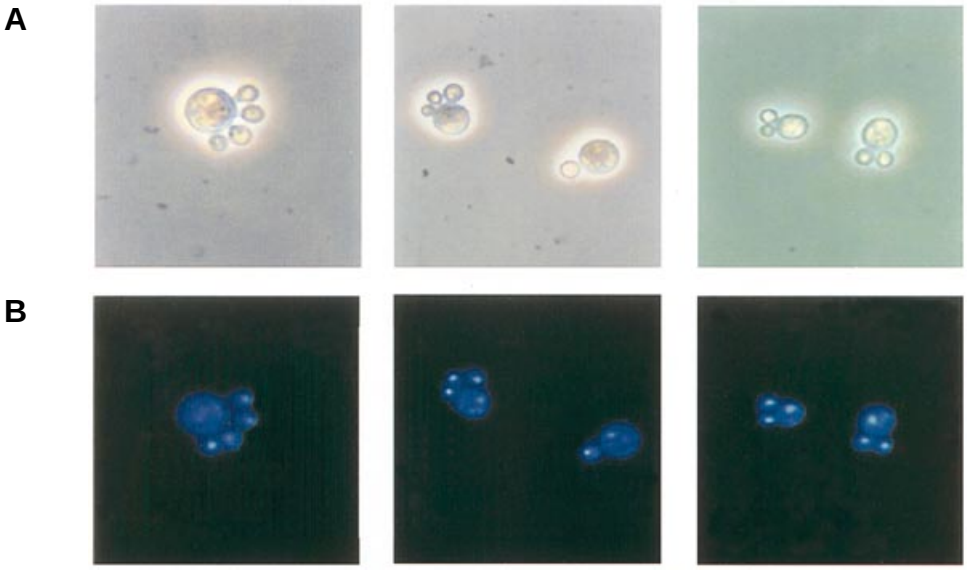

Figure 4. Analysis of the terminal morphology of WR-1 *dnl4*::*URA3* cells at the non-permissive temperature by fluorescence light microscopy. An overnight culture of WR-1 cells grown at 30°C on drop-out medium lacking tryptophan was diluted 1:50 in the same medium and then incubated at 37°C for 24 h. Aliquots (25 µl) were removed from the culture and diluted with 1 ml of deionized water. Cells were harvested, washed and then resuspended in 100 µl of deionized water. After the addition of 4′,6-diamino-2-phenolindole propidium iodide (DAPI) (5 µl of a 1 µg/ml solution), the suspension was incubated for 15 min at room temperature. Cells were harvested, washed twice with 1 ml of deionized water and then resuspended in 20 µl of deionized water. (**A**) Phase contrast microscopy (magnification 800×); (**B**) the same field visualized by epifluorescence using a Zeiss fluorescence microscope. Cell morphology was not altered by the DAPI staining protocol.

was incubated with zymolase, vortexed and then re-examined. Since growing buds are still linked by a cytoplasmic bridge to the mother cells, they are resistant to enzymes that degrade the yeast cell wall. Incubation with the cell wall-digesting enzyme zymolyase eliminated the population of cells that appeared to have more than one bud (Table 2). Thus, we conclude that these structures are not growing buds but instead are more likely to be inviable daughter cells that remain linked to the mother cell by the cell wall.

Inactivation of the yeast homologs that encode subunits of the heterodimeric DNA binding complex Ku also results in temperature-sensitive growth (33,34,36,37). In addition to participating in the NHEJ pathway, Ku is implicated in the maintenance of telomeres (33,34). Thus, it is possible that the growth defect in these strains is due to telomere shortening rather than a defect in NHEJ. The length of telomeres in cells from the WR-1 *dnl4*::*URA3* strain and its parental strain M1-2B were measured by hybridization of *Xho*I-digested genomic DNA with a labeled poly$(GT)_{20}$ probe (33). Since inactivation of the *DNL4* gene had no significant effect on telomere length (data not shown), we conclude that the temperature-sensitive growth of the WR-1 *dnl4*::*URA3* strain is not a consequence of progressive telomere shortening.

Table 3. Sporulation efficiency and quality of *dnl4*::*URA3* mutants at 30°C

Strain	Relevant genotype	No. of cells scored	Sporulation efficiency (%)	Sporulation quality (*r*)
CG2573	SideXΔ/SideXΔ *dnl4*/*dnl4*	3100	88.6	0.06
CG2579	SideYΔ/SideYΔ *dnl4*/*dnl4*	2100	89.6	0.08
CG2595	SideXΔ/SideYΔ *dnl4*/*dnl4*	800	90.3	0.04
CG2596	SideXΔ/SideYΔ *dnl4*/*dnl4*	800	88.5	0.07
CG2706	SideXΔ/SideYΔ *dnl4*/*dnl4*	1400	93.1	0.05
Subtotal	*dnl4*	8200	89.8	0.06
CG2597	SideXΔ/SideYΔ *dnl4*/*DNL4*	800	87.0	0.06
CG2583	SideXΔ/SideXΔ *DNL4*/*DNL4*	1100	86.8	0.07
CG2584	SideYΔ/SideYΔ *DNL4*/*DNL4*	2400	87.2	0.08
Subtotal	*DNL4*	4300	87.1	0.07

Sporulation experiments were performed as described in Materials and Methods. The quality of sporulation (*r*) was calculated as the ratio of one and two spore asci to three and four spore asci.

Table 4. Tetrad viability analysis of sporulation products of *dnl4*::*URA3* mutants

Strain	Relevant genotype	No. of tetrads	Spore viability (%)	Tetrad class (viable:inviable)				
				4:0	3:1	2:2	1:3	0:4
CG2573	SideXΔ/SideXΔ *dnl4*/*dnl4*	120	94.0	97	18	4	1	0
CG2579	SideYΔ/SideYΔ *dnl4*/*dnl4*	24	90	16	6	2	0	0
Subtotal	*dnl4* observed	144	93.4	113	24	6	1	0
	Theoretical random tetrad class distribution			110	31	3	0	0
CG2583	SideXΔ/SideXΔ *DNL4*/*DNL4*	120	91.5	92	16	11	1	0
CG2584	SideYΔ/SideYΔ *DNL4*/*DNL4*	24	90	16	6	2	0	0
Subtotal	*DNL4* observed	144	91.1	108	22	13	1	0
	Theoretical random tetrad class distribution			99	39	6	0	0

Spore viability was examined as described in Materials and Methods.

Inactivation of the *DNL4* gene has no effect on sporulation

The elevated levels of *LIG4* gene expression in human testis (6) suggests that DNA ligase IV may be involved in germ cell differentiation. Since yeast sporulation is functionally equivalent to gametogenesis in mammals, we have disrupted the *DNL4* gene in two different yeast strains and compared the sporulation behavior of these strains with isogenic parental strains. Inactivation of the *DNL4* gene had no significant effect on the efficiency or quality of sporulation (Table 3). Furthermore, we observed no difference in spore viability in either of the *dnl4*::*URA3* mutant strains compared with their isogenic wild-type parental strains (Table 4). These data demonstrate that normal meiotic chromosome behavior does not require the *DNL4* gene product.

Because disruption of the *DNL4* gene in the M1-2B genetic background (but not in the strain backgrounds used in the sporulation studies) results in temperature-sensitive vegetative growth, sporulation was examined at 35°C. The sporulation behavior of each of the *dnl4*::*URA3* strains was indistinguishable from that of its isogenic parental strain at the higher temperature (data not shown). In contrast to these results, it has been reported previously that *dnl4* mutants exhibit a delay during meiosis I, reduced sporulation efficiency and an increase in the proportion of inviable spores (22). Since the correction of this meiotic phenotype by reintroduction of the wild-type *DNL4* gene was not demonstrated, we suggest that the relatively subtle defects in sporulation previously ascribed to loss of Dnl4 activity (22) are more likely caused by secondary mutations introduced during disruption of the *DNL4* gene.

DISCUSSION

Previously we partially purified and characterized a DNA ligase activity from *S.cerevisiae* extracts that was distinct from Cdc9 DNA ligase (21). At the same time we reported that an open reading frame, YOR005c, which was identified during sequencing of the *S.cerevisiae* genome, exhibited homology with other eukaryotic DNA ligases, in particular human DNA ligase IV. To confirm that YOR005c does encode a DNA ligase, we have shown that expression of this polypeptide complements the temperature-sensitive phenotype of an *E.coli lig* mutant. To determine the relationship between YOR005c and the previously described yeast DNA ligase activities (20,21), the open reading frame was disrupted. This had no effect on Cdc9 DNA ligase but resulted in the loss of the activity that we originally designated DNA ligase II (21). Furthermore, this enzyme activity was restored by the introduction of a plasmid containing YOR005c into the *dnl4*::*URA3* mutant strain. Thus, we conclude that DNA ligase II activity is encoded by YOR005c and, because of the homology between YOR005c and human *LIG4*, we now refer to YOR005c as *DNL4* and the enzyme activity as Dnl4.

Disruption of the *DNL4* gene does not confer significant cellular sensitivity to DNA damage, indicating that this gene product does not play a critical role in the major DNA repair pathways operating in yeast. However, in the absence of Dnl4 activity, the cells exhibit a marked defect in the ability to recircularize transfected linear plasmid DNA molecules. These results are similar to those reported by other laboratories (22,24) and are consistent with the idea that Dnl4 functions in a non-homologous end joining pathway whose inactivation has a relatively minor effect on sensitivity to exogenous DNA damaging agents because homologous recombinational repair predominates in yeast. In support of this notion, mutation of the *DNL4* gene confers increased sensitivity to DNA damage only when the homologous recombination pathway has been inactivated (23,24). It appears that Dnl4 and Ku, which is a heterodimer that binds avidly to DNA double-strand ends, function in the same NHEJ pathway because disruption of both the *DNL4* and *HDF1* (which encodes one of the subunits of the Ku heterodimer) genes does not cause any further increase in sensitivity to DNA damage (23,24),

A distinguishing feature of one of the *dnl4*::*URA3* strains constructed in this study, WR-1, was its temperature-sensitive growth. It is unlikely that this phenotype is caused by other genetic changes introduced during gene disruption, since several independently isolated *dnl4*::*URA3* transformants in this genetic background exhibited the same phenotype and the temperature-sensitive growth defect was complemented by plasmid-borne copies of the *DNL4* gene. Crosses of WR-1 with a non-temperature-sensitive strain confirm our conclusion that the temperature-sensitive growth of WR-1 is a complex phenotype dependent upon both the *dnl4*::*URA3* mutation and additional epistatic interactions present in the M1-2B genetic background. Interestingly, *hdf* mutants also exhibit temperature-sensitive growth (33,34,36–38). It has been suggested that the abnormal growth of these strains is caused by their failure to maintain telomeres (33,34). This cannot account for the temperature-sensitive growth of the WR-1 *dnl4*::*URA3* strain, because telomere length

was found to be normal in these cells. Alternatively, it has been suggested that, in the absence of the NHEJ pathway, some forms of spontaneous DNA damage are not repaired and that the accumulation of this damage in the genome eventually triggers a cell cycle checkpoint (38). It is possible that the network of pathways which monitor genome integrity and transduce signals to the cell cycle apparatus after DNA damage are less efficient in the M1-2B cells and because of this difference these cells are extremely sensitive to the low levels of DNA damage that are normally repaired by the NHEJ pathway. It should be noted, however, that the terminal morphology observed in the *dnl4* strain at the non-permissive temperature is different from that observed in the *hdf* strains (38) and does not resemble that commonly associated with cell cycle arrest. Elucidation of the molecular basis for the terminal morphology of the *dnl4* strain awaits further investigation.

In humans, DNA ligase IV is highly expressed in the thymus and the testis (6). Given the predicted involvement of DNA ligase IV in V(D)J recombination (39,40), one would expect this gene to be highly expressed in lymphoid tissues but not in reproductive organs containing germ cells, which are characterized by a high frequency of homologous recombination. However, a previous study of yeast sporulation reported that inactivation of *DNL4* caused a delay during meiosis I, a reduced efficiency of sporulation and an increased proportion of inviable spores (22). In our study, inactivation of the *DNL4* gene in two different strains had no effect on sporulation. Based on these results we conclude that Dnl4 is dispensable for proper meiotic chromosome behavior and the production of haploid gametes in yeast. Since the previously reported meiotic defects were not shown to be uniquely conferred by the *dnl4* mutation (22), we suggest that they may be a consequence of secondary genetic changes introduced during gene disruption rather than Dnl4 deficiency.

The non-homologous end-joining pathway involving Dnl4 has been more extensively studied in mammalian cells. In these cells, the joining activity of DNA ligase IV is stimulated by an interaction with Xrcc4, a protein which is involved in NHEJ and V(D)J recombination (39–41). Xrcc4 also enhances DNA binding by Ku and the assembly of the DNA-dependent protein kinase (DNA-PK) (42). Although yeast lacks an obvious homolog of the catalytic subunit of DNA-PK, it does possess homologs of Ku (33–38) and, more recently, Lif1, a functional homolog of Xrcc4, has been identified (43). Thus, it appears that the fundamental mechanisms of NHEJ are conserved and so further biochemical and genetic studies of non-homologous end joining in yeast should continue to provide insights into the functionally homologous pathways in higher eukaryotes.

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REFERENCES

1 Tomkinson,A.E. and Levin,D.S. (1997) *BioEssays*, **19**, 893–901.
2 Tomkinson,A.E., Lasko,D.D., Daly,G. and Lindahl,T. (1990) *J. Biol. Chem.*, **265**, 12611–12617.
3 Husain,I., Tomkinson,A.E., Burkhart,W.A., Moyer,M.B., Ramos,W., Mackey,Z.B., Besterman,J.M. and Chen,J. (1995) *J. Biol. Chem.*, **270**, 9683–9690.
4 Chen,J., Tomkinson,A.E., Ramos,W., Mackey,Z.B., Danehower,S., Walter,C.A., Schultz,R.A., Besterman,J.M. and Husain,I. (1995) *Mol. Cell. Biol.*, **15**, 5412–5422.
5 Barnes,D.E., Johnston,L.H., Kodama,K., Tomkinson,A.E., Lasko,D.D. and Lindahl,T. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 6679–6683.
6 Wei,Y.-F., Robins,P., Carter,K., Caldecott,K., Pappin,D.J.C., Yu,G.-L., Wang,R.-P., Shell,B.K., Nash,R.A., Schar,P., Barnes,D.E., Haseltine,W.A. and Lindahl,T. (1995) *Mol. Cell. Biol.*, **15**, 3206–3216.
7 Robins,P. and Lindahl,T. (1996) *J. Biol. Chem.*, **271**, 24257–24261.
8 Barnes,D.E., Tomkinson,A.E., Lehmann,A.R., Webster,A.D.B. and Lindahl,T. (1992) *Cell*, **69**, 495–503.
9 Mackenney,V.J., Barnes,D.E. and Lindahl,T. (1997) *J. Biol. Chem.*, **272**, 11550–11556.
10 Levin,D.S., Bai,W. and Tomkinson,A.E. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 12863–12868.
11 Waga,S., Bauer,G. and Stillman,B. (1994) *J. Biol. Chem.*, **269**, 10923–10934.
12 Teo,I.A., Arlett,C.F., Harcourt,S.A., Priestly,A. and Broughton,B.C. (1983) *Mutat. Res.*, **107**, 371–386.
13 Thompson,L.H., Brookman,K.W., Jones,N.J., Allen,S.A. and Carrano,A.V. (1990) *Mol. Cell. Biol.*, **10**, 6160–6171.
14 Caldecott,K.W., McKeown,C.K., Tucker,J.D., Ljunquist,S. and Thompson,L.H. (1994) *Mol. Cell. Biol.*, **14**, 68–76.
15 Hartwell,L.H., Mortimer,R.K., Culotti,J. and Culotti,M. (1973) *Genetics*, **74**, 267–286.
16 Fabre,F. and Roman,H. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 4586–4588.
17 Game,J.C., Johnston,L.H. and von Borstel,R.C. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 4589–4592.
18 Johnston,L.H. and Naysmith,K.A. (1978) *Nature*, **274**, 891–893.
19 Johnston,L.H. (1979) *Mol. Gen. Genet.*, **170**, 89–92.
20 Tomkinson,A.E., Tappe,N.J. and Friedberg,E.C. (1992) *Biochemistry*, **31**, 11762–11771.
21 Ramos,W., Tappe,N.J., Talamantez,J., Friedberg,E.C. and Tomkinson,A.E. (1997) *Nucleic Acids Res.*, **25**, 1485–1492.
22 Schar,P., Herrman,G., Daly,G. and Lindahl,T. (1997) *Genes Dev.*, **11**, 1912–1924.
23 Teo,S.H. and Jackson,S.P. (1997) *EMBO J.*, **16**, 4788–4795.
24 Wilson,T.E., Grawunder,U. and Lieber,M.R. (1997) *Nature*, **388**, 495–498.
25 Saiki,R., Scharf,S., Faloona,F., Mullis,K., Horn,G., Ehrlich,H.A. and Arnheim,N. (1985) *Science*, **230**, 1350–1353.
26 Ron,D. and Dressler,H. (1992) *BioTechniques*, **13**, 866–868.
27 Bardwell,L., Cooper,A.J. and Friedberg,E.C. (1992) *Mol. Cell. Biol.*, **12**, 3041–3049.
28 Rothstein,R. (1991) *Methods Enzymol.*, **194**, 281–301.
29 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30 Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
31 Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
32 Kodama,K., Barnes,D.E. and Lindahl,T. (1991) *Nucleic Acids Res.*, **19**, 6093–6099.
33 Boulton,S.J. and Jackson,S.P. (1996) *EMBO J.*, **15**, 5093–5103.
34 Boulton,S.J. and Jackson,S.P. (1996) *Nucleic Acids Res.*, **24**, 4639–4648.
35 Milne,G.T., Jin,S., Shannon,K.B. and Weaver,D.T. (1996) *Mol. Cell. Biol.*, **16**, 4189–4198.
36 Feldmann,H., Driller,L., Meier,B., Mages,G., Kellermann,J. and Winnacker,E.-L. (1996) *J. Biol. Chem.*, **271**, 27765–27769.
37 Feldmann,H. and Winnacker,E.L. (1993) *J. Biol. Chem.*, **268**, 12895–12900.
38 Barnes,G. and Rio,D. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 867–872.
39 Grawunder,U., Wilm,M., Wu,X., Kulesza,P., Wilson,T.E., Mann,M. and Lieber,M.R. (1997) *Nature*, **388**, 492–495.
40 Critchlow,S.E., Bowater,R.P. and Jackson,S.P. (1997) *Curr. Biol.*, **7**, 588–598.
41 Taccioli,G.E., Gottlieb,T.M., Blunt,T., Priestley,A., Demengeot,J., Mizuta,R., Lehmann,A.R., Alt,F.W., Jackson,S.P. and Jeggo,P.A. (1994) *Science*, **265**, 1442–1445.
42 Leber,R., Wise,T.W., Mizuta,R. and Meek,K. (1998) *J. Biol. Chem.*, **273**, 1794–1801.
43 Herrmann,G., Lindahl,T. and Schar,P. (1998) *EMBO J.*, **17**, 4188–4198.