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Identification and Characterization of the *rhp23*$^+$ DNA Repair Gene in *Schizosaccharomyces pombe*[1]

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We have identified *rhp23*$^+$, the ortholog of the *Saccharomyces cerevisiae RAD23* and human *HHR23A* and *HHR23B* genes, in *Schizosaccharomyces pombe* and examined its role in cell survival and DNA repair. In *S. pombe* two repair mechanisms are operative on UV-induced photoproducts, i.e., UV damage repair (UVDR) and nucleotide excision repair (NER). Here we show that Rhp23 is solely involved in NER and study its role in DNA repair in the absence of the UVDR pathway. *S. pombe rhp23*-deficient cells are sensitive toward UV irradiation, although not as sensitive as complete NER-deficient cells. Furthermore we demonstrate that the residual survival observed in *rhp23*-deficient cells is NER dependent. Despite this NER-dependent survival, *uvde rhp23* double mutants are unable to repair cyclobutane pyrimidine dimers. The inability to remove these photolesions from both DNA strands clearly demonstrates that *rhp23*$^+$ is involved in transcription coupled repair as well as global genome repair.

Nucleotide Excision Repair (NER) is a versatile mechanism capable of removing a broad spectrum of unrelated lesions from the DNA, including UV-induced cyclobutane pyrimidine dimers (CPDs) (reviewed in (1)). Many proteins involved in this repair pathway have been identified from budding yeast and human cells and the molecular mechanism of NER has largely been elucidated during recent years. After a lesion in the DNA is recognized, dual incisions around the damage are generated and an oligonucleotide containing the lesion is removed. Subsequently, the remaining gap is filled. One of the genes involved in NER in *S. cerevisiae* is *RAD23* (2). *In vitro* and *in vivo* experiments demonstrated that *S. cerevisiae* Rad23 is complexed with Rad4 (3–5), as are their human homologs HHR23B and XPC (6, 7). Biochemical evidence suggests that Rad23-Rad4 complexes are involved in the assembly or disassembly of NER complexes (2, 5, 8). Both Rad23-Rad4 and HHR23B-XPC complexes are able to bind to UV- and to *N*-acetoxy-2-(acetylamino)-fluorene-damaged DNA *in vitro* (9–12) and most likely function as the damage detector (12, 13). Yeast *rad23* deficient cells exhibit a moderate sensitivity towards UV light, like *rad7* and *rad16* mutants. However, where *rad7* and *rad16* deficient cells are still able to remove lesions from the transcribed part of their genomes (14, 15), *rad23* mutants appear to be unable to remove any lesion from DNA (16). This demonstrates that Rad23 affects total NER. In human cells, two homologs of the *RAD23* gene, *HHR23A* and *HHR23B*, were identified (6). Both homologs are functionally interchangeable *in vitro* (13) although *in vivo*, only the HHR23B protein is bound to XPC (6). Surprisingly, in none of the human and rodent NER deficient cell lines deficiencies in *RAD23* homologs were identified.

In *S. pombe* many NER mutants were identified. In contrast to *S. cerevisiae* NER deficient cells, *S. pombe* NER mutants are still able to remove photolesions from their DNA (17, 18). Besides NER *S. pombe* possesses another repair mechanism capable of removing UV-induced lesions (reviewed in (19)). The *uvde*$^+$ gene, which is essential for this second repair mechanism called UV damage repair (UVDR), was identified recently (20). The study of repair of photolesions in *S. pombe* has been complicated by the redundancy of the two repair pathways, NER and UVDR. With the identification of the *uvde*$^+$ gene it became possible to study specifically NER in *S. pombe* since disruption of this gene results in a complete abrogation of the UVDR pathway (21, 22).

Abbreviations used: CPDs, cyclobutane pyrimidine dimers; GGR, global genome repair; NER, nucleotide excision repair; TCR, transcription coupled repair; UBA, ubiquitin associated; UBL, ubiquitin-like; UVDR, UV damage repair.

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TABLE 1

S. pombe Strains Used in This Study

Strain	Genotype[a]	Source
Y4	*ade6*-M216 *leu1*-32 *ura4*-D18	A. Yasui
Y21	*rad13*::*ura4*$^+$	A. Yasui
Y26	*rad13*::*ura4*$^+$ *uvde*::LEU2	A. Yasui
Y53	*uvde*::LEU2	A. Yasui
MGSP54	*rhp23*::hisG-*ura4*$^+$-hisG	This study
MGSP59	*uvde*::LEU2 *rhp23*::hisG-*ura4*$^+$-hisG	This study
MGSP60	*rad13*::*ura4*$^+$ *rhp23*::*hisG*	This study

[a] All strains are isogenic with Y4.

Here, we describe the identification and characterization of *rhp23*$^+$, the homolog of *RAD23* and *HHR23B*, in the fission yeast *S. pombe* and study its role in DNA repair *in vivo*.

MATERIALS AND METHODS

Screening the databases. The *S. pombe* genomic sequence database, produced by the *S. pombe* Sequencing Group at the Sanger Centre, was searched using the tblastn program (version 2.0a (23)). The *rph23*$^+$ DNA sequence data was retrieved from ftp://ftp.sanger.ac.uk/pub/yeast/sequences/pombe (Accession Number SPBC2D10.12). The EMBL Accession Number of the Rhp23 protein is CAA21170. The alignment of Rad23, Rhp23, and HHR23B was generated by the ClustalW program version 1.74 (24) using the blossum option as weight matrix.

Strains and media. *S. pombe* strains are maintained on complete medium (YES; 5 g/l yeast extract, 30 g/l glucose, 2% bacto agar supplemented with 75 mg/l adenine and 75 mg/l uracil). Cells were grown in liquid complete (YES) medium at 29°C under vigorous shaking. Complementation experiments in *S. cerevisiae* were done on YEPG plates (1% yeast extract, 2% bacto peptone, 2% galactose).

The *S. pombe* strains used in this study are given in Table 1. Strain MGSP54 was created by one step gene replacement using a LiAc based transformation method (25). MGSP59 results from a cross (26) between isogenic *h*$^-$ *uvde*::LEU2 and *h*$^+$ *rhp23*::hisG-*ura4*$^+$-hisG strains. An *rhp23*::hisG strain resulted from MGSP54 by selecting cells for 5-fluoro-orotic acid (5-FOA) resistance. In this strain the *rad13*$^+$ gene was disrupted by one step gene replacement with a linear *rad13*::*ura4*$^+$ construct. This *rad13*::*ura4*$^+$ disruption construct was kindly provided by Drs. A. Yasui and S. Yasuhira (Tohoku University, Japan).

Cloning of rhp23$^+$. The *rhp23*$^+$ gene was amplified by PCR and cloned into pUC18. An internal *Pst*I-*Bst*YI fragment was replaced by the hisG-*ura4*$^+$-hisG cassette (a gift of Dr. McNabb, Massachusetts Institute of Technology, USA, (27)) to give rise to pUCrhp23::hisG-*ura4*$^+$-hisG. This plasmid was used to create the *rhp23* deletion strain by one step gene replacement.

cDNA was made using oligo(dT)$_{25}$ beads (Dynal), according to the manufacturers instructions. Two PCR primers, rhp23start (5′ TTG GGG ATC CAA ATG AAT TTG ACA TTC AAA AAT CTA CAG CAG C) and rhp23stop (5′ TTC CCT CTA GAT TAA GGT TCA TCC TCA GAT TCA TGT CC), were designed to amplify *rhp23*$^+$ cDNA (*rhp23*$^+$ sequence underlined). A cDNA PCR product (digested with *Bam*HI and *Xba*I) was ligated in the *Bam*HI and *Xba*I sites of pUC19 for sequencing. From this plasmid a *Bam*HI and *Xba*I *rhp23*$^+$ fragment was cloned in pYET2 (a pYES2 derivative containing the *TRP1* selection marker) resulting in pYETrhp23 which was used for complementation experiments.

Survival experiments. Cells were grown to an optical density of 1.0. Serial dilutions of these cells were prepared in PBS and spread on YES plates. The plates were irradiated with increasing doses of UV light and incubated at 29°C. After 4 days colonies were counted and survival was calculated.

UV irradiation and DNA isolation. Yeast cells diluted in chilled PBS were irradiated with 40 J/m^2 at a rate of 3.5 J/m^2/s with 254 nm UV light (Philips TUV 30W). Cells were collected by centrifugation, resuspended in complete medium and incubated for various times at 29°C prior to DNA isolation (28). DNA samples were purified on CsCl gradients (29). Repair analyses at nucleotide resolution in the *sprpb2*$^+$ gene have been described elsewhere (22, 30). Shortly: DNA was digested with *Eco*RI and hybridised to biotinilated oligonucleotides complementary to the transcribed or non-transcribed strand. Single strand *sprpb2*$^+$ fragments were isolated using streptavidine coated magnetic beads. Isolated fragments were radio-labelled with super-TAQ (HT Biotechnology LTD). PCR generated anti-strand was added to make the fragment double stranded. After incubation with T4EndoV, which results in 5′ incisions of CPDs, the DNA was run on sequencing gels and autoradiograms were generated.

RESULTS

The Identification of rhp23$^+$

A sequence with extensive homology to both the *S. cerevisiae RAD23* gene and its human homologs *HHR23A* and *HHR23B*, was identified from the *S. pombe* database at the Sanger Centre and named *rhp23*$^+$ (*RAD23* homolog in *S. pombe*). The human homologs HHR23A and HHR23B are highly homologous (67% similar, 55% identical) however, because HHR23B is considered to be the functional homolog (6) this protein is used for comparison. The putative Rhp23 protein consists of 368 amino acids, comparable with the sizes of Rad23 and HHR23B (398 and 409 amino acids respectively). Overall Rhp23 is 35% identical and 51% similar with Rad23, and 38% identical and 55% similar with HHR23B. The alignment of Rad23, Rhp23 and HHR23B is given in Fig. 1. The *rhp23*$^+$ gene was cloned and cDNA sequencing confirmed the presence of four introns. The introns are present at positions +70 to +109, +183 to +223, +1066 to +1112 and +1182 to +1233 (relative to the first nucleotide of the putative start codon ATG = +1). Rhp23 harbors a ubiquitin like (UBL) domain at the amino terminus of the putative protein. Furthermore, two ubiquitin associated (UBA) domains are present (Fig. 1). In between both UBA domains a block of considerable homology is present in HHR23B and homologs in higher eukaryotes. Interestingly, this domain is well conserved in *S. pombe* Rhp23, but is less well conserved in *S. cerevisiae* Rad23.

Rhp23 Is Involved in NER

Disruption of the *rhp23*$^+$ gene in repair proficient *S. pombe* cells results in an enhanced sensitivity to UV irradiation (Fig. 2A). NER deficient *rad13* (the ortholog of *S. cerevisiae RAD2* and human *XPG* and one of the core NER genes) cells are sensitive to UV irradiation.

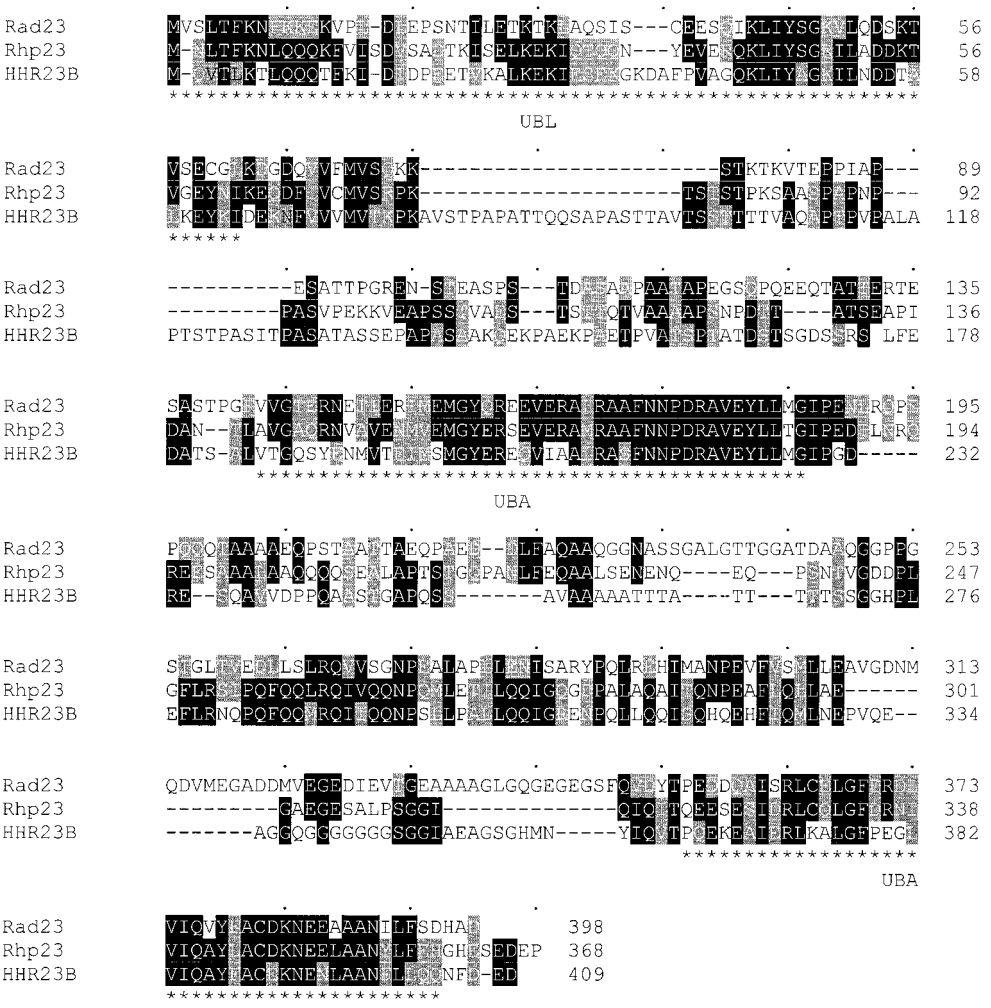

FIG. 1. The alignment of Rad23, HHR23B and Rhp23. Protein sequences were aligned with the ClustalW program version 1.74 (24) using the blossum option as weight matrix. Identical and similar residues are boxed black and gray respectively. There is 35% identity and 51% similarity between Rhp23 and Rad23, and 38% identity and 55% similarity between Rhp23 and HHR23B. Overall there is 20% identity and 36% similarity. The ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains in the protein sequences are indicated.

Disruption of *rhp23*$^+$ in NER deficient *rad13* cells does not result in a further enhancement of the sensitivity. This clearly demonstrates that Rhp23 is involved only in NER and not in the UVDR pathway. In *uvde* deficient cells, where the UVDR pathway is knocked out and lesions are exclusively removed by the NER machinery, disruption of *rhp23*$^+$ does result in a severe reduction in survival after UV irradiation. However, *uvde rhp23* deficient cells are more resistant to UV light than *uvde rad13* deficient cells (Fig. 2A). This demonstrates that the residual survival of *uvde rhp23* deficient cells is NER dependent.

Expression of rhp23$^+$ *Partially Complements S. cerevisiae rad23 Mutants*

The sequence of the *rhp23*$^+$ gene is homologous to that of the *S. cerevisiae RAD23* gene. To test whether Rhp23 is also the functional homolog of Rad23, we tried to rescue the sensitivity of *S. cerevisiae rad23* mutants. *rhp23*$^+$ cDNA was cloned behind the galactose driven Gal1 promoter in pYET2. Subsequently, the resulting plasmid was transformed to *S. cerevisiae rad23* deficient cells (MGSC101, (16)). Expression of Rhp23 in *rad23* deficient *S. cerevisiae* cells result in a partial rescue of the UV sensitivity (Fig. 2B). This heterologous complementation demonstrates that, indeed, *rhp23*$^+$ is the functional homolog of *RAD23*.

Lesion Removal by NER Is Dependent on the rhp23$^+$ *Gene*

Because there is NER dependent residual survival in *rhp23* deficient cells, we examined repair in *uvde* deficient cells. In this genetic background, the UVDR pathway is abrogated and UV-induced lesions are removed

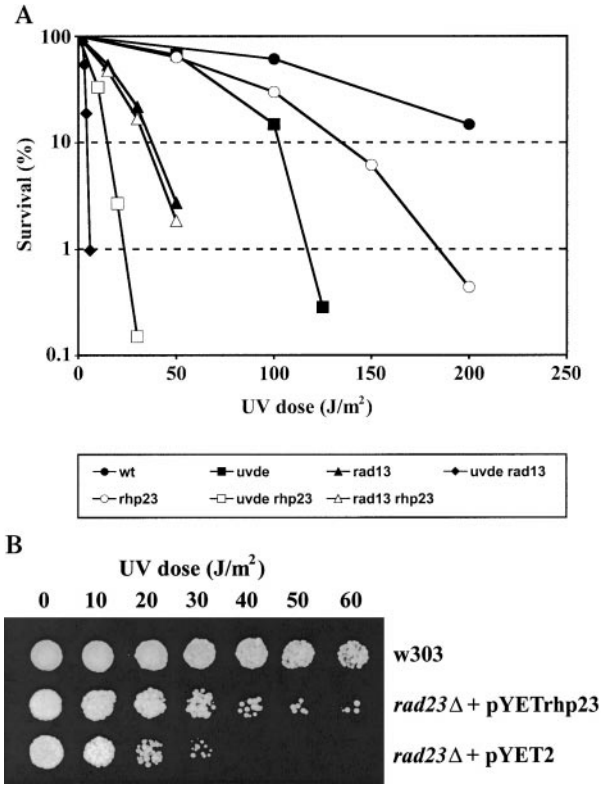

FIG. 2. (A) Survival curves of different *S. pombe* strains. Yeast cells were plated and irradiated with the indicated doses of UV light. After incubation at 29°C for 4 days, colonies were counted and survival was calculated. Disruption of *rhp23*$^+$ results in an enhanced sensitivity in repair-proficient (wt) and *uvde*-deficient cells, but not in *rad13*-deficient cells. (B) *S. pombe rhp23*$^+$ partially complements the UV sensitivity of *S. cerevisiae rad23*-deficient cells. Repair proficient w303 *S. cerevisiae* cells as well as isogenic *rad23*-deficient cells (MGSC101, (16)) transformed with *rhp23*$^+$ expression (pYETrhp23) or control (pYET2) plasmids were irradiated with the UV dose indicated. Cells were put on galactose containing plates to induce *rhp23*$^+$ expression. The plate was incubated at 28°C for 3 days.

exclusively by NER (21, 22). Within NER two subpathways are operative *i.e.* transcription coupled repair (TCR) which is the fast repair of transcribed DNA, and global genome repair (GGR) which removes lesions from the complete genome (reviewed in (31). Examination of the transcribed and non-transcribed DNA allows analysis of the role of Rhp23 in TCR and GGR. The removal of UV-induced CPDs was measured at nucleotide resolution in both the transcribed strand (TS) and non-transcribed strand (NTS) of the *sprpb2*$^+$ gene in *S. pombe*. In *uvde* deficient cells CPDs from the TS are removed efficiently by the NER machinery (Fig. 3A) because TCR rapidly removes lesions. Disruption of *rhp23* in this background completely abrogates repair of CPDs in the TS (Fig. 3B). Lesions from the NTS are removed in *uvde* deficient cells, however, less efficient as lesions from the TS, because the GGR subpathway of NER is rather inefficient in *S. pombe* (Fig. 3C and (22, 32)). In *uvde rhp23* mutants, even the slow repair of lesions from the NTS seen in *uvde* deficient cells, is completely abolished (Fig. 3D). These data clearly demonstrate that Rhp23 is involved in repair of

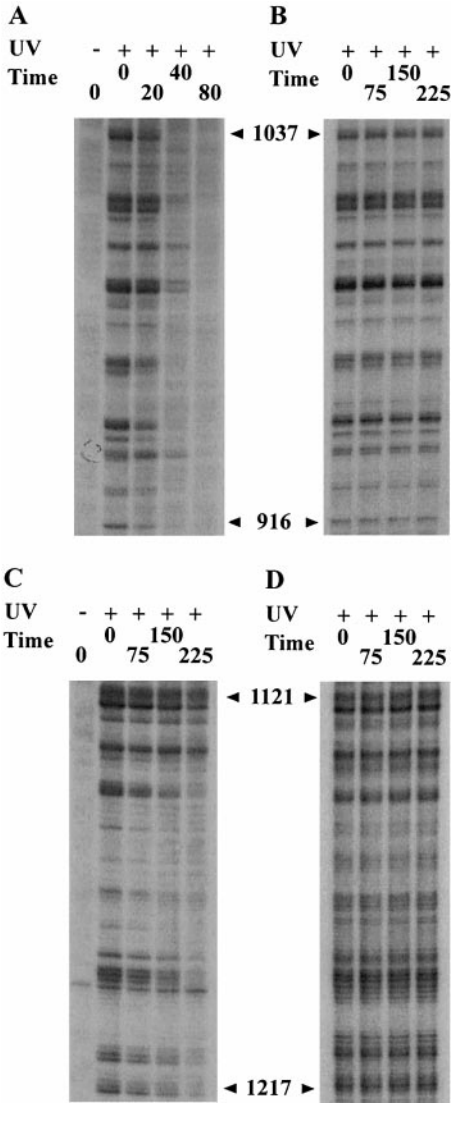

FIG. 3. Repair of CPDs from the transcribed and the nontranscribed strands of the *S. pombe sprpb2*$^+$ locus. Cells were irradiated with 40 J/m^2 of UV light and allowed to repair DNA for time periods indicated. DNA was isolated and the fragment of interest was labeled and treated with the CPD-specific enzyme T4EndoV. Lesion-specific bands in the TS (A and B, from positions +916 to +1037) and in the NTS (C and D, from positions +1121 to +1217) of *uvde* (A and C) and *uvde rhp23* (B and D)-deficient cells are shown in the autoradiograms. The positions indicated are relative to the start codon ATG designated +1. In lanes indicated "UV−" nonirradiated control DNA was treated with T4EndoV. The initial lesion distribution is given at 0 minutes. There is a rapid time-dependent decrease in the intensity of the bands in A. In C bands do disappear but at a much lower rate. The time-dependent decrease in the intensity of the bands in A and C indicates repair. In B and D no CPDs are repaired. Note the difference in repair times between A and B.

lesions from both the TS and the NTS, and therefore Rhp23 is involved in TCR and GGR.

DISCUSSION

In this study we describe the characterization of the *S. pombe* homolog of the *S. cerevisiae RAD23* and the human *HHR23A* and *HHR23B* genes. Both the sequence similarity between *S. cerevisiae* Rad23 and *S. pombe* Rhp23, and the heterologous complementation of *S. cerevisiae rad23* deficient cells upon introduction of the cloned *rhp23*$^+$ gene, demonstrate that the two genes are homologs.

Like the other Rad23 homologs, Rhp23 carries a ubiquitin like (UBL) domain at its N-terminus and two ubiquitin associated (UBA) domains, one is located in the central part and one is at the C-terminus of the protein. The UBA domains are found in several enzymes of the ubiquitination pathway and are thought to be involved in the ubiquitin metabolism (33), possibly by binding ubiquitin (34). The homology between these domains in different Rad23 homologs is very high. In the C-terminal UBA domain, HHR23A and HHR23B are 98% homologous and Rhp23 and HHR23B share 83% homology. The structure of the C-terminal UBA domain of HHR23A was resolved and consists of three alpha helices (35), however the function of the UBA domains remains enigmatic. Homology modeling of the C-terminal UBA domain of Rhp23 strongly suggests that it has the same structure as the known UBA domain of HHR23A (data not shown).

In between the two UBA domains a block of considerable homology is present in HHR23B and Rhp23. This putative domain is also present in homologs of Rhp23 in other eukaryotes but interestingly, it is less well conserved in Rad23. Furthermore, Masutani and coworkers demonstrated that (*in vitro*) the interaction between XPC and HHR23B locates in between both UBA domains (36), exactly covering the block of homology between Rhp23 and HHR23B. Analyses with secondary structure programs suggest that this domain in *S. pombe* Rhp23, human HHR23B and also *S. cerevisiae* Rad23 has a similar structure with a predominant alpha-helical character (data not shown).

Like *S. cerevisiae rad23* mutants, *S. pombe rhp23* deficient cells are sensitive towards UV irradiation, although not as sensitive as complete NER deficient cells. We demonstrate that Rhp23 is involved in NER and that the residual survival in *uvde rhp23* deficient cells is NER dependent. Furthermore, we show that there is no removal of CPDs from either the TS or the NTS in *uvde rhp23* deficient cells, demonstrating that *rhp23*$^+$ is involved in both transcription coupled and global genome repair. So, despite the NER dependent residual survival in *uvde rhp23* deficient cells, no NER is detected within 225 minutes after irradiation. One can speculate that in *rhp23* deficient cells there might be very slow repair of CPDs that is dependent on NER factors. Rhp23 possibly acts as an efficiency factor for the damage recognition step in NER and in its absence the NER machinery is only poorly targeted to the lesion resulting in removal of lesions that is undetectable but still significantly contributes to survival. An alternative explanation for the observed phenotype is that an as yet unidentified damage tolerance mechanism is operative that is dependent on (some) NER factors, but not on Rhp23.

Similar to *S. pombe* Rhp23, *S. cerevisiae* RAD23 is also involved in both subpathways of NER (16, 37). In contrast, the human counterpart HHR23B is suggested to play exclusively a role in the GGR subpathway of NER. This suggestion is based at the phenotype of XPC cells, which are specifically defective in GGR, but TCR is unaffected (38). However, *HHR23A* −/− *HHR23B* −/− cell-lines have not been tested for their repair capacity.

In men (6) and mice (39) two homologs of the *RAD23* gene do exist, whereas in *S. cerevisiae* only one copy of the gene is present. Until now, only one homolog of *RAD23* in *S. pombe* has been identified. In the databases no other homolog can be identified and also the sensitivity of *rhp23* mutants does not suggest the presence of a second homolog involved in NER. Most likely, the duplication of this gene occurred late in evolution. It is remarkable that also in (some) plants the *RAD23* homolog is duplicated (40). Apparently these two gene duplications have arisen independently, because both isoforms of the carrot *RAD23* genes and also both human homologs are more similar to each other than to the *S. cerevisiae* or *S. pombe* genes.

In both *S. cerevisiae* and human cells there is a molar excess of Rad23 or HHR23 to Rad4 or XPC respectively (10, 41). This suggests that Rad23 and its homologs possibly have functions in other processes than NER. One suggestion comes from the genetic interaction between Rad23 and Dsk2, demonstrating that Rad23 can (partially) take over the function of Dsk2 in spindle pole body duplication (42). Also, a genetic interaction between Rad23 and Rpn10 has been reported (43). Rpn10 is part of the 19S particle of the proteasome. Pleiotropic defects like cold sensitivity, growth deficiencies and proteolytic defects are seen in *rad23 rpn10* double mutants, however not in either single mutant (43). This suggests that besides a role in NER Rad23 might be involved in proteolysis.

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