Human and mouse homologs of the *Saccharomyces cerevisiae RAD54* DNA repair gene: evidence for functional conservation

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Background: Homologous recombination is of eminent importance both in germ cells, to generate genetic diversity during meiosis, and in somatic cells, to safeguard DNA from genotoxic damage. The genetically well-defined RAD52 pathway is required for these processes in the yeast *Saccharomyces cerevisiae*. Genes similar to those in the RAD52 group have been identified in mammals. It is not known whether this conservation of primary sequence extends to conservation of function.

Results: Here we report the isolation of cDNAs encoding a human and a mouse homolog of *RAD54*. The human (hHR54) and mouse (mHR54) proteins were 48 % identical to Rad54 and belonged to the SNF2/SWI2 family, which is characterized by amino-acid motifs found in DNA-dependent ATPases. The *hHR54* gene was mapped to chromosome 1p32, and the hHR54 protein was located in the nucleus. We found that the levels of *hHR54* mRNA increased in late G1 phase, as has been found for *RAD54* mRNA. The level of *mHR54* mRNA was elevated in organs of germ cell and lymphoid development and increased *mHR54* expression correlated with the meiotic phase of spermatogenesis. The *hHR54* cDNA could partially complement the methyl methanesulfonate-sensitive phenotype of *S. cerevisiae rad54Δ* cells.

Conclusions: The tissue-specific expression of *mHR54* is consistent with a role for the gene in recombination. The complementation experiments show that the DNA repair function of Rad54 is conserved from yeast to humans. Our findings underscore the fundamental importance of DNA repair pathways: even though they are complex and involve multiple proteins, they seem to be functionally conserved throughout the eukaryotic kingdom.

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Background

Homology-dependent repair of double-strand DNA breaks involves a complex recombinational repair pathway that requires a large number of proteins [1]. Double-strand breaks are induced by ionizing radiation which is often used in cancer therapy, and by radiomimetic agents, including endogenously produced radicals. Accurate repair of double-strand breaks is of pivotal importance in preventing chromosomal aberrations, such as translocations, which are often involved in carcinogenesis. In addition to the important role of double-strand break repair in mammalian somatic cells, the process also plays a vital role in germ cells. During meiosis, double-strand breaks are critical for homologous recombination, which is required for proper chromosome segregation and results in the generation of genetic diversity [2]. The processing of double-strand breaks is, therefore, an important cellular process, and its relevance extends beyond the repair of exogenously induced DNA damage.

Saccharomyces cerevisiae RAD52 epistasis group mutants are sensitive to ionizing radiation and to the alkylating agent methyl methanesulfonate (MMS), implicating the RAD52 group genes in double-strand break repair [3]. Mutations in the *RAD51*, *RAD52*, and *RAD54* genes cause the most sensitive phenotypes. The *rad51*, *rad52*, and *rad54* mutants are also defective in mating-type switching, a specialized mitotic recombination event [4]. The *RAD51*, *RAD52*, and *RAD54* genes have been isolated and characterized to some extent. The Rad51 protein is the homolog of the *Escherichia coli* recombination protein RecA [5]. These proteins, which are 22 % identical in amino-acid sequence, form a nucleoprotein filament with single-stranded DNA and mediate homologous DNA pairing and strand exchange [6–9]. The role of Rad52 in homologous recombination and double-strand break repair is less well understood. Rad52 can interact, either directly or indirectly, with Rad51 [5,10]. On the basis of its amino-acid sequence, Rad54 belongs

to the still expanding superfamily of DNA-dependent ATPases. Some members in this superfamily have DNA helicase activity. The superfamily is divided into subfamilies; Rad54 is a member of the SNF2/SWI2 subfamily [11]. Proteins in the superfamily are characterized by seven common motifs, including a bipartite purine nucleotide-binding motif. Proteins from a variety of species are found in the SNF2/SWI2 family and its members have been implicated in transcriptional regulation, recombination and a number of DNA repair pathways [12].

Recently, it has become clear that the primary sequence of some genes in the RAD52 epistasis group is conserved throughout evolution. Genes with sequence similarity to *RAD51* and *RAD52* have been found in numerous species, including *Schizosaccharomyces pombe*, chicken, mouse and humans [13–20]. Recently, a *S. pombe RAD54* (*rhp54*) and a human homolog of *S. cerevisiae MRE11* have been isolated [21,22]; mutants in *S. cerevisiae MRE11* are defective in meiotic recombination and the gene has been placed in the RAD52 epistasis group [23]. It is not known whether the function of any of the mammalian RAD52 group genes is conserved. Here we report the isolation of cDNAs encoding a human and a mouse homolog of Rad54, named hHR54 and mHR54, respectively. We show that *RAD54* and *hHR54* are true homologs because hHR54 can functionally substitute for Rad54 in repair of MMS-induced DNA damage. It appears that DNA repair pathways are of fundamental importance because even though they are highly complex multiprotein pathways they seem to be conserved throughout evolution.

Results

Isolation of human and mouse cDNAs encoding homologs of *RAD54*

We isolated a human and a mouse cDNA each encoding a protein with extensive similarity to Rad54. Amino-acid sequence conservation between the SNF2/SWI2 family member ERCC6 from humans and Rad54 was exploited to design two degenerate oligonucleotide primers corresponding to stretches of conserved amino acids near motif VI (see Materials and methods). Using RT-PCR (reverse transcribed polymerase chain reaction), we isolated a cDNA fragment from chicken bursal mRNA that encoded 41 amino acids with 83 % identity to Rad54 and 68 % identity to ERCC6 [24]. The PCR product was used to isolate a 1.3 kb DNA fragment from a chicken testis cDNA library that encoded 415 amino acids with 56 % identity and 72 % similarity to Rad54 (amino acids 389 to 810). The 1.3 kb DNA fragment was used to screen human and mouse testis cDNA libraries. The sequence of overlapping cDNA clones that hybridized to the chicken-derived probe was determined. One of the human cDNAs contained a 2241 bp open reading frame predicted to encode a protein of 747

amino-acids with a calculated molecular weight of 84.3 kDa. The putative start codon was not in the context of an optimal Kozak translation initiation sequence, but it was preceded by stop codons in all three reading frames at positions –26, –18, and –10, respectively. The mouse cDNA also contained a 2241 bp open reading frame, encoding a protein that was 94 % identical to the human homolog.

Figure 1 shows the comparison of the predicted amino-acid sequences of the proteins encoded by the human and mouse cDNAs with Rad54 and rhp54. Because of the high amino-acid sequence identity between the mammalian proteins and Rad54, we refer to them as hHR54 (for human homolog of Rad54) and mHR54 (for mouse homolog of Rad54). The amino-acid sequence identities and similarities are quantitated in Table 1. The identity and similarity between Rad54, rhp54, hHR54, and mHR54 extended over their entire length. The overall identity between the mammalian and yeast proteins was 48 % or higher. The seven motifs characteristic of SNF2/SWI2 family members were all present in hHR54 and mHR54 (Fig. 1). These motifs, including a Walker-type nucleotide-binding motif, spanned approximately 460 amino acids. We refer to this region as the DNA-dependent ATPase domain. The identity among the four proteins was highest in this domain, but the amino and carboxyl termini also showed significant
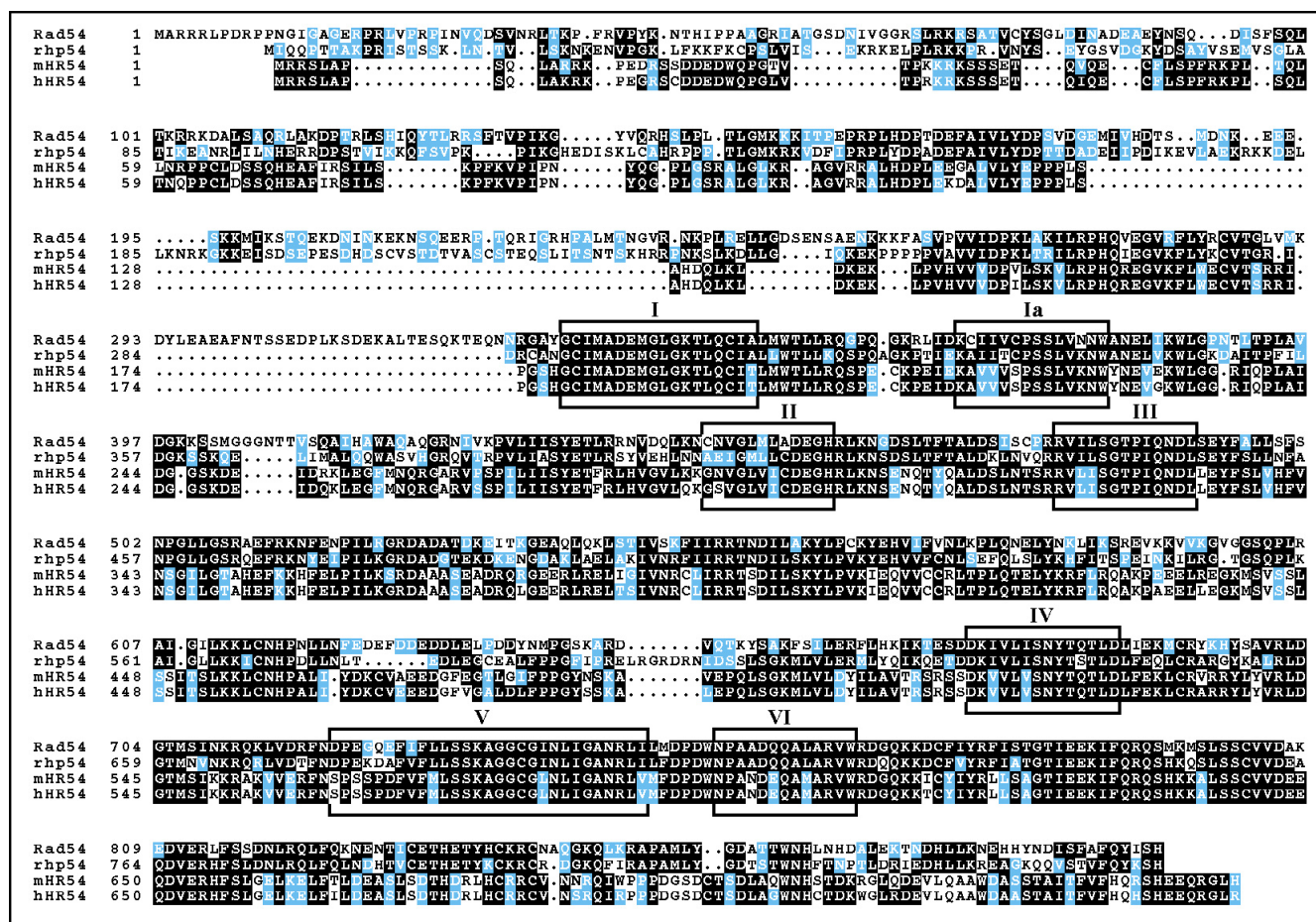
Table 1

Quantitation of amino-acid sequence identities and similarities among Rad54 homologs.

	S. pombe rhp54		hHR54	
	% Identity	% Similarity	% Identity	% Similarity
S. cerevisiae Rad54				
Overall	58	74	48	68
Amino terminus	34	60	32	55
DNA-dependent ATPase domain	69	81	57	75
Carboxyl terminus	66	79	34	56
S. pombe rhp54				
Overall			50	69
Amino terminus			33	57
DNA-dependent ATPase domain			59	76
Carboxyl terminus			40	62

The deduced amino-acid sequence of the proteins shown in the table were aligned pairwise to calculate the percent of identical and similar amino-acid positions. The following amino acids were considered to be similar: D, E, N and Q; R, K and H; I, V, L and M; A, G, P, S and T; F, Y and W. No penalty was used to correct for gaps in the alignments. The amino termini of Rad54, rhp54 and hHR54 encompass amino acids 1–329, 1–288 and 1–177, respectively. The DNA-dependent ATPase domains encompass amino-acids 330–798, 289–752 and 178–638. The carboxyl terminus encompasses amino-acids 799–898, 753–852 and 639–747. Comparison of mHR54 with Rad54 and rhp54 gave results similar to the ones shown here for hHR54.

Figure 1

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Rad54    1  MARRRLPDRPPNGIGACERPRLVPRPINVQDSVNRLLKP.FRVPYK.NTHIPPAACRLATGSDNIVGGRSLRKFSATVCYSGLLINADEAEYNSQ...DLSFSQL
rhp54    1       MIQQPTTAKPRISTSSK.LN.TV..L.KNKENVPGK.LFKKFKCPSLVIS...EKRKELPLRKKFR.VNYS..EYGSVDCKYDSAYVSEVSCLA
mHR54    1       MRRSLAP.............SQ..LARRK..PEDRSSDDEDWQPGTV.........TPKKKRKSSSET....QYQE..CFLSPFRKPL..IQL
hHR54    1       MRRSLAP.............SQ..LAKRK..PEGRSCDDEDWQPGLV.........TPRRKRKSSSET....QIQE...CFLSPFRKPL..SQL

Rad54  101  TKRRKDALSAQRLAKDPLRLSHIQYTIRRSFTVPIKG.....YVQRHSLPL.TLGMKKKITPEPRPLHDPTDEFAIVLYDPSVDGEMIVHDTS..MDNK..ELE.
rhp54   85  TILEANRLILNHERRDPSTVIKKQFSVPK....PIKGHEDISKLCHRPPP.TLGMKRKVDFIPRPLYDPADEFAIVLYDPTTDADEIIPDIKEVLAEKRKKDEL
mHR54   59  LNRPPCLDSSQHEAFIRSILS.......KPFKVPIPN.....YQG.PLGSRALGLKR..AGVRRLHDPLPEGALVLYFPPPLS...................
hHR54   59  TNQPPCLDSSQHEAFIRSILS.......KPFKVPIPN.....YQG.PLGSRALGLKR..AGVRRALHDPLPKDALVLYFPPPLS...................

Rad54  195  .....SKKMIKSTQEKDNINKEKNSQEERP.TQRICRHPALMTNGVR.NKPLRELLGDSENSAENKKKFASVPVVIDPKLAKILRPHQVEGVRFLYRCVTGLVLK
rhp54  185  LKNRKGKKEISDSEPESLHDSCVSTDTVASCSTEQSLITSNTSKHRRPNKSLKDLLG....IQKEKPPPPVAVVIDPKLPRILRPHQIEGVKFLYKCVTGR.I.
mHR54  128  ...................................AHDQLKL........DKEK..LPVHVVVDPVLSKVLRPHQREGVKFLWECVTSRRI.
hHR54  128  ...................................AHDQLKL........DKEK..LPVHVVVDPILSKVLRPHQREGVKFLWECVTSRRI.

                                                    I                                    Ia
Rad54  293  DYLEAEAFNTSSEDPLKSDEKALTESQKTEQNNRGAYGCIMADEMGLGKTLQCIALMWTLLRQGPQ.GKRLIDKCIIVCPSSLVNNWANELIKWLGPNTITPLAV
rhp54  284  ......................LRCANGCIMADEMGLGKTLQCIALLWTLLKQSPQAGKFTIEKAIITCPSSLVKNWANELVKWLGKDAITPFIL
mHR54  174  ......................PGSHGCIMADEMGLGKTLQCITLMWTLLRQSPE.CKPEIEKAVVVSPSSLVKNWYNEVEKWLGG.RIQPLAI
hHR54  174  ......................PGSHGCIMADEMGLGKTLQCITLMWTLLRQSPE.CKPEIDKAVVVSPSSLVKNWYNEVGKWLGG.RIQPLAI

                                               II                                           III
Rad54  397  DGKKSSMGGGNTTVSQATHAWAQAQGRNIVKPVLIISYETLRRNVDQLKNCNVGLYLLADEGHRLKNGDSLTFTALDSISCERRVILSGTPIQNDLSEYFALLSFS
rhp54  357  DGKLSSKQE.....IIMALQQWASVHGRQVTRPVLIASYETLRSYVEHLNNAEIGMLLCDEGHRLKNSDSLTFTALDKLNVQRRVILSGTPIQNDLSEYFSLLNFA
mHR54  244  DG.GSKDE.....IDRKLEGFMNQRGARVPSPLLIISYETFRLHVGVLKKGNVGLVICDEGHRLKNSENQTVQALDSLNTSRRVLISGTPIQNDLLEYFSLVHFV
hHR54  244  DG.GSKDE.....IDQKLEGFMNQRGARVSSPLLIISYETFRLHVGVLQKGSVGLVICDEGHRLKNSENQTVQALDSLNTSRRVLISGTPIQNDLLEYFSLVHFV

Rad54  502  NPGLLGSRAEFRKNFENPILRGRDADATLKEITKGEAQLQKLSTIVSKFIIRRTNDILAKYLPCKYEHVLFVNLKPLQNELYNKLIKSREVKKVVKGVGGSQPLR
rhp54  457  NPGLLGSRQEFRKNVETPILKGRDADGTEKDKENGLALLAELAKIVNRFIIRRTNDILSKYLPVKYEHVVFCNLSEFQLSLYKLFITSPEINKILRG.TGSQPLK
mHR54  343  NSGLLGTAHEFKKHFELPILKSRDAAASEADRQRGEERLRELLIVNRCLIRRTSDILSKYLPVKIEQVVCCRLTPLQTELYKRFIRQAKPEELEREGKMSVSSL
hHR54  343  NSGLLGTAHEFKKHFELPILKGRDAAASEADRQLGEERLRELTSIVNRCLIRRTSDILSKYLPVKIEQVVCCRLTPLQTELYKRFIRQAKPAEELLEGKMSVSSL

                                                                    IV
Rad54  607  AI.GILKKLCNHPNLLNFEDEFDDEDDLELPDDYNMPGSKARD.......VQTKYSAKFSILERFLHKIKLESDDKIVLISNYTQTLDLIEKMCRYKHYSAVRLD
rhp54  561  AI.GLLKKLCNHPDLLNLT.....EDLEGCEALFPPGFIFRELRGRDRNIDSSLSGKMLVLERLYQIKQETDDKIVLISNYTSTLDLFEQLCRARGYKALRLD
mHR54  448  SSITSLKKLCNHPALI.YDKCVAEEDGFEGTLGIFPPGYNSKA.......VEPQLSGKMLVLDYILAVTRSRSSDKVVLVSNYTQTLDLFEKLCVRRRYLYVRLD
hHR54  448  SSITSLKKLCNHPALI.YDKCVEEEDGFVGALDLFPPGYSSKA.......VEPQLSGKMLVLDYILAVTRSRSSDKVVLVSNYTQTLDLFEKLCRARRYLYVRLD

                                   V                          VI
Rad54  704  GTMSINKRQKLVDRFNDPEGQLFIFLLSSKAGGCGINLIGANRLILMDPDWNPAADQQALARVWRDGQKKDCFIYRFISTGTIEEKIFQRQSMKMSLSSCVVDAK
rhp54  659  GTMNVNKRQRLVDTFNDPEKDAFVFLLSSKAGGCGINLIGANRLILFDPDWNPAADQQALARVWRDQQKKDCFVYRFIATGTIEEKIFQRQSHKQSLSSCVVDEA
mHR54  545  GTMSIKKRAKVVERFNSPSSPDFVFMLSSKAGGCGLNLIGANRLVMFDPDWNPANDEQAMARVWRDGQKKICYIYRLLSAGTIEEKIFQRQSHKKALSSCVVDEE
hHR54  545  GTMSIKKRAKVVERFNSPSSPDFVFMLSSKAGGCGLNLIGANRLVMFDPDWNPANDSQAMARVWRDGQKKTCYIYRLLSAGTIEEKIFQRQSHKKALSSCVVDEE

Rad54  809  EDVERLFSSDNLRQLFQKNENTLCETHETYHCKRCNAQGKQLKRAPAMLY..GDALTWNHLNHDALEKTNDHLLKNEHHYNDISEAFQYISH
rhp54  764  QDVERHFSLDNLRQLFQLNPHTVCETHETYKCKRCR.DGKQFIRAPAMLY..GDTLTWNHFTNPTLDRIEDHLLKREAGKQQVSTVFQYISH
mHR54  650  QDVERHFSLGELKELFTLLDEALSLDTHLRLHCFRCV.NNRQIWPPPDGSDCTSDLAQWNHSTDKRGLQDEVLQAAWDAASTAITFVFHQLSHEEQRGLH
hHR54  650  QDVERHFSLGELKELFILEASLSDTHLRLHCFRCV.NSRQIRPPPDGSDCTSDLAGWNHCTDKWGLRDEVLQAAWDAASTAITFVFHQLSHEEQRGLH
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Comparison of the deduced amino-acid sequences of *S. cerevisiae* Rad54 [53], *S. pombe* rhp54 [21], mHR54 and hHR54. The complete amino-acid sequence of the proteins is shown in the single-letter code. Identical and similar amino acids to Rad54 in rhp54, mHR54 and hHR54 are shown in black and blue boxes, respectively. Dots denote gaps. Amino-acid positions are shown on the left. The seven motifs with similarity to the SNF2/SWI2 family are boxed [11]. The GenBank accession numbers for the cDNAs encoding mHR54 and hHR54 are X97796 and X97795, respectively.

levels of identity (Table 1). In particular, a potential nuclear localization signal was conserved in the amino terminus around amino- acid position 34 in the mammalian proteins.

Chromosomal and subcellular localization

We determined the chromosomal localization of the *hHR54* locus to see whether it was located on a human chromosome that complements ionizing radiation-sensitive Chinese hamster ovary (CHO) cell lines. *In situ* hybridization showed that the *hHR54* cDNA localized to human chromosome 1 near band p32 (Fig. 2). This chromosome cannot correct the ionizing radiation-sensitive phenotype of the repair-deficient CHO cell lines tested to date [25]. Thus, it is unlikely that the *RAD54* homolog is defective in any of these CHO mutants.

The subcellular localization of the hHR54 protein was determined using polyclonal antibodies generated against a part of hHR54 produced in *E. coli* (see Materials and methods). COS-1 cells were transfected with a pSVL-derived expression vector and with the same vector containing the *hHR54* cDNA. In transfected cells, the hHR54 protein was detected in the nucleus by *in situ* immunofluorescence staining using affinity-purified anti-hHR54 antibodies (Fig. 3a,b). The staining was specific for hHR54 because no staining was observed when the pre-immune serum was used on COS-1 cells transfected with the *hHR54* expression vector (Fig. 3c). In addition, no staining was found in non-transfected COS-1 cells or in COS-1 cells transfected with the pSVL-derived vector itself (data not shown). The specificity of the affinity-purified anti-hHR54 antibody was also confirmed by immunoblot experiments. Protein extracts were prepared from HeLa cells and from the COS-1 cells that had been used in the immunofluorescence experiment. A band of the expected molecular weight was only detected in the COS-1 cells transfected

Figure 2

Chromosomal localization of *hHR54* by *in situ* hybridization. **(a)** Metaphase spreads of human chromosomes were hybridized to a biotinylated *hHR54* cDNA probe. The hybridization signal, indicated by the arrowheads, was detected on chromosome 1p32. **(b)** DAPI (4,6-diamidino-2-phenylindole) fluorescence of the chromosome spread shown in (a) to reveal the chromosomal banding pattern. Both chromosomes 1 are indicated.
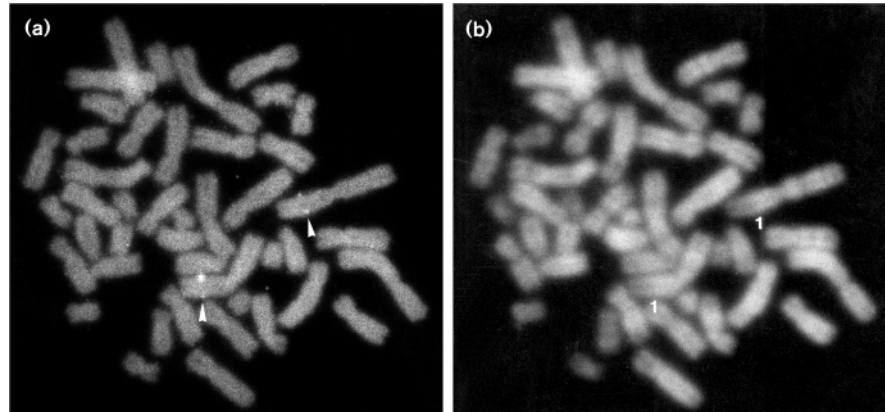
with the *hHR54* expression vector and in HeLa cells (Fig. 3d). However, the anti-hHR54 antibody was less specific under immunoblot conditions as compared to the immuno-fluorescence conditions, because some non-specific bands were observed on the immunoblot. We conclude that hHR54 resides in the nucleus. This observation is consistent with its potential nuclear localization signal and with a function of hHR54 in DNA metabolism.

Expression pattern of the mammalian *RAD54* homolog

We examined the expression of the mammalian *RAD54* homolog in different mouse tissues in order to determine where it might be active. The *mHR54* cDNA was used as

a probe in northern blot analysis of total RNA from a variety of mouse tissues (Fig. 4a). Transcripts of *mHR54* were hardly detectable in most tissues. However, the level of the 2.6 kb *mHR54* transcript was dramatically increased in thymus, spleen and testis. The 18S ribosomal RNA indicated the relative amount of RNA loaded in each lane.

The increase in the *mHR54* transcript level in these tissues is consistent with a role of *mHR54* in V(D)J recombination — the process that brings together the 'variable', 'diversity' and 'joining' regions of antibody genes — and meiotic recombination. However, it cannot be excluded that the increased expression is simply a result of cellular

Figure 3
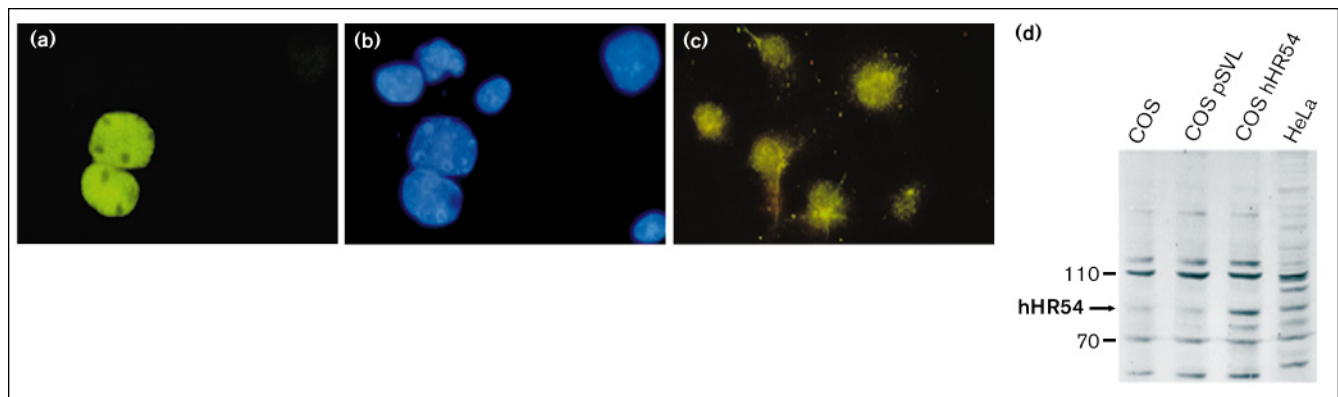
Subcellular localization of hHR54 by immunofluorescence staining. **(a)** hHR54 was detected in COS-1 cells transfected with a pSVL-derived expression vector containing the *hHR54* cDNA. Cells were fixed, stained with DAPI and treated with affinity-purified antibodies generated against part of hHR54 produced in *E. coli*. This primary antibody was detected with a goat anti-rabbit antibody coupled to fluorescin isothiocyanate (FITC). **(b)** DAPI fluorescence of the field of cells shown in (a) to identify the nuclei. **(c)** COS-1 cells transfected with the same hHR54 expression construct used in (a) and treated identically except that pre-immune serum was used as the source of primary antibodies. The photograph is highly overexposed compared to

the one shown in (a). **(d)** Immunoblot from the cells used in the immunofluorescence experiment. Protein extracts were prepared from COS-1 cells, COS-1 cells transfected with the pSVL-derived vector itself (COS pSVL), COS-1 cells transfected with a pSVL derivative containing the hHR54 cDNA (COS hHR54) and HeLa cells. The extracts were electrophoresed through a 8.0 % SDS–PAGE gel, transferred to nitrocellulose, and probed with affinity-purified anti-hHR54 antibodies. Detection was with alkaline phosphatase-coupled goat anti-rabbit antibodies. The arrow indicates the position of hHR54. The size of the protein molecular weight markers (in kDa) is indicated on the left.
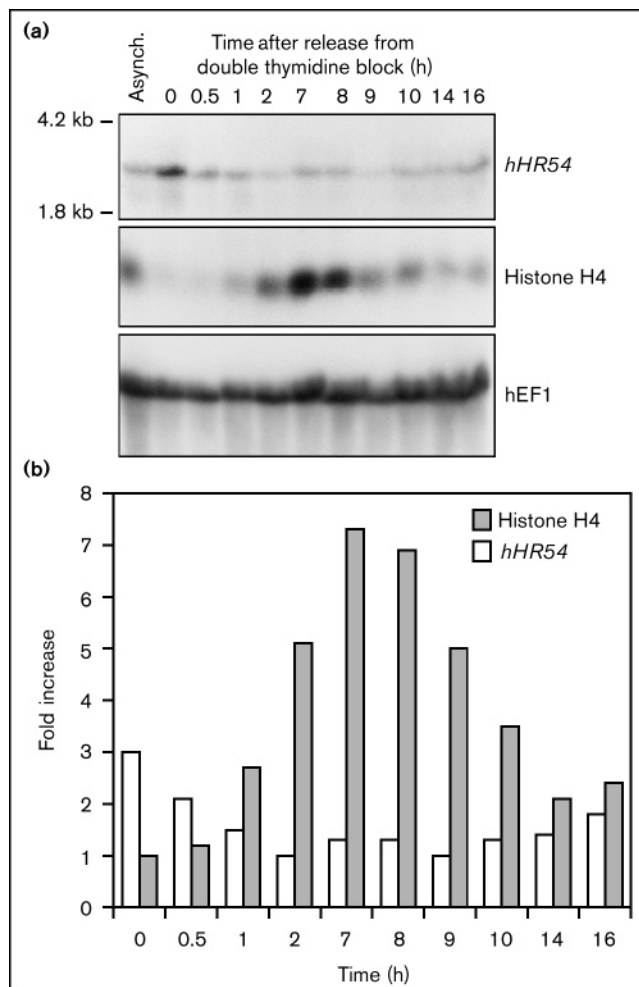
Figure 4
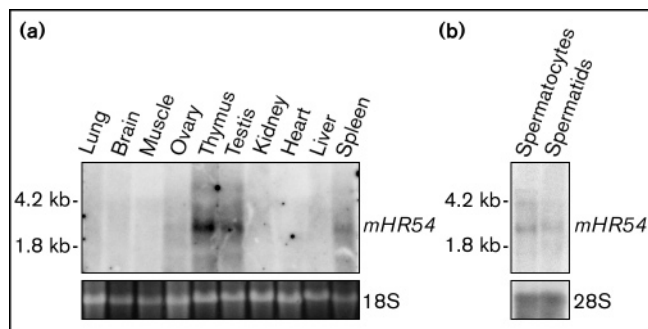
Expression of *mHR54* in different mouse tissues and during spermatogenesis. **(a)** Top panel: total RNA, isolated from the indicated mouse tissues, was electrophoresed through a 0.9 % formaldehyde–agarose gel, immobilized on nitrocellulose, and hybridized to a ^{32}P-labeled *mHR54* cDNA probe. Bottom panel: ethidium bromide-stained 18S ribosomal RNA in the gel used in the top panel to indicate relative amount of RNA loaded in each lane. **(b)** Top panel: total RNA, isolated from mouse spermatocytes and spermatids and analyzed as described above. Bottom panel: the membrane shown in the top panel was stripped and hybridized with a DNA probe derived from 28S ribosomal RNA to control for the amount of RNA loaded.

Figure 5

Determination of *hHR54* mRNA expression levels during the cell cycle. **(a)** HeLa cells were synchronized in late G1 phase by a double thymidine block. Total RNA was isolated from cells harvested at the indicated times after release from the second thymidine block, electrophoresed through a 0.9 % formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled *hHR54* cDNA probe (top panel). The first lane contains RNA isolated from a asynchronous population of HeLa cells. The membrane shown in the top panel was stripped and probed with a histone H4 cDNA to identify S phase (middle panel) and with a human elongation factor 1 (hEF1) cDNA to control for the amount of RNA loaded (bottom panel). **(b)** Quantitation of the autoradiograms shown in (a).

proliferation in the thymus, spleen and testis. To determine whether the increase in *mHR54* expression in testis correlated with meiosis we compared the level of *mHR54* mRNA in mouse spermatocytes and spermatids. Figure 4b shows a northern blot of total RNA isolated from meiotic spermatocytes and from post-meiotic spermatids [26]. The blot was probed with the *mHR54* cDNA; 28S ribosomal RNA was used as a loading control. Expression of *mHR54* was approximately 3-fold higher in spermatocytes compared to spermatids. In addition, two longer *mHR54*-derived transcripts seemed to be present only in spermatocytes. We conclude that increased expression of *mHR54* in testis coincides with the meiotic phase of spermatogenesis.

We next examined the regulation of *hHR54* expression during the cell cycle, as expression of *RAD54* in *S. cerevisiae* increases in late G1 phase [27]. HeLa cells were synchronized in late G1 by a double thymidine block [28]. Total RNA was isolated at different times after release from the block. The RNA was size-fractionated by gel electrophoresis, transferred to nitrocellulose, and sequentially hybridized with an *hHR54* cDNA probe, a histone H4 cDNA probe to identify S phase, and a human elongation factor 1 (hEF1) cDNA probe to control for RNA loading. Figure 5a shows the resulting autoradiograms. Quantitation of the *hHR54* and histone H4 mRNA levels is presented in Figure 5b. Expression of *hHR54* was increased approximately 3-fold in late G1 phase compared to other phases of the cell cycle. This increase was specific for *mHR54*, because no increase in late G1 phase was seen with hEF1 mRNA (Fig. 5a) and the transcripts of three other DNA repair genes: *mHR51*, *mHR52* (data not shown) and the mouse homolog of *S. pombe rad21+* [29]. Thus, the regulation of the expression of *hHR54* and *RAD54* during the cell cycle seems to be conserved between yeast and humans.

Complementation of *S. cerevisiae rad54Δ* cells by *hHR54*

We next tested whether conservation of primary structure between Rad54 and hHR54 extended to conservation of

function. In *S. cerevisiae*, mutations in *RAD54* cause pleiotropic phenotypes in DNA damage repair, homologous recombination and meiosis because of a defect in double-strand break repair [3]. A role for *RAD54* in meiosis is suggested by the reduced spore viability of *rad54* mutants. A convenient DNA damage–repair phenotype to assess is sensitivity to MMS. We tested whether the MMS sensitivity of *S. cerevisiae rad54Δ* cells could be rescued by expression of the *hHR54* cDNA. Multicopy plasmids expressing either *RAD54* or the *hHR54* cDNA from the GAL10 promoter were transformed into *rad54Δ* cells. The expression vector served as a background control. Rescue was quantitated by determining the survival of the transformed cells in the presence and absence of MMS. Figure 6a shows that *rad54Δ* cells harboring the expression vector itself displayed a marked sensitivity to MMS; survival was reduced to 17 ± 13 % at 0.005 % MMS. In contrast, 83 ± 9 % of the *rad54Δ* cells containing the vector expressing *RAD54* survived at this MMS concentration. Expression of the *hHR54* cDNA resulted in partial complementation of the MMS-sensitive phenotype of the *rad54Δ* cells. In this case, 44 ± 12 % of the cells survived at 0.005 % MMS.

We determined whether the observed rescue depended on enzymatically active hHR54. An enzymatic activity that has been clearly demonstrated for proteins in the SNF2/SWI2 family is DNA-dependent ATPase activity [30,31]. Therefore, we tested whether the observed rescue depended on the ability of hHR54 to hydrolyze ATP. We changed the lysine residue at position 189 in the putative GKT Walker-type nucleotide-binding motif to an arginine residue by site-directed mutagenesis; this protein is referred to as hHR54(K189R). For a number of DNA helicases, including *E. coli* UvrD and *S. cerevisiae* Rad3, conversion of this invariant lysine residue into an arginine residue impairs NTP hydrolysis, while leaving nucleotide binding unaffected [32,33]. In addition, elimination of the ATPase activity of SNF2/SWI2 disrupts its function [31]. The cDNA encoding hHR54(K189R) did not rescue the MMS-sensitive *rad54Δ* phenotype. Only 11 ± 13 % of the cells survived at 0.005 % MMS; this was not significantly different from the vector control (Fig. 6a). An immunoblot confirmed that the wild-type and mutant proteins were expressed at similar levels in these experiments (data not shown). The equivalent of the K189R mutation in Rad54, K341R, also eliminated rescue of the MMS sensitivity of *S. cerevisiae rad54Δ* cells (J. Schmuckli and W-D.H., unpublished observations).

We next tested whether overexpression of hHR54 was required to rescue MMS sensitivity. The data shown in Figure 6a were obtained in the presence of galactose to induce the GAL10 promoter. The particular promoter we used gives a low level of gene expression when cells are grown on glucose (B.C. and W-D.H., unpublished observations).

Figure 6
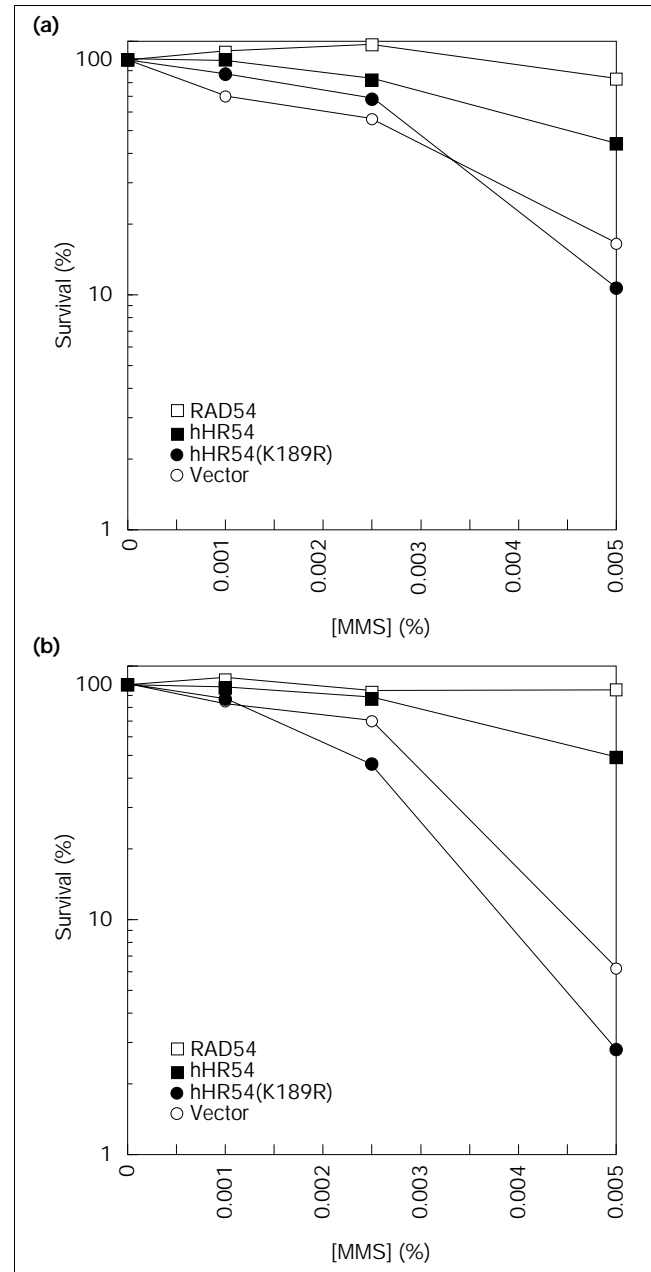
Partial complementation of the MMS-sensitive phenotype of *S. cerevisiae* rad54Δ cells by *hHR54*. MMS sensitivity was determined by plating serial dilutions of transformant colonies onto selective plates with or without MMS (see Materials and methods). The percentage of colony forming units is plotted against the MMS concentration. *S. cerevisiae* strain FF18973 (rad54Δ) was transformed either with vector DNA (pWDH129), vector DNA containing RAD54 (pWDH195), vector DNA containing *hHR54* (pWDH213), or vector containing *hHR54(K189R)* (pWDH352). Shown are averages of four to six determinations. **(a)** Data obtained on media containing galactose; **(b)** data obtained using glucose as the carbon source. The standard deviations for the values at 0.005 % MMS for vector and *hHR54(K189R)* are overlapping. All other standard deviations at this concentration are not overlapping.

We therefore repeated the experiment shown in Figure 6a using glucose instead of galactose as a carbon source. Figure 6b shows that the complementation results under these conditions were similar to those obtained in the presence of galactose. The *hHR54* cDNA partially complemented the MMS sensitivity of *rad54Δ* cells as judged by a survival of 49 ± 10.9 % in 0.005 % MMS. The vector alone resulted in a survival of 6.2 ± 5.4 % at this MMS concentration. This is similar to the 2.8 ± 3 % survival measured for cells transformed with the *hHR54(K189R)* cDNA. Expression of *RAD54* increased survival to 95 ± 13 %. Thus, the partial complementation of the MMS-sensitive phenotype of the *rad54Δ* by *hHR54* does not require overexpression of *hHR54*. In addition, we found no dominant-negative effect of *hHR54* in wild-type *S. cerevisiae* cells, either under induced or non-induced conditions (data not shown). The data presented in Figure 6 show that *RAD54* and *hHR54* have a similar function in the repair of DNA damage inflicted by MMS.

Discussion

To safeguard their DNA from damage by environmental and endogenously generated genotoxins cells have developed a number of DNA repair pathways. Extensive studies on UV-sensitive *S. cerevisiae* mutants placed a number of genes involved in nucleotide excision–repair in the RAD3 epistasis group [34]. From analyses of the hereditary human disorder xeroderma pigmentosum, it has become clear that the RAD3 pathway is conserved during evolution [35]. Studies on ionizing radiation-sensitive *S. cerevisiae* mutants have shown that a number of genes involved in homology-dependent double-strand break repair are in the RAD52 epistasis group [1]. The recent isolation of several mammalian genes with similarity to the RAD52 group genes suggests that this repair pathway is also conserved. Here we describe the isolation of human and mouse cDNAs encoding hHR54 and mHR54, which have extensive amino-acid sequence similarity to *S. cerevisiae* Rad54; we also show that *hHR54* is a functional homolog of *RAD54* with respect to repair of MMS-induced DNA damage.

Rad54 and the mammalian proteins share seven motifs, indicated in Figure 1, which are referred to as the DNA-dependent ATPase domain. A number of superfamilies of proteins containing this domain have been identified [11]. Division of these large superfamilies places hHR54 and mHR54 in the SNF2/SWI2 family [11,12]. The sequence identity and similarity between Rad54 and hHR54 is highest within the ~460 amino-acid DNA-dependent ATPase domain (Table 1). However, it is likely that Rad54, hHR54 and mHR54 are homologs and not just different members of the SNF2/SWI2 family, because the sequence identity within their DNA-dependent ATPase domains is higher than that found among other family members. In addition, the amino and carboxyl termini contain a significant number of identical amino acids (Table 1). Similar levels of identity and similarity are found between the human and yeast homologs of *ERCC6*, which are SNF2/SWI2 family members involved in transcription-coupled nucleotide excision–repair [36].

Proteins in the SNF2/SWI2 family are involved in many aspects of DNA metabolism, including transcription, repair and recombination. The nuclear localization of hHR54 is consistent with a role for this protein in DNA metabolism (Fig. 3). The only enzymatic activity that has been demonstrated for proteins in the SNF2/SWI2 family is DNA-dependent ATPase activity [30,31]. It has been suggested that proteins within this subfamily are not DNA helicases, as is the case for some proteins within the superfamily, and that the conserved motifs that define the superfamily provide a function of which helicase activity is a subset [37]. A clear biochemical activity has been demonstrated for Mot1 from *S. cerevisiae*. This SNF2/SWI2 family member is capable of removing TATA-binding protein (TBP) from DNA, resulting in the global repression of RNA polymerase II transcription [30]. The protein complex containing SNF2/SWI2 and its human counterpart have been shown to help transcription factors overcome the repressive effects of chromatin, possibly by rearranging chromatin structure [31,38]. The biochemical activities of Rad54 and its mammalian homologs remain to be determined. DNA unwinding or chromatin-rearranging activities are attractive possibilities for proteins involved in homology-dependent double-strand break repair. Interestingly, other DNA repair pathways, such as nucleotide excision-repair and post-replication repair, also require at least one SNF2/SWI2 family member.

The existence of mammalian homologs of *RAD54* suggests that the RAD52 recombination pathway from *S. cerevisiae* is conserved in mammals. Of the *S. cerevisiae* genes that have been placed in the RAD52 epistasis group, four putative human homologs have now been identified. The amino-acid sequences of the human homologs of Rad51, Rad52, Rad54 and Mre11 are 67 %, 28 %, 48 % and 37 % identical to their *S. cerevisiae* counterparts, respectively ([15,19,22]; Table 1). Given the large evolutionary distance between yeast and humans this high degree of sequence identity strongly suggests conservation of the mechanism of homology-dependent double-strand break repair.

Using fluorescence *in situ* hybridization we localized the *hHR54* locus to chromosome 1p32 (Fig. 2). This chromosomal region has been implicated in the development of neuroblastoma. Tumor cells derived from 70 % of patients with neuroblastoma exhibit a deletion of part of the short arm of chromosome 1 [39]. Molecular analysis of a number of neuroblastoma cell lines indicates that the

deletions cluster at position 1p32 [40]. However, it remains to be determined whether *hHR54* plays a role in the development of neuroblastoma. The chromosomal position of the *hHR54* locus eliminates the possibility that any of the known ionizing radiation-sensitive CHO mutants, for which the correcting human chromosome has been identified, have a defect in its *hHR54* homolog, because none of these mutants can be complemented by chromosome 1 [25]. We tested six other ionizing radiation-sensitive CHO cell lines for correction of their sensitivity to the crosslinking agent mitomycin C by transfection of the *hHR54* cDNA. None of the lines tested (V-C8, V-H4, V4B3, V5B, V7B and V8B) could be corrected by the cDNA (data not shown).

Northern blot experiments showed that *mHR54* expression is increased in organs of lymphoid and germ cell development (Fig. 4a). The expression pattern of *mHR54* coincides with that of other mammalian RAD52 epistasis group genes. Expression of the mouse homolog of *RAD51* is increased in thymus, spleen and testis [15,16]. Expression of the *MRE11* homolog is elevated in spleen and testis [22]; expression in the thymus was not investigated in these experiments. Expression of the chicken and mammalian homologs of *RAD52* is increased in thymus and testis, but not in spleen [18,19]. The overlap in the expression pattern of these genes is consistent with a function of the encoded proteins in the same pathway. The increased expression of *mHR54* in thymus, spleen and testis is consistent with a role of *mHR54* in V(D)J and meiotic recombination. If *mHR54* is involved in V(D)J recombination, its role in this process is currently unclear. V(D)J recombination does not involve homologous recombination, but *mHR54* could mediate a substrate preparation step that V(D)J and meiotic recombination have in common, such as changing the chromatin structure of the loci that will be rearranged. A role for *mHR54* in meiotic recombination is suggested by its elevated expression in testis. To rule out the possibility that the increased *mHR54* expression in testis results from the high cell proliferation in this tissue and to better correlate *mHR54* expression with meiosis we determined *mHR54* mRNA levels during spermatogenesis. Expression of *mHR54* was approximately 3-fold higher in spermatocytes compared to spermatids (Fig. 5). This indicates that within testis tissue, *mHR54* expression is highest in cells undergoing meiosis.

In *S. cerevisiae*, expression of *RAD54* is cell-cycle regulated. Expression is increased approximately 4-fold in late G1 phase [27]. Because many genes required for S phase are highly expressed in late G1, *RAD54* could have a role in repairing DNA damage after replication [21,41]. We showed that the cell-cycle regulation of *hHR54* expression is similar to that of *RAD54*; *hHR54* is expressed throughout the cell cycle and its expression increases approximately 3-fold in late G1 phase (Fig. 5). Because V(D)J

recombination seems to be initiated in G1 phase, our finding is consistent with a role for *hHR54* in processing the double-strand breaks involved in V(D)J recombination [42]. In contrast to the conservation of cell-cycle regulated expression between *RAD54* and *hHR54*, expression induced by ionizing radiation is not conserved, at least in primary fibroblasts. Although *RAD54* expression is induced by DNA-damaging agents such as MMS and γ rays, we found no increase in *hHR54* mRNA upon irradiation of primary fibroblasts with γ rays (data not shown). However, the relevance of *RAD54* induction is unclear, because *S. cerevisiae* strains that have lost *RAD54* inducibility are no more sensitive to DNA-damaging agents than the wild-type strain [43].

An important question that needs to be addressed when genes from different species are isolated based on amino-acid sequence conservation of the encoded proteins is whether these genes perform a similar function. Therefore, we tested whether *hHR54* could correct the DNA repair defect of *S. cerevisiae rad54Δ* cells. The repair defect in these cells is manifested by sensitivity to MMS. Figure 6 shows that expression of the *hHR54* cDNA does indeed complement the MMS sensitivity caused by deletion of *RAD54*. The final level of rescue by the *hHR54* cDNA is greater than 50 % of that achieved by expression of *RAD54*. The observed rescue depends on enzymatically active *hHR54*. A *hHR54* mutant, in which the lysine residue in the putative ATP-binding loop has been replaced by an arginine residue, is unable to rescue MMS sensitivity (Fig. 6). Based on biochemical studies, this mutation is likely to interfere with ATP hydrolysis [32,33].

Cross-species complementation has also been investigated for other RAD52 group genes. Reports on the rescue of MMS sensitivity of *S. cerevisiae rad51* mutants by the human and mouse *RAD51* homologs are conflicting. It seems that the human cDNA cannot complement a *rad51Δ* mutation [15]. However, the mouse cDNA might be able to suppress the repair defect of the *rad51-1* point mutation [16]. The *RAD52* homolog from chicken can partially rescue the MMS sensitivity and mating-type switching defects of the *S. pombe rad22-67* mutant (K. Ostermann and H. Schmidt, personal communication). Our complementation data show that *RAD54* and *hHR54* have a similar function in the repair of DNA damage inflicted by MMS. Taken together with the high conservation of *Rad54* and *hHR54* at the amino-acid level, it is likely that *hHR54* is indeed the functional homolog of *RAD54*.

It is informative to compare and contrast two fundamentally different mechanisms through which double-strand breaks can be repaired. In the yeast *S. cerevisiae*, double-strand breaks are repaired efficiently through a homology-dependent recombination mechanism [3]. From analyses

of cell lines deficient in double-strand break repair it seems that a homology-independent DNA end-joining mechanism plays a prominent role in mammals [25,44]. This apparent preference for different double-strand break repair mechanisms in yeast and mammals could have arisen because of their different genome organization. Repair by recombination ensures accurate restoration of sequences around the break, whereas repair by DNA end joining does not. In a unicellular organism such as *S. cerevisiae*, repair of double-strand breaks needs to be accurate to ensure survival, particularly because most of the *S. cerevisiae* genome contains coding information. Multicellular organisms such as mammals may not be critically dependent on accurate double-strand break repair, as mutations in their somatic cells may be tolerated more easily, especially given their large amount of non-coding DNA.

There is some evidence for two double-strand break repair pathways in mammalian cells [45]. Cell lines containing mutations in genes involved in DNA end joining are not completely deficient in the repair of double-strand breaks. In addition, mutant cell lines from the XRCC4 group show reduced sensitivity to ionizing radiation in late S–G2 phase [44]. It is possible that this reflects DNA repair through the homology-dependent recombination mechanism and requires the mammalian RAD52 group genes. Alternatively, the RAD52 group genes in mammals may have a more important function in meiosis than in the repair of double-strand breaks. These and other issues can be addressed when defined mutants in these mammalian genes become available.

Conclusions

The correct processing of double-strand breaks is an essential cellular process, both in germ cells during meiosis, and in somatic cells during repair of certain types of DNA damage. In *S. cerevisiae*, double-strand breaks are processed by the RAD52 recombination pathway. A number of genes similar to those in the RAD52 pathway have been isolated from mammals. For one of these genes, the *RAD54* homolog, we have shown here that this conservation of primary sequence extends to conservation of function.

We have provided three lines of evidence to show that *RAD54* has a mammalian homolog. First, mouse and human homologs are over 48 % identical to Rad54 from the yeasts *S. cerevisiae* and *S. pombe*. Given the large evolutionary distance between yeasts and mammals this degree of identity is highly significant. Second, the increase of *hHR54* and *RAD54* expression in late G1 phase is conserved. This cell-cycle regulated expression is shared with many genes required for S phase and is consistent with a function of *RAD54* and its mammalian homologs in post-replication repair [21,41]. Third, hHR54

can repair MMS-induced DNA damage in *S. cerevisiae* cells lacking Rad54. This result shows that hHR54 and Rad54 are functional homologs with respect to their DNA repair function. In addition, a role for *mHR54* in recombination is suggested by the demonstration that expression of the gene is essentially limited to organs with high recombination activity: thymus, spleen and testis. Moreover, increased expression in the testis coincided with the meiotic phase of spermatogenesis.

A number of DNA repair pathways, including the RAD3 nucleotide excision–repair pathway and the RAD52 recombinational repair pathway, require the close cooperation of many proteins to accurately execute the events leading to repair of DNA damage. It is clear that the RAD3 pathway is functionally conserved from yeast to humans. The observation that hHR54 functions in yeast cells suggests that this may also be the case for the RAD52 pathway. Therefore, our finding underscores the fundamental importance of DNA repair pathways — even though they involve multiple proteins, they seem to be functionally conserved throughout the eukaryotic kingdom.

Materials and methods

General procedures
Purification of nucleic acids, restriction enzyme digestions, gel electrophoresis, DNA ligation, synthesis of radiolabeled probes using random oligonucleotide primers, Southern, northern and western hybridizations, PCR, DNA sequencing and site-directed mutagenesis were all performed according to standard procedures [46]. Levels of mRNA were quantitated using a Molecular Dynamics phosphorimager.

Cell culture
COS-1 and HeLa cells were grown in F10–DMEM containing 5 % FCS. Transfection of plasmid DNA to COS-1 cells was carried out using the DEAE dextran method. HeLa cells were synchronized in late G1 phase by a double thymidine block as described [28].

Isolation of cDNAs encoding human and mouse homologs of RAD54
The following degenerate oligonucleotides were used to isolate a cDNA fragment of the chicken *RAD54* homolog: 5′–GGAATTC GAC/T CCI GAC/T TGG AAC/T CC and 5′–GGAATTC AA/G ATC/T TTC/T TCC/T TCI ATI GT. RT-PCR was performed as described [18]. Human and mouse testis cDNA libraries were hybridized at 61 °C in 3 × SSC, 10 × Denhardt's solution, 0.1 % SDS, 9 % dextran sulfate and 50 μg ml⁻¹ salmon sperm DNA.

Accession numbers
The GenBank accession numbers for the cDNAs encoding mHR54 and hHR54 are X97796 and X97795, respectively.

In situ *hybridization*
Treatment of human lymphocyte metaphase spreads prior to hybridization was as described [47]. A *hHR54* cDNA fragment labeled with biotin was hybridized to the metaphase spreads as described [48]. Slides were incubated sequentially with 5 μg ml⁻¹ avidin D–FITC (Vector, USA) and biotinylated goat anti-avidin D antibody, washed, dehydrated with ethanol and air-dried. Chromosomes were either counterstained with propidium iodide in antifade media or banded with DAPI and actinomycin D.

Immunofluorescent staining

A 1.72 kb *Hinc*I fragment from the *hHR54* cDNA was subcloned into the *Sma*I site of a pGEX-3X derivative. The fusion protein derived from this plasmid containing amino acids 146–684 of hHR54 fused to glutathione-S-transferase was produced in *E. coli* strain DH5α. The protein was purified from the insoluble fraction by preparative SDS–PAGE and used to immunize two rabbits. The resulting polyclonal antibodies were affinity purified using fusion protein immobilized on a nitrocellulose filter [49].

COS-1 cells were transfected with a pSVL (Pharmacia) derivative containing the *hHR54* cDNA to determine the subcellular localization of *hHR54*. As a negative control the pSVL-derived vector by itself was transfected to COS-1 cells. After 24 h the transfected COS-1 cells were transferred to slides for 24 h. The slides were sequentially incubated in PBS, PBS containing 2 % paraformaldehyde, and methanol. After washing in PBS containing 20 mM glycine and 5 mg ml^{-1} BSA the cells were incubated with primary antibodies. After washing, the cells were incubated with goat anti-rabbit FITC-conjugated antibodies. Slides were washed and preserved in Vectashield mounting media (Brunschwig). The DNA was stained with DAPI. Fluorescence microscopy was performed using an Aristoplan laser beam microscope.

Complementation of a S. cerevisiae rad54Δ *strain by* hHR54

The expression vector pWDH129, a derivative of pRDK249 [50], was used to direct the expression of *RAD54*, *hHR54*, or *hHR54(K189R)* in *S. cerevisiae* from the galactose-inducible GAL10 promoter. The *Cfr*10I–*Nhe*I *RAD54* fragment of Yep13-RAD54-216A [51] was ligated into the *Xho*I–*Nhe*I-digested pWDH129 together with a double-stranded oligonucleotide adapter composed of the sequences 5′–TCG AGA CAC CAT GGC AAG ACG CAG ATT ACC AGA CAG ACC ACC AAA TGG AAT AGG AG and 5′–CCG GCT CCT ATT CCA TTT GGT GGT CTG TCT GGT AAT CTG CGT CTT GCC ATG GTG TC, resulting in plasmid pWDH195. The *Dra*I–*Xba*I cDNA fragment of *hHR54* was cloned into the *Xho*I–*Nhe*I-digested pWDH129 after filling in the *Xho*I site using Klenow fragment of *E. coli* DNA polymerase I, resulting in plasmid pWDH213. The *hHR54(K189R)* mutation was constructed by oligonucleotide-directed mutagenesis. The *Xho*I–*Sac*I fragment encompassing the mutation was used to replace the corresponding fragment in pWDH213 to result in pWDH352.

Functional complementation of the *RAD54* deletion allele *rad54::LEU2* by vector DNA, *RAD54*, *hHR54*, and *hHR54(K189R)* was measured by determining the MMS sensitivity of strain FF18973 (*MATα, leu2-3 122, trp1-289, ura3-52, his7-2, lys1-1, rad54::LEU2*). Cell survival was determined in the presence and absence of increasing concentrations of MMS. All standard *S. cerevisiae* methods and media were as described [52]. Strain FF18973 was transformed with either pWDH129, pWDH195, pWDH213, or pWDH352 and after three days of incubation on SD-ura plates four to six independent transformants of each strain were picked into sterile water. 100 μl of each of four serial two-fold dilutions were plated on SD-ura plates with or without MMS, and with either 2 % galactose or 2 % glucose as the carbon source. Plates were incubated at 30 °C and colonies were counted after six days.

Acknowledgments

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