New factors in nucleotide excision repair : a study in saccharomyces cerevisiae

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Chapter

4.1

Supplement: Further analysis of the two Rad4 homologues in *Saccharomyces cerevisiae*

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Abstract

Previously, we reported that in the yeast *Saccharomyces cerevisiae* two Rad4 homologues are present. Both Rad4 and Rad34 function in NER, but at distinct chromosomal loci and are unable to replace each other. Rad4 is essential for all GGR and for TCR only in RNA polymerase II (RNA pol II) transcribed regions. Rad34 on the other hand is specifically involved in the preferential repair of the RNA pol I transcribed *rRNA* genes. Here we show that Rad34, like Rad4, interacts directly with Rad23 and Rad33. The homology of Rad4 to Rad34, and to all other Rad4 homologues, is concentrated in the carboxy terminal pFAM domain. This might suggests that the pFAM domains of Rad4 and Rad34 are functionally required for the NER reaction whereas the poorly conserved amino terminal half determines in which chromosomal loci the proteins operate. However, we demonstrate that the conserved domains cannot be interchanged between Rad4 and Rad34. We discuss the possible roles of the two Rad4 proteins and argue whether the role in NER is responsible for the evolutionary conservation of the Rad34 protein.

Introduction

In *Saccharomyces cerevisiae*, the heterodimeric Rad4-Rad23 complex is the initial damage recognition factor of the Nucleotide Excision Repair (NER) pathway. In RNA pol II transcribed regions the Rad4-Rad23 complex is essential for both NER subpathways Global Genome Repair (GGR) and Transcription Coupled Repair (TCR). Rad4 and its human homologue XPC belong to the group of NER proteins that are essential for the *in vitro* reconstituted NER reaction and are therefore defined as core-NER proteins (Guzder *et al.*, 1995; He *et al.*, 1996; Mu *et al.*, 1995). Rad4 homologues are present in all eukaryotes studied thus far. The homology between Rad4 and XPC, like in all Rad4 proteins, is concentrated in the carboxy terminal domain whereas the sequences of the amino terminal regions display substantial divergence. Biochemical studies imply that XPC and Rad4 have generally similar functions in NER since both proteins preferentially bind to damaged DNA (Batty *et al.*, 2000; Guzder *et al.*, 1998; Jansen *et al.*, 1998; Sugasawa *et al.*, 1998). However, although the basic function is conserved, the roles of the Rad4 homologues differ with regard to their involvement in the two NER sub-pathways GGR and TCR. In human cells the XPC protein is essential for all GGR but not required for the TCR reaction in RNA pol II transcribed regions. In *S. cerevisiae* however two Rad4 homologues are present with specific, not overlapping tasks: Rad4 is essential for both GGR and TCR of RNA pol II transcribed genes whereas Rad34 (the protein we previously referred to as YDR314C) is specifically required for preferential repair of RNA pol I transcribed rDNA (den Dulk *et al.*, 2005). The fission yeast *Schizosaccharomyces pombe* also contains two functional Rad4 homologues, Rhp41 and Rhp42 (Fukumoto *et al.*, 2002; Marti *et al.*, 2003). These proteins have overlapping roles with regard to their involvement in GGR or TCR, although their relative contribution is not clear. The involvement of the *S. pombe* Rad4 homologues clearly is organized in a different way compared that of the *S. cerevisiae* Rad4 and Rad34 proteins, which are unable to substitute for each other.

The differences between the Rad4 homologues described above demonstrate that in some forms of NER the action of the Rad4 homologues is either not required (XPC independent TCR human cells), is dependent on a second homologue within the same organism (Rad34 dependent rDNA repair in *S. cerevisiae*) or can optionally be substituted by a second homologue (Rhp41 and Rhp42 in *S. pombe*). Understanding the nature of these yet enigmatic differences may reveal more about the actual mechanism by which the Rad4 homologues function in NER.

Here we present data that further establish the homology between Rad4 and Rad34 and show that the non-conserved amino terminal regions can not be interchanged between these proteins. We discuss these results in the light of the recently published crystal structure of Rad4 (Min and Pavletich, 2007).

Results

Rad34 binds directly to both Rad23 and Rad33.

The Rad4 homologue Rad34 was previously shown to act in preferential repair of the RNA pol I transcribed strand (den Dulk *et al.*, 2005), suggesting that the action of Rad34 is comparable to that of Rad4 in TCR of RNA pol II transcribed DNA. It is therefore expected that Rad34, like Rad4, will also have direct interactions with both Rad23 and Rad33. This idea is supported by data from large scale screens that reported interaction of Rad34 with both Rad23 and Rad33 (Gavin *et al.*, 2002; Ito *et al.*, 2001).

To examine whether Rad33 and Rad23 directly bind Rad34 we performed a two-hybrid test. **Figure 1** shows that Rad34 binds Rad23 via a direct interaction. Analogous to the situation in the Rad4 complex (den Dulk *et al.*, 2008), Rad33 does not bind Rad23, but we do find interaction between Rad33 and Rad34. This suggests that *S. cerevisiae* has two similar damage recognition complexes: Rad4-Rad23-Rad33 and Rad34-Rad23-Rad33. Rad23 is required for functioning of both complexes as the *RAD23* deletion completely disables Rad4 and Rad34 dependent NER (Verhage *et al.*, 1996a; Verhage *et al.*, 1996b). In *rad33* cells Rad34-Rad23 appears not functional as no repair of rDNA is observed (**Figure 2,** (den Dulk *et al.*, 2006)). In contrast, the Rad4-Rad23 complex lacking Rad33 can still facilitate TCR in RNA pol II transcribed regions, albeit with a reduced efficiency (den Dulk *et al.*, 2006).

UV survival of rad34 cells with a reduced number of rDNA repeats.

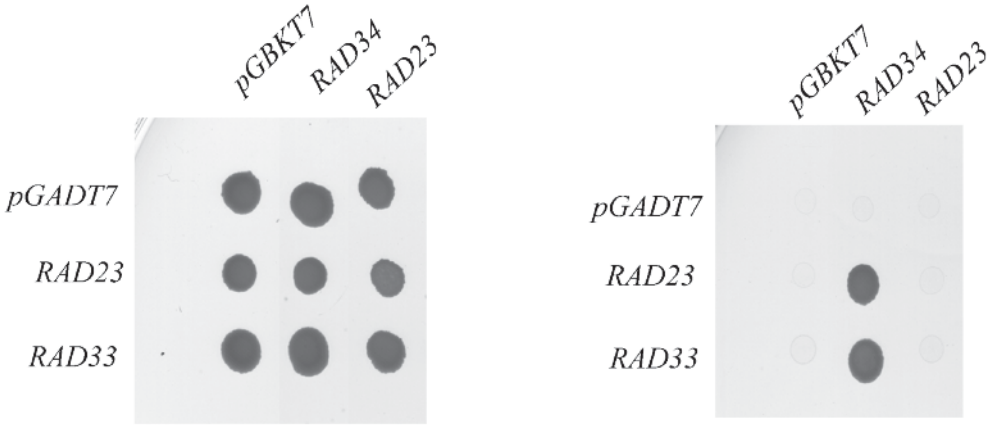


Figure 1:
Two Hybrid test. The coding regions of *RAD23, RAD33* and *RAD34* were cloned in the pGBKT7 or pGADT7 vectors (Clonetech Matchmaker 3) as indicated in the figure. The empty vectors and constructs were transformed to Y187 and AH109 cells as described in materials and methods. The cells were assayed on growth on YNB medium selective for the presence of both plasmids (left panel) and on medium selective for transcription activation of the reporter genes (*HIS3* and *ADE2) (*Right panel), which is indicative for interaction of the tested proteins.

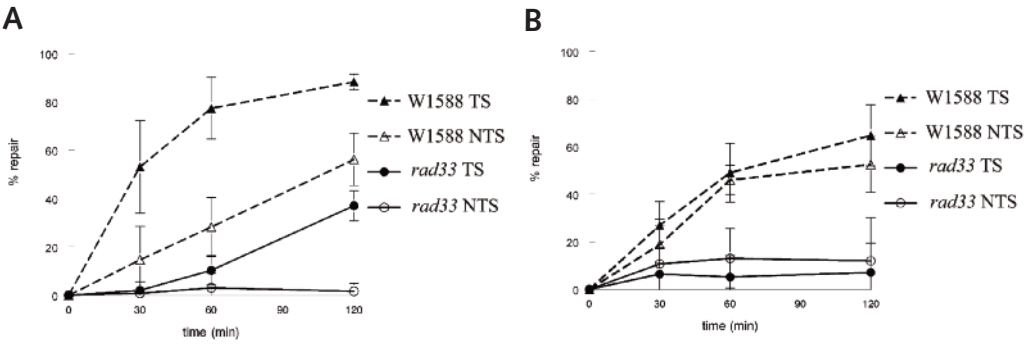


Figure 2:
Repair analysis.
(**A**) Strand specific repair analysis of the *RPB2* gene in wildtype (W1588) and *rad33* cells.
(**B**) Strand specific repair analysis of the RNA pol I transcribed rDNA locus. See also chapter 5.

Cells deleted for *RAD4* or *RAD34* display a significant difference in survival after UV irradiation. Whereas *rad4* cells are severely UV sensitive, we found no conditions in which deletion of the *RAD34* gene leads to increased UV sensitivity (den Dulk *et al.*, 2005). The involvement of Rad34 in NER is restricted to repair in the RNA pol I transcribed region of the rDNA locus. Possibly, the high number of *rRNA* genes (typically 100 to 200 copies are present in *S. cerevisiae* (Carmo-Fonseca *et al.*, 2000; Dammann *et al.*, 1993; French *et al.*, 2003)) has a bearing on the UV survival of *rad34* cells. The effect of the rDNA copy-number on UV survival is examined in two types of *rad4* deficient cells with a different number of *rRNA* genes; NOY1064*rad4* (~190 *rRNA* genes) and NOY1071*rad4* (~25 *rRNA* genes) (Cioci *et al.*, 2003). Due to the *rad4* deletion in these cells all remaining NER activity is confined to the RNA pol I transcribed strand and dependent on the Rad34 protein (den Dulk *et al.*, 2005). The method by which the number of *rRNA* genes has been altered is described in the materials and methods section.

UV dose response curves of NOY1064*rad4,* NOY1064*rad4rad34,* NOY1071*rad4* and NOY1071 *rad4rad34* are shown in **Figure 3.** Clearly, the deletion of *RAD34* does not sensitize NOY1064 *rad4* cells (~190 *rRNA* genes) for UV irradiation. Also, the NOY1071 *rad4rad34* cells (harboring ~25 *rRNA* genes) are not, or only marginally, more sensitive than the *RAD34*$^{+}$ NOY1071 *rad4* cells. This finding shows that even in cells with a reduced number of *rRNA* genes, that are solely dependent on Rad34 for the removal of UV induced lesions, deletion of the *RAD34* gene does not significantly affect the survival after UV irradiation.

Exchanging the conserved domains of Rad4 and Rad34.
The sequence homology between Rad4 homologues is mainly present in the so called pFAM domain (Bateman *et al.*, 2004; Bunick *et al.*, 2006; Sonnhammer *et al.*, 1997), which constitutes most of the carboxy terminal half of the Rad4 proteins (**Figure 4A**). The pFAM domain harbors several interaction sites that are essential for the function of Rad4/XPC in NER. We recently showed the Rad4 pFAM domain includes binding sites for Rad23 and Rad33 (den Dulk *et al.*, 2008). In the XPC protein the pFAM re-

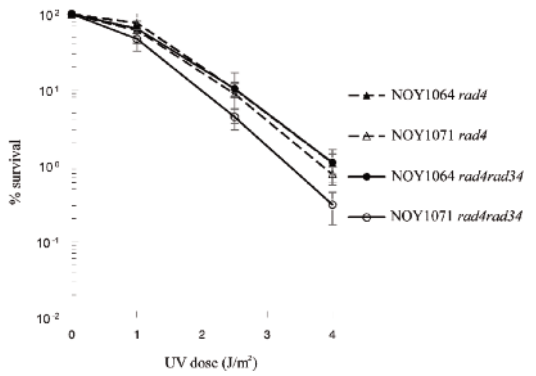


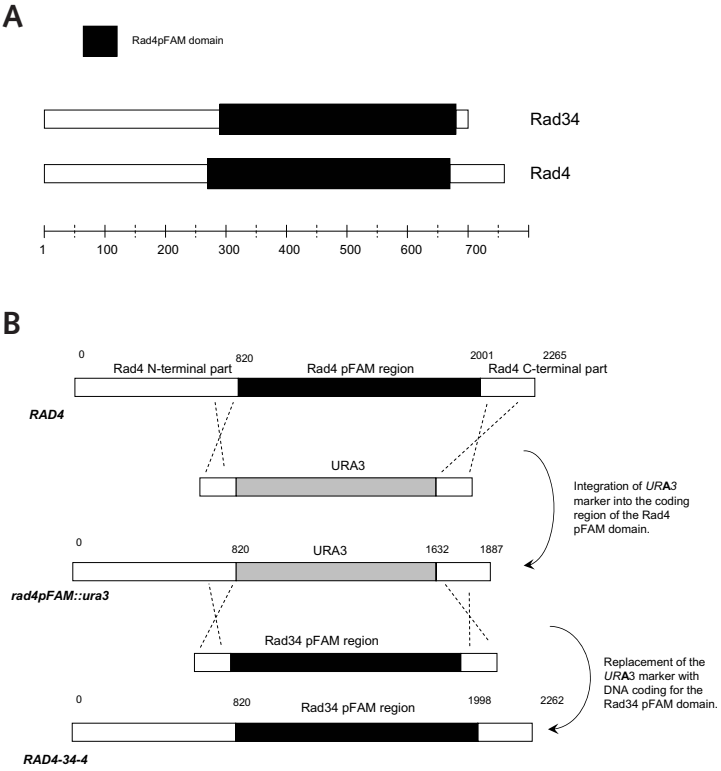
Figure 3
UV dose response curve. Suitable dilutions of exponentially growing cells were irradiated with the doses indicated. Plates were incubated 72 hours in the dark at 30°C. Colonies were counted and the percentage of surviving cells was calculated. Average of 4 independent experiments are shown.

gion is also essential for the interaction with Rad23, as well as for binding to TFIIH and DNA (Bunick *et al.*, 2006; Uchida *et al.*, 2002). The amino terminal half varies considerably between Rad4 homologues and the contribution of this region to NER is unclear. The factor that determines that Rad4 and Rad34 operate in distinct chromosomal regions might therefore be the amino terminal region. According to this hypothesis, the Rad4 protein would retain its specific function in NER when its conserved domain will be exchanged for that of Rad34, and *vice versa*.

To examine this possibility we constructed a Rad4 protein in which the conserved pFAM domain is replaced by the pFAM domain of Rad34 and a Rad34 protein that contains the pFAM domain of Rad4 (Figure4B), as described in the materials and methods. The Rad4 protein containing the conserved pFAM domain of Rad34 is referred to as Rad4-34-4, The Rad34 protein containing the pFAM domain of Rad4 is referred to as Rad34-4-34. To specifically monitor the role of the hybrid Rad4/Rad34 proteins in NER the *RAD4-34-4* gene was introduced in *rad34* cells and the *RAD34-4-34* gene in *rad4* cells. Expression of the hybrid proteins was demonstrated as described in materials and methods (**figure 4C**).

If the Rad4-34-4 protein is functional, our hypothesis predicts that it will (partially) substitute for the Rad4 protein, but not for the Rad34 protein. *RAD4-34-4 rad34* cells will thus be (partially) UV resistant but defective in rDNA repair. For *rad4* cells expressing the Rad34-4-34 protein we expect that these cells will remain UV sensitive since the Rad4 protein is absent, but will be proficient in preferential repair of the rDNA TS due to the presence of the Rad34-4-34 protein.

In contradiction to our hypothesis however, not only *rad4 RAD34-4-34* cells, but also the *rad34 RAD4-34-4* cells show UV sensitivity similar to that of complete NER deficient strains (**data not shown**), indicating that Rad4-34-4 cannot substitute for functional Rad4. To test whether one of the hybrid proteins can substitute for Rad34 in NER, repair of the RNA pol I transcribed strand was analyzed. The results in **Figure 4D** show that lesions are persistent in both the *rad4 RAD34-4-34* and *rad34 RAD4-34-4* mutants, demonstrating that the Rad34-4-34 hybrid protein is unable to fulfill the role of Rad34 in rDNA repair.


A
Rad4pFAM domain
Rad34
Rad4
1
100
200
300
400
500
600
700
B
0
Rad4 N-terminal part
820
Rad4 pFAM region
2001
2265
Rad4 C-terminal part
RAD4
URA3
Integration of URA3 marker into the coding region of the Rad4 pFAM domain.
0
820
URA3
1632
1887
rad4pFAM::ura3
Rad34 pFAM region
Replacement of the URA3 marker with DNA coding for the Rad34 pFAM domain.
0
820
Rad34 pFAM region
1998
2262
RAD4-34-4
C
Rad4-TAP
TAP-Rad4
TAP-Rad4 20%
TAP-Rad4-34-4
TAP-Rad4 10%
Rad34-TAP
Rad34-TAP 20%
Rad34-4-34-TAP
Rad34-TAP 10%
100
80
60
40
20
0

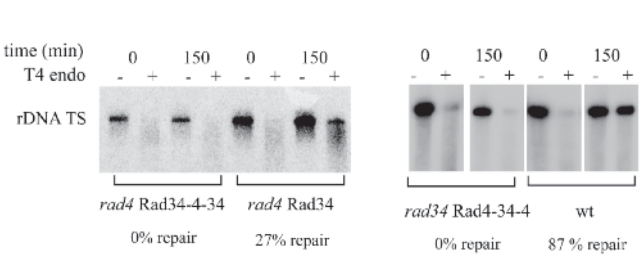


Figure 4
(**A**) Schematic representation of the location of the pFAM domain in the Rad4 and Rad34 proteins.
(**B**) Schematic representation of the construction of the *RAD4-34-4* gene. The *URA3* gene was amplified using primers with 5' flanks homologous to the flanking region of the pFAM region of *RAD4*. The PCR product was transformed into yeast yielding cells with a deletion of the *RAD4* pFAM region (*rad4pFAM::URA3* cells). A similar PCR was performed, now amplifying the *RAD34* pFAM domain. This construct was introduced in the *rad4pFAM::URA3* cells, generating mutants in which the coding region for the Rad4 pFAM domain is exchanged for the coding region of the Rad34 pFAM domain (*RAD4-34-4* cells).
(**C**) Expression of the Rad4/Rad34 hybrid proteins. Upper panel: Westernblot analysis of the TAP tagged Rad4, Rad34, Rad4-34-4, Rad34-4-34 proteins. Rad4 and Rad4-34-4 bear an N-terminal TAP-tag, Rad34 and Rad34-4-34 are C-terminally TAP tagged. Lane 2,3 and 5 contain different quantities of N-TAP-Rad4 extracts as indicated; analogously, lane 6,7 and 9 contain different quantities of Rad4CTAP extract. Lane 1 shows a C-terminally tagged Rad4 protein to illustrate the difference in migration between the N and C terminal tagged proteins. Lower panel: Quantification of the westernblot showing the relative intensities of the protein bands. The first 5 columns are all related to TAP-Rad4 (column 2), the last 4 columns are related to Rad34-TAP (column 6).
(**D**) Southern blot showing the removal of dimers from the rDNA TS at 0 and 150 minutes in *rad4* Rad34 cells, *rad4* Rad34-4-34, *wildtype* and *rad34* Rad4-34-4 cells. Samples mock-treated or treated with the dimer-specific enzyme T4endoV are denoted with – and + respectively. Percentage lesion removal is indicated below the blots.

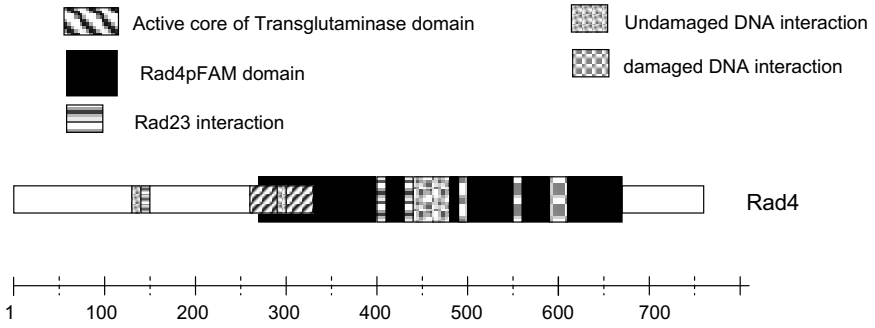


Figure 5
Schematic representation of the approximate location of the residues in Rad4 required for interaction with Rad23, undamaged dsDNA segment and the damaged DNA segment (Min and Pavletich, 2007).

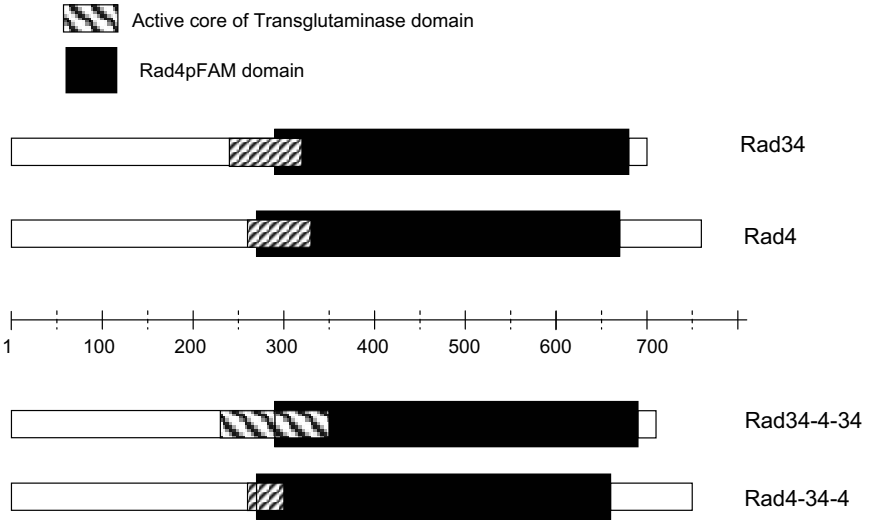


Figure 6:
Schematic representation of location of the pFAM domain and the TGD in Rad4, Rad34, Rad4-34-4 and Rad34-4-34 proteins.

Discussion

We previously reported on the presence of a Rad4 homologue, now known as Rad34, in the yeast *S. cerevisiae* (den Dulk *et al.*, 2005). Both yeast Rad4 proteins are involved in damage recognition in NER, but function in different DNA regions and are unable to substitute for each other. Here we study the role of Rad34 in more detail and focus on the difference between Rad4 and Rad34. We demonstrate that Rad34, like Rad4, binds directly to both Rad23 and Rad33 whilst Rad23 and Rad33 do not interact with each other, suggesting that Rad4 and Rad34 function in similar complexes. Absence of Rad23 in either complex results in a complete defect in CPD removal.

As for all Rad4 homologues, the homology of the Rad4 and Rad34 proteins is concentrated in a conserved domain referred to as Rad4-pFAM domain, located in the carboxy-terminal half of the Rad4-proteins (Bateman *et al.*, 2004; den Dulk *et al.*, 2005).

For XPC it is shown that the binding sites for DNA, hHR23B and TFIIH are all located in this domain (Uchida *et al.*, 2002) and we recently demonstrated that the binding sites on Rad4 for Rad23 and Rad33 are also located in the carboxy terminal domain (den Dulk *et al.*, 2008).

In contrast to the Rad4 pFAM domain the sequences of the amino terminal domains of Rad4 and Rad34 (and all other Rad4 orthologues) display considerable diversity. The sequence disparity in the amino terminal region of the Rad4 homologues implies that the precise composition of this region is of minor importance for the role in NER.

The sequence divergence of the amino-terminal region might be related to the distinct roles of the various Rad4 homologues. Possibly, in Rad4 homologues the conserved pFAM domain is required for the damage recognition in NER whilst the divergent amino terminal region regulates where in the genome this function is applied.

However, our results do not support this hypothesis. We find that the exchange of the pFAM domains between Rad4 and Rad34 does not generate functional Rad4 proteins.

Results from the recently published crystal structure of the Rad4 protein bound to damaged DNA (Min and Pavletich, 2007) might explain the observation that the Rad4-34-4 or Rad34-4-34 hybrid proteins are not functional. It was previously assumed that the Rad23 and DNA binding domains were present on the conserved pFAM domain (Uchida *et al.*, 2002). However, Min and Pavletich show that for the binding of DNA and the Rad23 peptide, residues on both the amino terminal part and the carboxy terminal part of Rad4 are involved (**Figure 5**). The fact that these residues are separated in our Rad4-34-4/Rad34-4-34 hybrid proteins may disturb the capability of the proteins to bind DNA and/or Rad23. In addition, the 45 residue core-transglutaminase fold which has a structure stabilizing role (Min and Pavletich, 2007) is present on the boundary of the amino-terminal part of Rad4 and the pFAM domain. Since the position of the TGD-core relative to the pFAM domain is different for Rad4 and Rad34, the TGD-core is scrambled in the hybrid proteins (**Figure 6**), thereby possibly affecting the stability of the proteins as might be reflected by the observation that the levels of Rad4-34-4 and Rad34-4-34 protein are ~10 to ~20 fold lower compared to that of Rad4 and Rad34. We do not believe that these reduced protein levels are solely the cause of the inactivity of the hybrid proteins however, since it has been shown that cells with a significantly reduced amount of Rad4 are only mildly or moderately UV sensitive (den Dulk *et al.*, 2006; Ortolan *et al.*, 2004), whereas expression of the Rad4-34-4 results in a complete NER defect.

The question why different Rad4 homologues are required for different loci remains unanswered. The substrates of Rad4 and Rad34 differ on three important points: (1) The RNA pol involved in the region, (2) the intracellular localization and (3) the chromatin structure. The fact that Rad4 is involved in GGR of rDNA (den Dulk *et al.*, 2005; Verhage *et al.*, 1996a) excludes the possibility that a physical barrier restrains the Rad4 protein from entering the nucleolus. In addition, the inability of Rad34 to replace Rad4 is not due to nucleolar confinement as GFP tagged Rad34 is present throughout the nucleus (Huh *et al.*, 2003). The involvement of Rad4 in GGR in rDNA also shows that whatever different properties of the rDNA chromatin compared to those of RNA pol II transcribed regions do not hinder Rad4 in performing GGR. This does not exclude the possibility that Rad34 is blocked by the chromatin structure outside the nucleolus. However, Rad34 is not involved in GGR of rDNA either, but specifically required for preferential repair of the RNA pol I template strand. The most likely explanation for the separate roles of Rad4 and Rad34 is therefore that differences in the RNA polymerases determine the requirement of either Rad4 or Rad34. We propose that Rad34 is a TCR factor required for RNA pol I transcribed regions.

Nonetheless, Rad34 does not contribute to the cellular survival after DNA damage induction. Since the presence of Rad34 as NER factor has no clear evolutionary advantage it is uncertain that Rad34 is intrinsically a NER factor. The conservation of Rad34 may be due to its involvement in processes other than NER. For example, the Rad34-Rad23-Rad33 complex might use the biochemical activities that are also present in the homologous Rad4-Rad23-Rad33 complex, for a distinct purpose. The recognition of disturbed DNA helix structures and subsequent induction of a conformational change in the DNA (Min and Pavletich, 2007) could also be applicable in other DNA

metabolic processes. Similar to the human TCR factor CSB which is involved both in TCR and in transcription elongation (Balajee *et al.*, 1997; Selby and Sancar, 1997), Rad34 might modulate RNA pol I transcription. Nevertheless, in contrast to CS-B cells, *rad34* knockout cells behave like wildtype cells and do not exhibit any transcription related defects (den Dulk *et al.*, 2005). Nevertheless, Rad34 *is* conserved in *S. cerevisiae*. If the role in NER is not the basis of its conservation, the question remains what the contribution of Rad34 to cellular survival is. A distinctive feature of the role of Rad34 in NER is its specific involvement in the relatively small rDNA locus. The stability of the rDNA locus is involved in the ageing of yeast cells, as the accumulation of rDNA circles, formed by recombination, eventually cause lethality (Sinclair and Guarente, 1997). Transcription dependent fork collisions are reported to induce extrachromosomal rDNA circle formation. Given the involvement of Rad34 in TCR, it might be worthwhile to examine rDNA circle formation in *rad34* cells.

Methods and materials

Strains

The strains used in this study are listed in table 1. MGSC769 (*rad4 RAD34-4-34*) and MGSC876 (*rad34 RAD4-34-4)* were constructed by targeting the sequences in the *RAD34* and *RAD4* genes coding for the pFAM domains with an *URA3* construct. This integration construct was created by PCR on the *URA3* gene using primers with 50bp 5' flanks homologous to the flanking regions of the pFAM regions of either *RAD4* or *RAD34* as shown in **Figure 4A**. The cells deleted for either the *RAD4* or *RAD34* regions coding for the pFAM domains were subsequently targeted with constructs containing the coding regions of the pFAM domains of *RAD4* and *RAD34*. These constructs were flanked by sequences homologous to the neighboring DNA of the *RAD34* and *RAD4* pFAM regions respectively, thus using the same flanking sequences as were employed in the introduction of the *URA3* gene. Correct exchange of the pFAM domains was confirmed by sequencing. To detect protein expression TAP tag constructs were created as described previously by Puig *et al.* (2001). For technical reasons the Rad4-34-4 protein was combined with an amino terminal TAP tag whereas the Rad34-4-34 protein fused to a carboxy-terminal TAP tag. To verify the expression of the TAP-Rad4-34-4 and Rad34-4-34-TAP proteins, cell extracts were analyzed on western blot (**Figure 3C**). The results show that the proteins are produced, but that the protein levels of the hybrid proteins are lower than that of native Rad4 or Rad34. From the western blot it is estimated that the amount of the Rad4-34-4 protein is roughly ~5% compared to Rad4, the level of Rad34-4-34 is around 10% of that of Rad34. These results may indicate that the hybrid proteins are unstable.

Despite the Rad4-34-4 protein is one residue larger than Rad4, the Rad4-34-4 protein runs significantly lower on gel (**Figure 3C**). Similarly, Rad34-4-34 is smaller than Rad34, but runs higher on the gel. This phenomenon is not unfamiliar; Rad4, an 88kD protein, appears at a height corresponding to a ~110kD protein. The data here shows that the pFAM domain of Rad4 is responsible for the still unexplained behavior of the protein when analyzed on protein gels.

The *fob1* mutants with ~25 rDNA units (NOY1071) or ~190 (NOY1064) (Cioci *et al.*, 2003) rDNA repeats were a gift of Dr. Masayasu Nomura (Department of Biological chemistry, University of California). These strains are derivatives of W303 with an altered number of *rRNA* genes. It has been established that the *rRNA* gene repeat is contracted in cells lacking RNA pol I transcription. The disabling of RNA pol I transcription was realized by the introduction of a deletion in the *RPA135* gene, encoding the largest subunit of RNA pol I. These *rpa135* mutants are only viable in the presence of helper plasmid containing one copy of the ribosomal genes behind a strong RNA pol II promoter in order to continue the supply of the ribosome subunits (Brewer *et al.*, 1992; Kobayashi *et al.*, 1998). The ~100-200 rDNA repeats that are normally present in yeast cells are gradually decreased in the *rpa135* mutant, to as few as 11 *rRNA* genes (Johzuka and Horiuchi, 2007). Reintroduction of the *RPA135* gene leads to the reversal of the decrease, up to the normal ~100-200 repeats (Kobayashi *et al.*, 1998). The underlying mechanisms of the contraction in *rpa135* cells is not fully clarified, but it is assumed that active transcription restrains the recombinational activity between the tandemly repeated *rRNA* genes (Buck *et al.*, 2002). The contraction of the rDNA re-

peat is dependent on the Fob1 protein, which is required for recombinational hotspot activity in the rDNA region (Kobayashi and Horiuchi, 1996). When *FOB1* is deleted in cells containing a contracted rDNA repeat, the *RPA135* gene can be reintroduced, and the 35S helper plasmid removed, without altering the rDNA copynumber.

Table 1: *S. cerevisiae* strains used

Strain	*Genotype*	Source
W1588-4a	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1*	R. Rothstein
W303-1a	*MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100*	*(Brill and Sternglanz, 1988)*
NOY1064	same as W303-1a, but *fob1::HIS3;* rDNA copy number ~190	*(Cioci et al., 2003)*
NOY1071	same as NOY1064, but rDNA copy number ~25	*(Cioci et al., 2003)*
MGSC 901	Same as NOY1064, but *rad34::LEU2*	This study
MGSC 905	Same as NOY1071, but *rad34::LEU2*	This study
MGSC 900	Same as NOY1064, but *rad4::HisG-URA3-HisG*	This study
MGSC 904	Same as NOY1071, but *rad4::HisG-URA3-HisG*	This study
MGSC 902	Same as NOY1064, but *rad4::HisG-URA3-HisG, rad34::LEU2*	This study
MGSC 906	Same as NOY1071, but *rad4::HisG-URA3-HisG, rad34::LEU2*	This study
MGSC 737	Same as W1588, but *N-TAP-RAD4*	This study
MGSC 542	Same as W1588, but *RAD34-C-TAP*	This study
MGSC 479	Same as W1588, but *rad4::HisG*	This study
MGSC 517	Same as W1588, but *rad34::LEU2*	This study
MGSC 769	as W1588, but *rad4::HisG RAD34-4-34-TAP::URA3KL*	This study
MGSC 876	Same as W1588, but *rad34::LEU2, N-TAP-RAD4-34-4*	This study

Two hybrid experiments

For protein interaction studies the Clontech Matchmaker 3 system was used. The full length *RAD23* and *RAD33* genes were fused to the *GAL4* activating domain (AD) in pGADT7. The full length *RAD23* and *RAD34* genes were fused to the *GAL4* binding domain (BD) in pGBKT7. The AD and BD vectors and constructs were introduced into Y187 and AH109 respectively by LiAc transformation. Mating was used to create diploid cells with combinations of AD and BD plasmids. Cultures of diploids were spotted on plates selecting for the presence of two plasmids and on plates indicating expression of the reporter genes ($GAL1_{UAS}$-$GAL1_{TATA}$-*HIS3;* $GAL2_{UAS}$-$GAL2_{TATA}$-*ADE2*). Plates were incubated for 3 days at 30°C.

Western blot analysis

Cells containing TAP-tagged proteins (*TAP-RAD4, RAD34-TAP, rad4 RAD34-4-34-TAP* or *TAP-RAD4-34-4*) were grown to an optical density of 10 before extracts were prepared. Cells were pelleted and proteins were extracted using 20%TCA as described previously (den Dulk *et al.*, 2006; Falconi *et al.*, 1993). The protein extracts were run on SDS page gels and transferred to a PVDF transfer membrane (GE healthcare Hybond-P) using a semi-dry western blotting set (Sigma-Aldrich). The presence of TAP-

tagged proteins was shown using rabbit Peroxidase-anti-Peroxidase antibodies (American Qualex, P2250).

UV survival curve
For UV survival analysis overnight cultures were diluted in YPD and grown for 6 hours in YPD medium. Cells were then diluted in water to appropriate OD_{600} values, plated on YPD plates and irradiated with the doses indicated. Cells were grown for 3 days in the dark at 30°C, colonies were counted and survival was calculated. The values depicted in the graphs are averages of 4 independent experiments; error-bars represent standard deviations.

Repair analysis
Cells were grown in YPD to an OD_{600} of 4.0, pelleted and resuspended in ice-cold PBS at an OD_{600} of 1.4. The cells were irradiated to 100 J/m^2 at a rate of 2.9 J/m^2/s. The irradiated cells were pelleted and resuspended in YPD and kept at 30°C to allow repair. After 0 and 150 minutes cells were pelleted, resuspended in ice-cold water to stop repair, pelleted and frozen at -20°C prior to DNA isolation. DNA was isolated as described previously (den Dulk *et al.*, 2005; Li and Smerdon, 2002). Repair of rDNA was measured as described by Verhage *et al.* (1996a). The Southern blots were quantified using a Bio-Rad Molecular Imager and Quantity One software.

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