RESEARCH ARTICLE

A yeast-based genetic screening to identify human proteins that increase homologous recombination

Anita Collavoli, Laura Comelli, Giuseppe Rainaldi & Alvaro Galli

Laboratorio di Terapia Genica e Molecolare, Istituto di Fisiologia Clinica CNR, Area della Ricerca CNR Pisa, Italy

Correspondence: Alvaro Galli, Laboratorio di Terapia Genica e Molecolare, Istituto di Fisiologia Clinica CNR, Area della Ricerca CNR, via Moruzzi 1, 56125 Pisa, Italy. Tel.: +050 3153094; fax: +050 3153327; e-mail: a.galli@ifc.cnr.it

Received 26 July 2007; revised 11 October 2007; accepted 26 November 2007.
First published online 31 January 2008.

DOI:10.1111/j.1567-1364.2007.00343.x

Editor: Hyun Kang

Keywords
homologous recombination; human cDNA library; RAD52; methyl metanesulfonate.

Abstract

To identify new human proteins implicated in homologous recombination (HR), we set up 'a papillae assay' to screen a human cDNA library using the RS112 strain of *Saccharomyces cerevisiae* containing an intrachromosomal recombination substrate. We isolated 23 cDNAs, 11 coding for complete proteins and 12 for partially deleted proteins that increased HR when overexpressed in yeast. We characterized the effect induced by the overexpression of the complete human proteasome subunit β2, the partially deleted proteasome subunits α3 and β8, the ribosomal protein L12, the brain abundant membrane signal protein (*BASP1*) and the human homologue to v-*Ha-RAS* (H*RAS*), which elevated HR by 2–6.5-fold over the control. We found that deletion of the *RAD52* gene, which has a key role in most HR events, abolished the increase of HR induced by the proteasome subunits and H*RAS*; by contrast, the *RAD52* deletion did not affect the high level of HR due to *BASP1* and *RPL12*. This suggests that the proteins stimulated yeast HR via different mechanisms. Overexpression of the complete β2 human proteasome subunit or the partially deleted α3 and β8 subunits increased methyl methanesulphonate (MMS) resistance much more in the *rad52Δ* mutant than in the wild-type. Overexpression of *RPL12* and *BASP1* did not affect MMS resistance in both the wild-type and the *rad52Δ* mutant, whereas H*RAS* decreased MMS resistance in the *rad52Δ* mutant. The results indicate that these proteins may interfere with the pathway(s) involved in the repair of MMS-induced DNA damage. Finally, we provide further evidence that yeast is a helpful tool to identify human proteins that may have a regulatory role in HR.

Introduction

The budding yeast *Saccharomyces cerevisiae* is one of the most powerful genetic systems for analysing the basic functions of eukaryotic genes and studying the cellular factors involved in the fundamental processes of the cell (Botstein & Fink, 1988). Significant progress in understanding DNA replication, recombination and repair has been made on the basis of yeast genetics. Homologous recombination (HR) is essential in maintenance of genome stability in all organisms (Aylon & Kupiec, 2004a, b; Krogh & Symington, 2004). In mitotic cells, the primary role of HR is to repair double-strand breaks (DSBs). Most information regarding the genetic control and mechanism of HR comes from studies on the yeast *S. cerevisiae*, in which deletion of the *RAD52*, *RAD51* and *RAD54* genes profoundly impairs HR (Paques & Haber, 1999). Specifically, our understanding of the HR machinery is beginning to be elucidated by recent advances in the biochemical and structural characterization of recombinases and other factors that help the process of HR (Sung & Klein, 2006). Moreover, as HR is fundamental to genome integrity, it must be tightly regulated to avoid dangerous and potentially lethal events. Mutations in the tumour suppressor genes *BRCA1* or *BRCA2*, which have a regulatory function in HR, may lead to cancer. The *BLM* gene, found in mutated form in Bloom's syndrome, codes for a DNA helicase that may regulate the outcome of HR, leading to this cancer-linked disease (Karow *et al.*, 1997; Sung & Klein, 2006).

The frequency with which mitotic HR occurs can be modulated by several factors and processes. In yeast, mutations in genes primarily involved in transcription, DNA

replication or chromatin remodelling increase HR, suggesting that these processes may have a role in the regulation of HR (Aguilera *et al.*, 2000; Aguilera, 2002).

DSBs can be also repaired by another pathway, distinct from HR, named nonhomologous end-joining (NHEJ), which seems to compete with HR (Sonoda *et al.*, 2006). The balance and therefore the choice between HR and NHEJ in DSB repair seems to vary from yeast to vertebrate cells. In yeast, HR plays a dominant role, whereas in vertebrate cells, the contribution of NHEJ to repair of DSBs is much greater (Di Primio *et al.*, 2005; Sonoda *et al.*, 2006). To date, the reasons for this difference are not completely understood. Moreover, although the key regulators of HR are highly conserved from yeast to humans, the mutants behave differently. For example, the yeast *rad51*, *rad52* and *rad54* deletion mutants are viable and exhibit similar HR defects; by contrast, in higher eukaryotes, the *rad51* deletion is lethal and disruption of *RAD52* or *RAD54* has only an extremely mild phenotype (Sung *et al.*, 2000; Symington, 2002; Sung & Klein, 2006; Brugmans *et al.*, 2007).

Competition between HR and NHEJ was suggested by genetic evidence and by the ability of HR proteins RAD51 and RAD52, and the NHEJ protein Ku to bind the DNA ends (Allen *et al.*, 2003; Di Primio *et al.*, 2005). However, the nature and the control of this competition remain obscure. In yeast, deletion of the human counterpart NHEJ protein Ku70 has an influence also in HR, suggesting an interaction between HR and NHEJ (Cervelli & Galli, 2000). The characterization and the study of molecular and biochemical factors involved in the competition between HR and NHEJ could also have considerable impact on the therapeutic application of gene targeting mediated by HR. It has been demonstrated that a specific downregulation of key factors of the NHEJ or the overexpression of the yeast HR recombination *RAD52* protein improves the gene targeting efficiency in human cells (Pierce *et al.*, 2001; Di Primio *et al.*, 2005).

The identification of new proteins which could increase HR in human cells could help to understand how the cell can make the choice between the two pathways and, consequently, to improve genetic technologies such as gene targeting. To this end, we set up a genetic assay to screen a human cDNA expression library in yeast which allows us to identify clones with a high level of intrachromosomal HR. This paper describes the genetic system and the characterization of several clones with elevated HR frequency.

Materials and methods

Yeast strain

The diploid strain RS112 of *S. cerevisiae* (*MAT**a**/MATα, ura3-52/ura3-52 leu3-3,112/leu2-Δ98, trp5-27/TRP5 arg4-3/ARG4, ade2-40/ade2-101, ilv1-92/ilv1-92, HIS3::pRS6/his3Δ200 LYS2/lys2-801*) was provided by Robert Schiestl. The RS112 strain contains an intrachromosomal recombination substrate consisting of two terminally deleted *his3* alleles separated by the *LEU2* marker. As the two alleles share 400 bp in common, HR can occur, leading to *HIS3* reversion (Schiestl *et al.*, 1988).

Complete (YPAD), synthetic complete (SC) and drop-out (SD) media were prepared according to standard procedures.

Human cDNA library

The expression cDNA library was constructed from HeLa cells and inserted in the yeast expression plasmid pYES2 (InVitrogen). This vector carries the *URA3* marker to select for transformants and the 2 μ DNA replication origin for autonomous episomal replication. Moreover, the pYES2 plasmid allows cDNA expression on medium containing galactose because the cDNAs are placed under the control of the *GAL1* promoter. The library was a gift of Marco Foiani (IFOM Milan, Italy).

Genetic techniques

Yeast transformation with plasmid DNA was performed according to the procedure described by Gietz *et al.* (1995). The diploid RS112*rad52*Δ strain was constructed by the 'one-step gene replacement' technique (Rothstein, 1991). Briefly, the parental RSY6 (*MAT**a** ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5′-pRS6-his3Δ3′*) and Y433 *(MATα ura3-52 leu2-Δ98 ade2-101 ilv1-92 his3-Δ200 lys2-801)* strains were transformed with the pRad52Δ vector, provided by Akira Shinohara (Institute for Protein Research, Department of Biology, Osaka University, Osaka, Japan) and containing the *HisG-URA3-HisG* cassette flanking the *RAD52*-disrupted gene, linearized with EcoRI and SalI. Transformants were selected on SC-URA plates. The *URA3*$^+$ transformants were plated in media containing methyl methanesulphonate (MMS); only clones highly sensitive to 0.01% MMS were analysed further. Thereafter, the MMS-sensitive *ura3* clones were selected in medium containing 5-fluoro-orotic acid (5-FOA) as follows: five independent *URA3*$^+$ clones from each transformed strain were inoculated in 5–10 mL of YPAD medium and incubated under constant shaking for 24–48 h at 30 °C. Cells were then washed, counted and aliquots of $2–4 \times 10^5$ cells were plated on to SC medium containing 100 mg mL^{-1} 5-FOA (Boeke *et al.*, 1984). As the frequency of *URA3* pop-out is about 1×10^{-4}, we counted 20–40 colonies per plate (Boeke *et al.*, 1984). The loss of *URA3* marker was checked by streaking the 5-FOA-resistant clones in SC-URA plates. The diploid strain RS112*rad52*Δ*ura3*Δ was generated by 'mating' the two haploid parental strains according to standard procedures. The *RAD52* deletion was finally checked in the diploid strain by PCR amplification of the genomic DNA. The diploid

RS112*rad52*Δ*ura3*Δ was inoculated in 5–10 mL YPAD and incubated at 30 °C for 24 h. Genomic DNA was then extracted after zymolyase treatment, phenol/chloroform extraction and ethanol precipitation. DNA was quantified by gel electrophoresis. PCR amplification was performed using two *RAD52*-specific primers that allow the amplification of a 500-bp band. The primer sequence is available upon request.

Screening of the cDNA library

Saccharomyces cerevisiae RS112 was transformed with the cDNA library and transformants were selected by plating onto SC-URA 2% glucose medium. *URA3*$^+$ clones were then replicated onto SC-URA 5% galactose, in which the cDNAs were overexpressed, and onto SC-URA 2% glucose. Plates were incubated for 48 h at 30 °C, replicated onto SC-HIS plates and grown for 2–3 days at 30 °C to visualize microcolonies, termed 'papillae'. We compared levels of *HIS3* papillae derived from replica plating of SC-URA glucose vs. galactose plates and picked colonies with a greater number of papillae after galactose plating. Colonies showing an elevated frequency of intrachromosomal recombination were stored in 20% glycerol at − 80 °C for further analysis.

Intrachromosomal recombination assay

To confirm the increased HR level of the *URA3* clones, we determined the frequency of spontaneous intrachromosomal recombination in the RS112 diploid strain. This strain contains an intrachromosomal recombination synthetic substrate consisting of two terminal *his3* deletions that share 400 bp in common and are separated by the *LEU2* marker. An intrachromosomal recombination event leads to *HIS3* reversion and loss of *LEU2* (Schiestl *et al.*, 1988). Therefore, to determine the frequency of spontaneous intrachromosomal recombination, single *URA3*$^+$ colonies were inoculated into SC-URA-LEU and incubated at 30 °C for 24 h under constant shaking. Thereafter, the transformants were inoculated into SC-URA-LEU glucose and into SC-URA-LEU galactose and grown for 48 h at 30 °C. Finally, cultures were washed twice, counted and appropriate numbers were plated onto YPAD and SC-HIS plates to determine the surviving fraction and the frequency of intrachromosomal recombination, respectively. We calculated the HR frequency in at least four independent experiments and collected the data using the IBIOCAL software (kindly provided by Riccardo Favilla).

Plasmid DNA extraction and sequencing

URA3$^+$ clones were inoculated onto SC-URA and grown for 17–24 h at 30 °C. Briefly, cells were collected by centrifugation at 4000 ***g*** for 5 min and resuspended in 1 mL zymolyase solution (2 mg mL^{-1} 20T zymolyase, 100 mM KH_2PO_4, pH 7.5; 1.2 M sorbitol). Cell suspension was then incubated for up to 1 h at 37 °C. We then added 0.2 mL of lysis solution (Promega Wizard Minipreps), which was incubated for 5 min at 65 °C. Later, 0.2 mL of neutralization solution was added and samples were chilled for 15 min on ice. Plasmid DNA was purified from yeast lysate following the Promega Wizard Miniprep protocol and amplified by transformation in *Escherichia coli* DH5α. Plasmids were purified from *E. coli* using the Promega Wizard Minipreps kit. Finally, cDNAs were sequenced and analysed using the BLAST – human genome program (National Center for Biotechnology Information).

MMS survival assay

RS112 wild-type and *rad52*Δ strains expressing human cDNAs or containing the vector pYES2 were inoculated into SC-URA galactose at 30 °C for 24 h. Thereafter, cells were washed, counted and resuspended in 1 mL of distilled sterile water. Serial dilutions (10^{-1}–10^{-6}) were spotted onto galactose SC-URA medium lacking or containing MMS. Plates were incubated at 30 °C for 2–4 days. To evaluate the percentage of survivors, cells of the wild-type RS112 or strain RS112*rad52*Δ were inoculated at 30 °C for 24 h into SC-URA galactose medium lacking or containing different concentrations of MMS (50–400 μg mL^{-1}). Thereafter, cells were washed, counted and an appropriate number were plated onto YPAD. Plates were incubated at 30 °C for 2–3 days.

Data comparison and statistical analysis

Results were statistically analyzed using the Student's *t*-test.

Results

Human proteins that increased yeast HR when overexpressed

To identify new human proteins affecting HR, we set up a 'papillae' assay to screen a cDNA library of HeLa cells using the RS112 strain of *S. cerevisiae*, which allowed us to score intrachromosomal recombination events occurring between two truncated alleles of the *HIS3*$^+$ gene (Fig. 1a) (Schiestl *et al.*, 1988). The *HIS3*$^+$ recombinants can be visualized onto SC-HIS plates as microcolonies, or 'papillae'. As the cDNAs are inserted in the pYES2 vector under the control of the *GAL1* promoter, we compared the level of *HIS3* papillae after replica plating onto galactose to glucose, and we picked up those clones showing a higher level of papillae after replica plating in galactose as compared with that from glucose plating (Fig. 1b). We analysed 250 000 colonies and isolated 279 clones with an elevated level of *HIS3*$^+$ papillae.

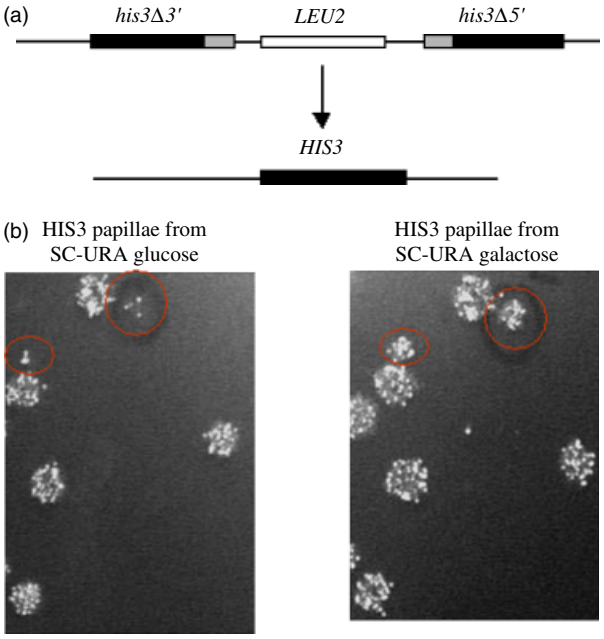

Fig. 1. Screening of a human cDNA library in *Saccharomyces cerevisiae* RS112. (a) The RS112 strain contains an intrachromosomal recombination substrate that measures deletion events between two *his3* terminally truncated alleles sharing 400 bp in common. The intrachromosomal recombination event leads to reversion to *HIS3* and loss of *LEU2* marker. (b) The human cDNA library was transformed in strain RS112 and *URA3* colonies were replicated in SC-URA 2% glucose and SC-URA 5% galactose plates (for details see 'Materials and methods'). Then, *URA3* colonies were replicated in SC-HIS 2% glucose to visualize the level of *HIS3* papillae. Clones giving a higher level of *HIS3* papillae after replica plating in SC-URA 5% galactose, in which the human proteins are overexpressed, as compared with the *HIS3* papillae derived from *URA3* colonies grown and replicated in SC-URA 2% glucose, in which the human proteins are repressed, were taken as hyper-recombinant. Here, we show how the hyper-recombinant colonies were chosen from the plates. In the red circles, two colonies with a higher level of *HIS3* after galactose growth are shown. The two clones were picked up from the SC-URA glucose plate. Papillae from the galactose sometimes appeared larger than those from glucose plates. This may be related to the higher concentration of galactose (5%) used in the plates as compared with glucose (2%). Regardless, only those clones with a visible higher level of *HIS3* after galactose growth were taken and the recombination frequency then measured as described in the 'Materials and methods'.

We also measured the intrachromosomal recombination frequency after growing the strains in glucose and in galactose and compared clone frequency. Forty-six clones had an increased HR level based on the intrachromosomal recombination frequency being significantly higher in galactose than in glucose ($P \leq 0.05$). Thereafter, plasmids were extracted and cDNAs sequenced. The sequences were further analysed to be certain of the correct frame. Finally, we were able to identify the proteins that, when overexpressed, increased intrachromosomal recombination in yeast. We found that 11 complete (Table 1) and 12 incomplete proteins (Table 2) induced a significant increase in HR in our diploid strain. We characterized the effect induced by the expression of four complete proteins, the human homologue to V-*Ha-RAS*, termed H*RAS2*, the brain abundant membrane attached signal protein (BASP1), the ribosomal protein L12 (*RPL12*) and the β2 human proteasome subunit, and two partially deleted proteins, the α3 and β8 human proteasome subunits. We chose to investigate the effect induced by the expression of these human proteasome subunits because recent studies have indicated that proteasome is involved in both HR and DSB repair in yeast and mammalian cells (Krogan *et al.*, 2004; Gudmundsdottir *et al.*, 2007; Murakawa *et al.*, 2007).

Effect of *RAD52* deletion on HR induced by human proteins

In *S. cerevisiae*, most HR events are caused by DSBs and are *RAD52*-dependent (Symington, 2002). *RAD52* deletion greatly reduces the frequency of intrachromosomal deletion events between homologous DNA sequences but the hyper-recombination phenotype conferred by the *pol3-t* or *rfa* mutations is only partially *RAD52*-dependent, suggesting that several mechanisms may account for DNA deletion events between repeats (Smith & Rothstein, 1995; Saparbaev *et al.*, 1996; Galli *et al.*, 2003). Therefore, to characterize the mechanism involved in the HR increase due to the overexpression of human proteins, we constructed a diploid strain, isogenic to RS112, carrying the *rad52* deletion. Complete deletion of the *RAD52* gene was confirmed by PCR analysis (see Fig. 3a below). Strain *rad52Δ* was then transformed with the plasmids encoding the entire β2 or the partially deleted α3 and β8 subunits of the proteasome as well as the complete proteins *BASP1* and H*RAS* or the ribosomal protein L12 (*RPL12*). Expression of the complete β2 or the deleted α3 and β8 proteasome subunits increased HR 2.6- to 2.6-fold and expression of H*RAS*, *BASP1* or *RPL12* induced HR 6.5-, 2.1- and 2-fold in the parental RS112 strain (Tables 1 and 2). Overexpression of each proteasome subunit as well as the *H*RAS protein did not lead to any increase of HR in the *rad52Δ* mutant, whereas the *RPL12* and the *BASP1* proteins increased HR in the *rad52Δ* as much as in the *RAD52* mutant (Tables 1 and 3). This suggests that these proteins increased intrachromosomal recombination in yeast by different mechanisms.

The human proteasome subunits increased the resistance to MMS

The main DNA lesion produced by MMS is methylation, primarily producing 3-methyladenine (3MeA). 3MeA is mainly repaired by base excision repair (BER), but some lesions can be converted to DSBs, which are repaired by NHEJ or HR (Chlebowicz & Jachymczyk, 1979; Beranek,

Table 1. Effect of the over-expression of several entire human proteins on intrachromosomal recombination in the RS112 strain of *Saccharomyces cerevisiae*

Clone	RF, Glucose	RF, Galactose	AI	*P*	Protein (gene)
1	0.52 ± 0.11	1.4 ± 0.13	2.7	0.0091	Benzodiazepine receptor, peripheral type (*BZRP*)
3	0.52 ± 0.05	2.7 ± 0.74	5.2	0.001	Ribosomal protein L18 (*RPL18*)
4	0.57 ± 0.18	1.49 ± 0.25	2.6	0.0005	Human proteasome, subunit β2
11	0.64 ± 0.2	1.49 ± 0.75	2.3	0.0400	Mitochondrial ribosomal protein L51 (*mtRPL51*)
16	0.3 ± 0.13	0.96 ± 0.34	3.2	0.0055	ATP synthase, H+transporting, mitochondrial F0 complex, subunit g (*ATP5L*)
18	0.35 ± 0.1	2.27 ± 1.17	6.5	0.0236	Harvey rat sarcoma viral oncogene, V-*Ha-RAS*, homolog isoform 2 (*HRAS*)
A15	1.54 ± 0.33	4.0 ± 0.6	2.6	0.024	Brain Abundant membrane signal protein (*BASP1*)
B1	1.5 ± 0.6	3.0 ± 1.3	2.0	0.047	Ribosomal protein L12 (*RPL12*)
D16	1.7 ± 0.4	4.82 ± 2.29	2.8	0.0179	Ribosomal protein P1 (*RPP1*)
D21	1.8 ± 0.8	6.2 ± 1.2	3.4	0.04	CD63 Antigen
E23	0.9 ± 0.2	2.0 ± 0.8	2.2	0.0175	Histine triad nucleotide binding protein 1 (*HINT1*)
	0.70 ± 0.13	0.60 ± 0.03	< 1	NS	pYES2

Experiments were carried out as follows: the cDNA containing vectors were transformed in the RS112 strain as described in the 'Materials and methods'. *URA*+transformants were picked up as single clones which were first grown in glucose SC-URA-LEU medium. Then, from each culture the same amount of cells was inoculated in parallel in glucose and galactose SC-URA-LEU and grown for 24 h. Thereafter, cultures were washed, counted, diluted and an appropriate number of cells were plated onto SC-HIS and complete media to score for recombinants and total cells respectively. Recombination frequency (RF), reported as number of *HIS3* colonies per 10^4 total cells. Results are reported as mean of five or more independent experiments ± SD. The absolute increase (AI) is calculated as the ratio between the RF in galactose and the RF in glucose. The *P* value was determined by the Student' *t*-test. The name of the protein and/or the cDNA was reported as found in the human genome database. NS, not statistically significant. $P \leq 0.05$ is considered statistically significant.

Table 2. Effect of the over-expression of several human partially encoded proteins on intrachromosomal recombination in the RS112 strain of *Saccharomyces cerevisiae*

Clone	RF, Glucose	RF, Galactose	AI	p	Protein (gene)	Encoded/total (aa)
2	0.57 ± 0.16	2.62 ± 0.84	4.6	0.0048	G1 to S phase transition factor (*GSPT1*)	222/628
5	0.41 ± 0.06	1.76 ± 0.23	4.3	0.0076	Ferritin heavy chain	69/190
6	0.48 ± 0.17	1.7 ± 1.02	3.5	0.0149	Ribosomal Protein L9 (*RPL9*)	116/192
9	0.41 ± 0.18	2.14 ± 1.32	5.2	0.0204	Transcription factor bHLH twist	91/202
19	0.34 ± 0.1	2.7 ± 1.47	7.9	0.0093	Cofilin 1	52/166
23	0.9 ± 0.1	1.99 ± 0.18	2.2	0.0087	Histone H1	85/213
B5	1.20 ± 0.13	2.5 ± 0.18	2.1	0.003	Replication Protein A (*RPA*)	60/180
D5	0.57 ± 0.18	1.48 ± 0.25	2.6	0.002	Proteasome β8 subunit	260/276
E4	1.1 ± 0.39	3.2 ± 0.7	2.9	0.036	Proteasome α3subunit	138/255
E15	1.2 ± 0.7	3.4 ± 1.2	2.8	0.0076	SKB1 methyl transferase	108/637
E37	2.2 ± 0.2	6.6 ± 2.3	3.0	0.003	ADP–ATP Carrier protein 2	269/292
E75	0.4 ± 0.08	0.9 ± 0.13	2.2	0.038	Ribosomal Protein L31 (*RPL31*)	72/125
	0.70 ± 0.13	0.60 ± 0.03	< 1	NS	pYES2	

See legend of Table 1. The portion of the protein encoded by the cDNA is reported.

1990; Kupiec, 2000; Lewis & Resnick, 2000). Recently, it has been shown that MMS does not induce DSBs in both yeast and mammalian cells (Lundin *et al.*, 2005). The number of alkylated sites converted to single-strand breaks and DSBs, however, may be too few to be detected by their assay, but enough to require the involvement of the DSB repair pathway. The authors also suggested that alkylation damage may stall the replication fork, leading to the formation of a chicken-foot structure, which resembles a Holliday junction (Lundin *et al.*, 2005). This may explain why the DSB repair mutants that are also deficient in recombination are sensitive to MMS (Lundin *et al.*, 2005). To determine whether overexpression of the proteasome subunits affects MMS resistance, two assays were carried out: a plate assay in which cells were forced to grow in a medium containing MMS, and a survival assay in which cells were exposed to different concentrations of MMS and then plated. Overexpression of the entire β2 or the partially deleted α3 and β8 proteasome subunits did not seem to alter the MMS resistance of the RS112 wild-type strain in the plate assay (Fig. 2a). However, in the survival assay, a weak increase in MMS resistance was induced by overexpression of the entire β2 or the partially deleted α3 and β8 proteasome subunits (Fig. 2b). At the highest MMS dose of $400\,\mu g\,mL^{-1}$, the proportion of

Table 3. Effect the over-expression of human proteins on intrachromosomal recombination In the RS112*rad52Δ* strain of *Saccharomyces cerevisiae*.

	His3 colonies/10^4survivors		
Protein	Glucose	Galactose	AI
*β*2 subunit, proteasome	0.17 ± 0.06	0.08 ± 0.01	< 1
α3 subunit, proteasome	0.14 ± 0.02	0.06 ± 0.03	< 1
*β*8 subunit, proteasome	0.13 ± 0.03	0.12 ± 0.04	< 1
BASP1	0.43 ± 0.013	0.96 ± 0.02	2.2
RPL12	0.68 ± 0.03	2.5 ± 0.02	3.6
HRAS	0.05 ± 0.01	0.09 ± 0.06	1.8
Control, pYES2	0.10 ± 0.03	0.08 ± 0.02	< 1

Experiments were carried out as follows: the cDNA containing vectors were transformed in the RS112*rad52Δ* strain as described in the 'Materials and methods'. *URA*+ transformants were picked up as single clones which were first grown in glucose SC-URA-LEU medium. Then, from each culture the same amount of cells was inoculated, in parallel, in glucose and galactose SC-URA-LEU and grown for 24 h at 30 °C. Thereafter, cultures were washed, counted, diluted and an appropriate number of cells were plated onto SC–HIS and complete media to score for recombinants and total cells respectively. Results are reported as mean of five or more independent experiments ± SD. AI is the absolute increase calculated as the ratio between the recombination frequency in Galactose and in Glucose.

survivors was $5.4 \pm 0.5\%$ for the control (pYES2), $35.3 \pm 7.8\%$ for the β8 subunit, $40.3 \pm 3.7\%$ for the β2 subunit and $24.8 \pm 5.8\%$ for the α3 subunit (Fig. 2b). We also measured the effect of overexpression of each proteasome subunit on MMS resistance in the RS112*rad52Δ* strain. As expected, the *rad52Δ* strain is very sensitive to MMS. All three subunits greatly increased MMS resistance in the *rad52Δ* mutant (Fig. 3b). At the highest MMS dose of 400 μg mL^{-1}, the proportion of survivors was $0.13 \pm 0.1\%$ for the control (pYES2), $26.5 \pm 5.2\%$ for the β8 subunit, $20.9 \pm 9.2\%$ for the β2 subunit and $5.2 \pm 3.0\%$ for the α3 subunit (Fig. 3c). Thus, when HR repair is defective, the α3, β2 and β8 subunits of the human proteasome could permit repair of MMS-induced DNA damage by another pathway. We also determined the effect of *BASP1*, H*RAS* and *RPL12* on MMS resistance in both wild-type and *rad52Δ* strains. In RS112, none of the proteins affected MMS resistance in the plate assay as compared with the control (pYES2) (Fig. 4a). In the *rad52Δ* mutant, expression of H*RAS* greatly decreased MMS resistance as compared with the control (pYES2); by contrast, *BASP1* and *RPL12* did not affect MMS resistance (Fig. 4b).

Discussion

Many proteins implicated in DSB repair by HR were identified using the yeast *S. cerevisiae* as a model eukaryote (Aylon & Kupiec, 2004a, b; Krogh & Symington, 2004). Moreover, HR must be regulated throughout the cell cycle and DNA replication to ensure genetic stability. As the HR machinery is widely conserved with evolution, we took advantage of yeast genetics to identify new human proteins which may increase HR in human cells and, therefore, could have a role in HR control and/or regulation. We identified 23 cDNAs, 11 coding for complete proteins and 12 C-terminal truncated proteins. It is surprising that we did not obtain any protein directly involved in HR or DNA repair. Indeed, overexpression of the main key HR proteins, Rad51 and Rad52, does not increase DSB-induced HR in yeast (Dornfeld & Livingston, 1991; Paffett *et al.*, 2005). We isolated six ribosomal proteins, three human proteasome subunits (the complete β2 and the partially deleted α3 and β8 subunits), the oncogene homologue H*RAS* and a transcriptional cosuppressor (*BASP1*) that have not previously been shown to have an influence on HR. The proteins most well represented were ribosomal proteins. Several ribosomal proteins are reported to have a secondary role independent of their involvement in protein biosynthesis (Wool, 1996). For instance, overexpression of the human ribosomal proteins L13a and L7 induces apoptosis and, in yeast, the deletion of the L13 homologue causes growth defects (Chen & Ioannou, 1999). Moreover, the *Drosophila* ribosomal protein S3 is involved in DNA BER (Cappelli *et al.*, 2003a, b). In yeast, we showed that overexpression of human ribosomal protein L12 increased HR by an *RAD52*-independent mechanism. Indeed, it would be tempting to investigate if overexpression of the yeast counterpart of each human ribosomal protein identified by this screening increases HR in yeast. However, ribosomal proteins are highly expressed in different human tissues and cancer cell lines, suggesting that in our cDNA library from HeLa cells, these proteins may be highly concentrated (Vaarala *et al.*, 1998; Bortoluzzi *et al.*, 2001). Moreover, in a study in which the gene expression level of multiple cDNA libraries was compared, three ribosomal genes were reported among the 21 most highly expressed (Stekel *et al.*, 2000). Therefore, we cannot rule out the possibility that identification of several ribosomal proteins in our screening is an artefact. Nevertheless, a study of whether overexpression of ribosomal proteins affects genome stability and/or DSB repair in human cells would be the best next step to validate our results.

Overexpression of *BASP1* increased yeast HR and this increase was *RAD52*-independent. *BASP1* is considered to be a signal protein participating in neurite outgrowth and, in fact, it is abundant in nerve terminals (Zakharov *et al.*, 2003). More recently, Basp1 has been shown to be a transcriptional cosuppressor for the WT1 protein, which was found to be related to Wilm's tumour (Carpenter *et al.*, 2004). Thus, Basp1 could interact with some yeast transcription factor and modulate HR. A similar *RAD52*-independent hyper-recombination phenotype has been previously observed for the *pol3-t* and *rfa1* mutants (Smith &

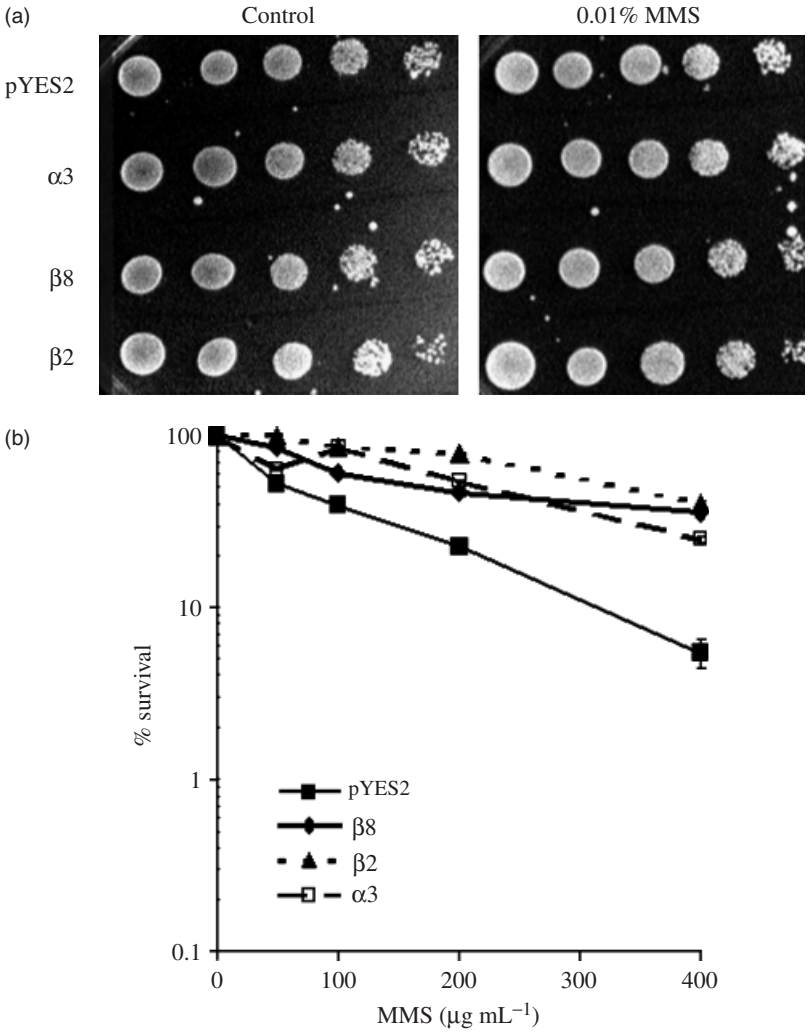

Fig. 2. Effect of expression of human proteasome subunits on MMS resistance in *Saccharomyces cerevisiae* RS112. (a) Serial dilutions of cultures of strain RS112 overexpressing the entire α3, or the partially truncated β2 or β8 proteasome subunits were spotted onto solid galactose media with or without (control) MMS. Plates were incubated at 30 °C for 2–3 days. (b) Then, 1×10^7 cells of the strain expressing each proteasome subunit were grown in 5 mL of SC-URA galactose in the presence of different MMS doses. Thereafter, cells were washed, counted and plated to score for survivors. Results are the mean ± SD of four independent experiments. Cells carrying the empty pYES2 vector were used as negative control.

Rothstein, 1995; Galli *et al*., 2003). The authors proposed that the *rfa*-mediated hyper-recombination is most probably due to a single-strand annealing mechanism (Smith & Rothstein, 1995).

The elevated HR induced by overexpression of the oncogene v-*Ha-RAS* homologue H*RAS* is *RAD52*-dependent. In mammalian cells, expression of H*RAS* increases the frequency of micronuclei and other chromosomal aberrations, indicating a chromosomal instability phenotype (Denko *et al*., 1994). More recently, it has been shown that H*RAS* induced defects in DNA damage and mitotic checkpoints, which may be the cause of this genomic instability (Knauf *et al*., 2006). Here, we have also provided further indications that overexpression of H*RAS* could stimulate HR between repeated DNA sequences.

We have also characterized the effect on HR induced by overexpression of the complete cDNA encoding the β2 subunit and the partially deleted cDNAs encoding the α3 and the β8 subunits of human proteasome. Deletion of the *RAD52* gene completely abolished the increased level of HR due to each human proteasome subunit. This suggests that the main functions involving Rad52 protein, such as DNA strand annealing and exchange, are required for the occurrence of this stimulation effect on yeast recombination. In yeast, it has been shown that some proteasome subunits are recruited to a DSB, suggesting a role for these components in DBS repair (Krogan *et al*., 2004). Recently, in human cells *BRCA2*, a DSB repair protein, was shown to interact with two proteasome subunits determining the choice of DSB repair pathway (Gudmundsdottir *et al*., 2007). Moreover, proteasome inhibitors have been demonstrated to suppress HR in mammalian cells, suggesting that the proteasome is required for the promotion of HR at an early step (Murakawa *et al*., 2007). Here, we show for the first time that overexpression of the entire β2 or the α3 and β8 deleted human proteasome subunits increase HR in yeast, confirming that human proteasome may have a role in regulating HR.

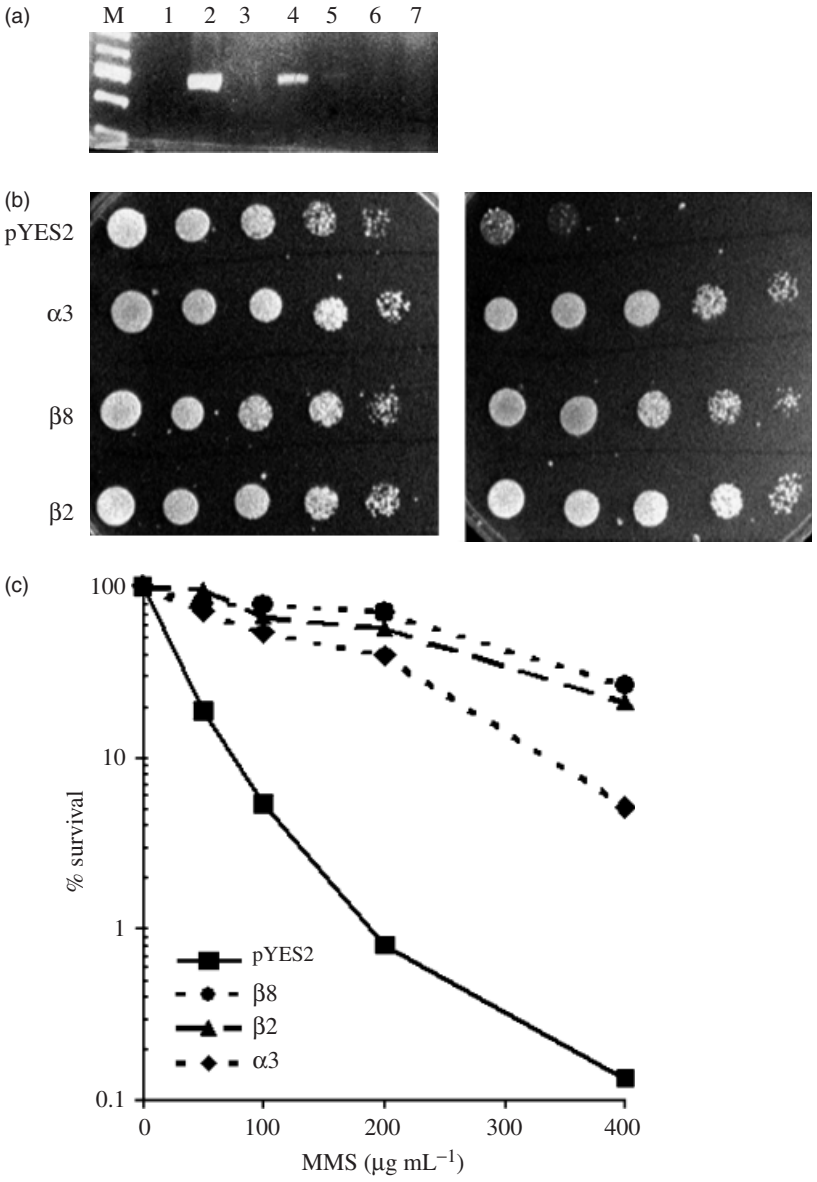

Fig. 3. Effect of expression of human proteasome subunits on MMS resistance in *Saccharomyces cerevisiae* RS112*rad52Δ*. (a) Complete deletion of the *RAD52* gene was confirmed by PCR analysis. Genomic DNA was extracted by three independent diploid strains derived by mating of highly MMS-sensitive RSY6 and Y433 strains, which had undergone selection for 5-FOA. PCR was carried out using two *RAD52*-specific primers that amplified a 500-bp band. PCR was then analysed by gel electrophoresis. Lanes: M, marker; 1, negative control; 2, amplification from a plasmid containing the entire *RAD52* gene; 3, from genomic DNA of a *rad52* deletion strain; 4, from genomic DNA of a RAD52 strain; 5–7, from genomic DNAs of three independent RS112*ura3* and MMS-sensitive strains. The strains whose DNA was run in lanes 6 and 7 contained the *rad52* deletion. (b) Serial dilutions of strain RS112*rad52Δ* overexpressing the entire β2, or the partially truncated α3 or β8 proteasome subunits were spotted onto solid SC-URA with or without (control) MMS. Plates were incubated at 30 °C for 2–3 days. (c) Then, 1×10^7 cells of the strain expressing each proteasome subunit were grown in 5 mL of SC-URA galactose in the presence of different MMS doses in the presence of different MMS doses. Thereafter, cells were washed, counted and plated to score for survivors. Results are the mean ± SD of four independent experiments. Cells transformed with the empty pYES2 vector were used as negative control.

We also investigated the effect of overexpression of the human proteasome subunits, *BASP1*, H*RAS* and *RPL12* on MMS resistance. MMS is a methylating agent that is thought to induce DNA breaks only because yeast mutants defective in the HR, such as *rad52Δ*, are very sensitive to MMS (Krogh & Symington, 2004). Recent experiments have shown that MMS does not produce detectable DSB *in vivo*, and therefore the authors speculated that the HR-deficient yeast mutants are highly MMS-sensitive because the MMS damage blocks the replication fork leading to the formation of structures resembling Holliday junctions that could be repaired by HR (Lundin *et al*., 2005). Moreover, we have previously showed that MMS induces more HR events in cell cycle-arrested than in dividing yeast cells, confirming that MMS does not directly produce DSB (Galli & Schiestl, 1999). Thus, the high level of HR we determined could not be related to an effect on MMS resistance. The expression of each subunit of the human proteasome, identified by this screening, conferred a higher MMS resistance in yeast, particularly when *RAD52* is deleted. This may suggest that when HR is defective the MMS-induced DNA damage is repaired by another pathway, possibly BER.

Among the other proteins identified, histone H1 is perhaps directly involved in HR. In yeast, the deletion of the gene coding for the histone H1, *hho1*, determines an increase in both HR and MMS resistance, suggesting an active role in controlling HR (Downs *et al*., 2003). When the human histone H1 is overexpressed in yeast, this could have

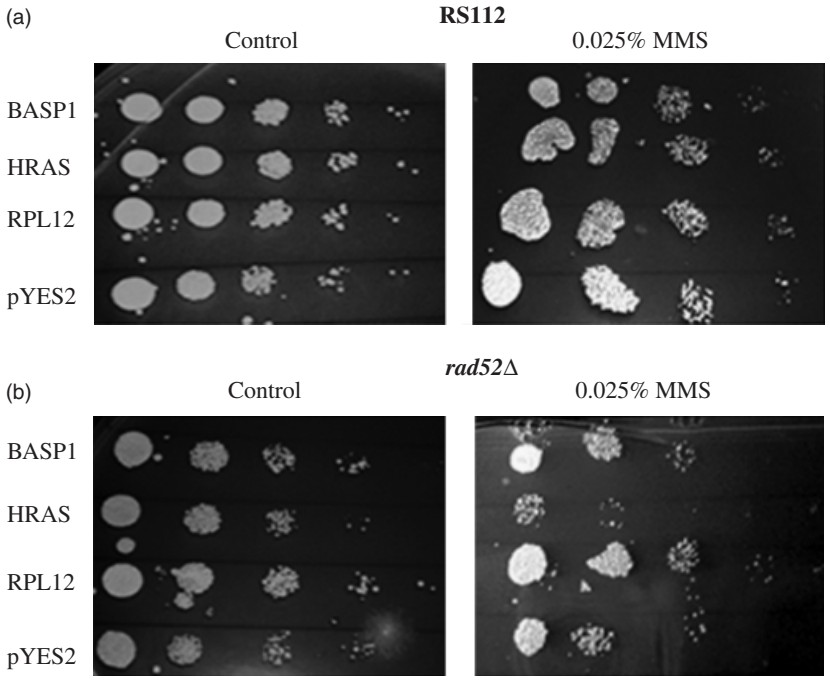

Fig. 4. Effect of the expression of BASP1, HRAS or RPL12 on MMS resistance in the *Saccharomyces cerevisiae* RS112 and RS112*rad52Δ*. Serial dilutions of yeast culture from (a) RS112 or (b) RS112*rad52Δ* expressing BASP1, HRAS or RPL12 were spotted onto galactose media containing MMS. The plates were then incubated at 30 °C for 2–3 days. Cells transformed with the empty pYES2 vector were used as negative control.

an impact on chromatin assembly and, consequently, affect the genome stability with an ultimate effect on HR. We also showed that overexpression of a small portion (60 amino acids out of 180) of Replication Protein A (RPA) increased HR in yeast. RPA is a single-strand DNA-binding protein that acts as a mediator of the Rad51 recombinase (Sung *et al*., 2003). Although *RPA* is not directly involved in HR, it is thought to be a DNA damage sensor protein which recognizes a DSB end (Lisby & Rothstein, 2005). Therefore, the elevated HR could result from activation of the DNA damage checkpoint.

In conclusion, we have set up a genetic screen to identify new proteins that may have a regulatory role in HR. Further characterization of six of 23 cDNAs indicated that these proteins may stimulate HR by different mechanisms and affect the MMS-induced DNA damage repair at different levels. Importantly, in the *rad52Δ* strain, the proteasome subunits greatly increased MMS resistance, but did not induce any increase in HR, suggesting that HR is not required to repair the MMS-induced DNA damage when these proteins are overexpressed. By contrast, in the *rad52Δ* strain, overexpression of H*RAS* decreased both MMS resistance and HR, indicating that HR is involved in the repair of MMS-induced lesions when H*RAS* is overexpressed. Therefore, we can hypothesize that these proteins may be involved in channelling the MMS-induced lesions in different DNA damage repair pathways, when HR is defective.

Taken together, the results obtained by this genetic screening may be important in initiating new analyses to determine whether these proteins affect HR and DSB repair in human cells. Moreover, the recent evidence that the proteasome is involved in HR in mammalian cells not only provides a validation to our genetic assay, but again confirms that *S. cerevisiae* is an excellent system to study how HR is regulated in eukaryotes.

Acknowledgements

We wish to thank Robert Schiestl, Marco Foiani and Akira Shinohara for the yeast strains, cDNA library and plasmids. This study was supported by an FIRB grant from the 'Ministero dell'Istruzione, Università e Ricerca' of the Italian Government.

References

Aguilera A (2002) The connection between transcription and genomic instability. *EMBO J* **21**: 195–201.

Aguilera A, Chavez S & Malagon F (2000) Mitotic recombination in yeast: elements controlling its incidence. *Yeast* **16**: 731–754.

Allen C, Halbrook J & Nickoloff JA (2003) Interactive competition between homologous recombination and non-homologous end joining. *Mol Cancer Res* **1**: 913–920.

Aylon Y & Kupiec M (2004a) DSB repair: the yeast paradigm. *DNA Repair (Amsterdam)* **3**: 797–815.

Aylon Y & Kupiec M (2004b) New insights into the mechanism of homologous recombination in yeast. *Mutat Res* **566**: 231–248.

Beranek DT (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat Res* **231**: 11–30.

Boeke JD, LaCroute F & Fink GR (1984) A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* **197**: 345–346.

Bortoluzzi S, d'Alessi F, Romualdi C & Danieli GA (2001) Differential expression of genes coding for ribosomal proteins in different human tissues. *Bioinformatics* **17**: 1152–1157.

Botstein D & Fink GR (1988) Yeast: an experimental organism for modern biology. *Science* **240**: 1439–1443.

Brugmans L, Kanaar R & Essers J (2007) Analysis of DNA double-strand break repair pathways in mice. *Mutat Res* **614**: 95–108.

Cappelli E, D'Osualdo A, Bogliolo M, Kelley MR & Frosina G (2003a) *Drosophila* S3 ribosomal protein accelerates repair of 8-oxoguanine performed by human and mouse cell extracts. *Environ Mol Mutagen* **42**: 50–58.

Cappelli E, D'Osualdo A, Bogliolo M, Xu Y, Kelley MR & Frosina G (2003b) Repair of 8 oxoguanine in mammalian cells expressing the *Drosophila* S3 ribosomal/repair protein. *Teratog Carcinog Mutagen Suppl* **1**: 113–121.

Carpenter B, Hill KJ, Charalambous M *et al.* (2004) BASP1 is a transcriptional cosuppressor for the Wilms' tumor suppressor protein WT1. *Mol Cell Biol* **24**: 537–549.

Cervelli T & Galli A (2000) Effects of HDF1 (Ku70) and HDF2 (Ku80) on spontaneous and DNA damage-induced intrachromosomal recombination in *Saccharomyces cerevisiae*. *Mol Gen Genet* **264**: 56–63.

Chen FW & Ioannou YA (1999) Ribosomal proteins in cell proliferation and apoptosis. *Int Rev Immunol* **18**: 429–448.

Chlebowicz E & Jachymczyk WJ (1979) Repair of MMS-induced DNA double-strand breaks in haploid cells of *Saccharomyces cerevisiae*, which requires the presence of a duplicate genome. *Mol Gen Genet* **167**: 279–286.

Denko NC, Giaccia AJ, Stringer JR & Stambrook PJ (1994) The human Ha-ras oncogene induces genomic instability in murine fibroblasts within one cell cycle. *Proc Natl Acad Sci USA* **91**: 5124–5128.

Di Primio C, Galli A, Cervelli T, Zoppe M & Rainaldi G (2005) Potentiation of gene targeting in human cells by expression of *Saccharomyces cerevisiae* Rad52. *Nucleic Acids Res* **33**: 4639–4648.

Dornfeld KJ & Livingston DM (1991) Effects of controlled RAD52 expression on repair and recombination in *Saccharomyces cerevisiae*. *Mol Cell Biol* **11**: 2013–2017.

Downs JA, Kosmidou E, Morgan A & Jackson SP (2003) Suppression of homologous recombination by the *Saccharomyces cerevisiae* linker histone. *Mol Cell* **11**: 1685–1692.

Galli A & Schiestl RH (1999) Cell division transforms mutagenic lesions into deletion-recombinagenic lesions in yeast cells. *Mutat Res* **429**: 13–26.

Galli A, Cervelli T & Schiestl RH (2003) Characterization of the hyperrecombination phenotype of the pol3-t mutation of *Saccharomyces cerevisiae*. *Genetics* **164**: 65–79.

Gietz RD, Schiestl RH, Willems AR & Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA /PEG procedure. *Yeast* **11**: 355–360.

Gudmundsdottir K, Lord CJ & Ashworth A (2007) The proteasome is involved in determining differential utilization of double-strand break repair pathways. *Oncogene* **26**: 7601–7606.

Karow JK, Chakraverty RK & Hickson ID (1997) The Bloom's syndrome gene product is a 3′–5′ DNA helicase. *J Biol Chem* **272**: 30611–30614.

Knauf JA, Ouyang B, Knudsen ES, Fukasawa K, Babcock G & Fagin JA (2006) Oncogenic RAS induces accelerated transition through G2/M and promotes defects in the G2 DNA damage and mitotic spindle checkpoints. *J Biol Chem* **281**: 3800–3809.

Krogan NJ, Lam MH, Fillingham J *et al.* (2004) Proteasome involvement in the repair of DNA double-strand breaks. *Mol Cell* **16**: 1027–1034.

Krogh BO & Symington LS (2004) Recombination proteins in yeast. *Annu Rev Genet* **38**: 233–271.

Kupiec M (2000) Damage-induced recombination in the yeast *Saccharomyces cerevisiae*. *Mutat Res* **451**: 91–105.

Lewis LK & Resnick MA (2000) Tying up loose ends: nonhomologous end-joining in *Saccharomyces cerevisiae*. *Mutat Res* **451**: 71–89.

Lisby M & Rothstein R (2005) Localization of checkpoint and repair proteins in eukaryotes. *Biochimie* **87**: 579–589.

Lundin C, North M, Erixon K, Walters K, Jenssen D, Goldman AS & Helleday T (2005) Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable *in vivo* DNA double-strand breaks. *Nucleic Acids Res* **33**: 3799–3811.

Murakawa Y, Sonoda E, Barber LJ *et al.* (2007) Inhibitors of the proteasome suppress homologous DNA recombination in mammalian cells. *Cancer Res* **67**: 8536–8543.

Paffett KS, Clikeman JA, Palmer S & Nickoloff JA (2005) Overexpression of Rad51 inhibits double-strand break-induced homologous recombination but does not affect gene conversion tract lengths. *DNA Repair (Amsterdam)* **4**: 687–698.

Paques F & Haber JE (1999) Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **63**: 349–404.

Pierce AJ, Hu P, Han M, Ellis N & Jasin M (2001) Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. *Genes Dev* **15**: 3237–3242.

Rothstein R (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol* **194**: 281–301.

Saparbaev M, Prakash L & Prakash S (1996) Requirement of mismatch repair genes MSH2 and MSH3 in the RAD1-RAD10 pathway of mitotic recombination in *Saccharomyces cerevisiae*. *Genetics* **142**: 727–736.

Schiestl RH, Igarashi S & Hastings PJ (1988) Analysis of the mechanism for reversion of a disrupted gene. *Genetics* **119**: 237–247.

Smith J & Rothstein R (1995) A mutation in the gene encoding the *Saccharomyces cerevisiae* single-stranded DNA-binding protein Rfa1 stimulates a RAD52-independent pathway for direct-repeat recombination. *Mol Cell Biol* **15**: 1632–1641.

Sonoda E, Hochegger H, Saberi A, Taniguchi Y & Takeda S (2006) Differential usage of non-homologous end-joining and homologous recombination in double strand break repair. *DNA Repair (Amsterdam)* **5**: 1021–1029.

Stekel DJ, Git Y & Falciani F (2000) The comparison of gene expression from multiple cDNA libraries. *Genome Res* **10**: 2055–2061.

Sung P & Klein H (2006) Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nat Rev Mol Cell Biol* **7**: 739–750.

Sung P, Trujillo KM & Van Komen S (2000) Recombination factors of *Saccharomyces cerevisiae*. *Mutat Res* **451**: 257–275.

Sung P, Krejci L, Van Komen S & Sehorn MG (2003) Rad51 recombinase and recombination mediators. *J Biol Chem* **278**: 42729–42732.

Symington LS (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol Mol Biol Rev* **66**: 630–670.

Vaarala MH, Porvari KS, Kyllonen AP, Mustonen MV, Lukkarinen O & Vihko PT (1998) Several genes encoding ribosomal proteins are over-expressed in prostate-cancer cell lines: confirmation of L7a and L37 over-expression in prostate-cancer tissue samples. *Int J Cancer* **78**: 27–32.

Wool IG (1996) Extraribosomal functions of ribosomal proteins. *Trends Biochem Sci* **21**: 164–165.

Zakharov VV, Capony JP, Derancourt J, Kropolova ES, Novitskaya VA, Bogdanova MN & Mosevitsky MI (2003) Natural N-terminal fragments of brain abundant myristoylated protein BASP1. *Biochim Biophys Acta* **1622**: 14–19.