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

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Chapter

5

Rad33, a new factor involved in nucleotide excision repair in Saccharomyces cerevisiae

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Rad33, a new factor involved in Nucleotide Excision Repair in *Saccharomyces cerevisiae*

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Abstract

In *Saccharomyces cerevisiae* the Rad4-Rad23 complex is involved in initial damage recognition and responsible for recruiting the other NER proteins to the site of the lesion. The Rad4-Rad23 complex is essential for both NER subpathways, Transcription Coupled Repair (TCR) and Global Genome Repair (GGR). Previously, we reported on the role of the Rad4 homologue YDR314C in NER. YDR314C is essential for preferential repair of the transcribed strand in RNA pol I transcribed rDNA. In large scale interaction studies it was shown that YDR314C physically interacts with a small protein encoded by the ORF *YML011C*. In the present study we show that YML011C is involved in NER and we propose to designate the YML011C ORF *RAD33*. Cells deleted for *RAD33* display intermediate UV sensitivity that is epistatic with NER. Strand specific repair analysis shows that GGR in RNA pol II transcribed regions is completely defective in *rad33* mutants whereas TCR is still active, albeit much less efficient. In RNA pol I transcribed rDNA both GGR and TCR are fully dependent on Rad33. We show that in both *rad23* and *rad33* cells Rad4 and YDR314C protein levels are significantly reduced. Although the *rad23rad33* double mutant is considerably more UV sensitive than a *rad23* or *rad33* single mutant, deletion of *RAD33* in a *rad23* background does not lead to a further reduction of Rad4 or YDR314C protein levels. This suggests that the role of Rad33 is not solely the stabilization of Rad4 and YDR314C but that Rad33 has an additional role in NER.

5.1 Introduction

Nucleotide Excision Repair (NER) is involved in removing many structurally unrelated helix distorting lesions from DNA (de Laat *et al.*, 1999; Prakash and Prakash, 2000; Wood, 1997). NER is the main pathway for repair of UV light induced lesions such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PPs). Extensive studies lead to the identification and isolation of the proteins involved in the basic NER reaction and enabled the reconstitution of the NER reaction *in vitro* (Aboussekhra *et al.*, 1995; Guzder *et al.*, 1995; He *et al.*, 1996; Mu *et al.*, 1995). *In vivo,* eukaryotic NER consists of two sub-pathways Global Genome Repair (GGR), involved in repair throughout the entire genome, and Transcription Coupled Repair (TCR), specifically implicated in repair of lesions in the transcribed strand of active genes.

The *Saccharomyces cerevisiae* Rad4-Rad23 complex is involved in initial damage recognition by binding preferentially to damaged DNA (Guzder *et al.*, 1998; Jansen *et al.*, 1998). In the human system it has been shown that XPC-HHR23B is essential for the recruitment of the other NER proteins to the site of the lesion (Sugasawa *et al.*, 1998; Volker *et al.*, 2001). Whereas the affinity of Rad4 and XPC for damaged DNA was shown (Batty *et al.*, 2000; Guzder *et al.*, 1998), the role of Rad23 in NER is not clear yet. The human Rad23 homologue HHR23B is not essential for the *in vitro* NER reaction, but stimulates the efficiency (Reardon *et al.*, 1996; Sugasawa *et al.*, 1996).The yeast Rad23 protein also appears to have an accessory role since cells deleted for the *RAD23* gene are only moderately UV sensitive compared to the UV sensitivity of mutants lacking functional NER (e.g. *rad2* or *rad14 cells)*. However, lesion removal in *rad23* mutants is almost as severely impaired as in complete NER deficient cells (Mueller and Smerdon, 1996; Verhage *et al.*, 1996c). The basis for the relative high survival after UV irradiation in the light of the virtual absence of any repair in *rad23* cells is yet unknown.

Multiple studies reported that in *rad23* cells Rad4 is degraded by the proteasome, indicating that the NER defect in *rad23* mutants is caused by a decrease of Rad4 protein levels (Ortolan *et al.*, 2004; Ramsey *et al.*, 2004; Sweder and Madura, 2002; Xie *et al.*, 2004). Experiments in mice showed that a similar effect occurs in mammalian cells (Ng *et al.*, 2003; Okuda *et al.*, 2004). Importantly, both the UV sensitivity and the low Rad4 protein levels in *rad23* cells can be suppressed significantly by mutating the 19S regulatory subunit of the 26S proteasome or by deletion of genes encoding the ubiquitin conjugating enzyme Ubc4 (Lommel *et al.*, 2002; Ng *et al.*, 2003; Ortolan *et al.*, 2004). This suggests that the main role of Rad23 is protecting Rad4 from ubiquitylation and subsequent degradation by the proteasome (Ortolan *et al.*, 2004). However, the Rad4-binding domain of Rad23 is sufficient for restoration of wildtype Rad4 protein levels but not for complete suppression of the UV sensitivity, suggesting that Rad23 has an additional role in NER. Moreover, overexpression of Rad4 did not suppress the UV sensitivity nor the repair defect of *rad23* cells (Lommel *et al.*, 2002; Xie *et al.*, 2004), consistent with the notion that stabilization of Rad4 might not be the only role of Rad23.

We reported recently that in *Saccharomyces cerevisiae* the Rad4 homologue YDR314C fulfills the role of Rad4 in repair of rDNA (den Dulk *et al.*, 2005). Results

from a large scale interaction study showed that YDR314C interacts with Rad23 (Gavin *et al.*, 2002). This interaction appears essential for the function of YDR314C as repair of rDNA is completely absent in *rad23* cells (Verhage *et al.*, 1996a).

In the present study we focus on the role of a second protein reported to interact with YDR314C, encoded by the ORF *YML011C* (Ito *et al.*, 2001). Cells devoid of YML011C were identified as UV sensitive in a large scale competitive growth assay (Hanway *et al.*, 2002). Here we demonstrate that the UV sensitivity of y*ml011c* mutants is epistatic with that of NER deficient cells and we propose to designate the *YML011C* ORF *RAD33*. Strand specific repair analysis of both RNA pol I and RNA pol II transcribed regions shows that NER in *rad33* cells is severely defective. Interestingly, the protein-levels of both Rad4 and YDR314C are strongly reduced in *rad33* mutants, indicating that Rad33, like Rad23, is involved in stabilization of Rad4 and YDR314C.

5.2. Materials and methods

5.2.1. Strains and plasmids

The strains used in this study are listed in table 1. The strains used for the experiments depicted in Figure 1A were derived from the BY4741 (Euroscarf) and Y5565 strains. Strains MGST2057, 2059, 2061 and 2131 were constructed by transforming the Euroscarf deletion strains with EcoRI digested p4348, mating with Y5565, sporulation and haploid isolation. P4348 and Y5565 were a gift from A.tong (Tong *et al.*, 2001). All other experiments were conducted in the W1588-4a (Mortensen *et al.*, 2002) background, provided by R. Rothstein. All mutants in the W1588-4a background were generated by lithium acetate transformation (Gietz *et al.*, 1992) using linear disruption constructs.

YML011C deletions were constructed by transforming target strains with a deletion construct obtained from the *yml011c* mutant of the Euroscarf collection by PCR using primers 5' CATATCAGGCGGGTCACCTTTAGA 3' and 5' CCATGCATTTGTGTATCAGCTTACC 3'.

TAP-tag constructs were created as described previously (Puig *et al.*, 2001). Cells containing the TAP-tagged genes were checked for the expected UV phenotype indicative for correct gene expression. The *YDR314C* disruption construct was created as described previously (den Dulk *et al.*, 2005). The W1588 *rad23::HisG* and *rad26::HIS3* mutants were created using disruption constructs previously used in our laboratory to delete these genes in the W303 background *(van Gool et al., 1994; Verhage et al., 1996c)*. *YCpTEF2RAD33* was created by cloning a PCR generated *RAD33* fragment using primers 5' CGGGATCCTCAATGAGCAAATCCACTAACGT and 5' GAGAATTCTTCGCTTCACATCTTTAAGTAACCTAG in *YCpTEF2*. Underlined sequences contain a BamHI and EcoRI restriction site respectively by which the *RAD33* fragment was cloned into the YCpTEF2 plasmid.

5.2.2 UV survival curves

Cells were grown for 3 days in YPD and diluted in water to appropriate OD_{600} values. The diluted cells were plated on YPD. The *rad4, rad4rad33* and *rad23rad33* cells were irradiated with 0, 2, 4 or 6 J/m^2, all other cells were treated with 0, 15, 30 or 40 J/m^2. 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 at least 3 independent experiments; error-bars represent standard deviations.

5.2.3 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 84 J/m^2 at a rate of 2.9 J/m^2/s. The irradiated cells were pelleted, resuspended in YPD and kept at 30°C to allow repair. After 0, 30, 60 and 120 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 by previously (den Dulk *et al.*, 2005; Li and Smerdon, 2002) Analysis of repair of the *RPB2* gene was performed as described by Jansen *et al.* (Jansen *et al.*, 2000) repair of rDNA was measured as described by Verhage *et al. (Verhage et al., 1996a)*. The Southern blots were quantified using a Bio-Rad Molecular Imager and Quantity

One software. The values depicted in the graphs are the average of at least three independent experiments and the error-bars indicate standard deviations.

5.2.4 Western blot analysis

Several cultures of cells harboring different TAP-tagged NER proteins were grown for three days in YPD or in selective medium for the complementation assays. Optical densities were checked for equality and corrected when necessary. Cells were pelleted and proteins were extracted as described previously (Falconi *et al.*, 1993). The protein extracts were run on SDS page gels and transferred to a PVDF transfer membrane (Amersham Hybond-P) using a semi-dry western blotting apparatus (Sigma-Aldrich). The presence of TAP-tagged proteins was shown using rabbit Peroxidase-anti-Peroxidase antibodies (American Qualex, P2250). To allow loading control, blots were stripped and re-probed with an antibody against alpha tubulin (Genetex, GTX76511).

5.2.5 mRNA level analysis

RNA was isolated as described (Schmitt *et al.*, 1990). A 80 g aliquot of total RNA as a dried pellet was dissolved in 6% formaldehyde, 17% formamide, and 2 * SSC. After heat denaturation and dilution in 20*SSC the sample was transferred to a nylon membrane (Amersham Hybond-N+) under vacuum using a slot-blot apparatus (Schleicher & Schuell Minifold II). RNA was UV-crosslinked to the membrane, blots were hybridized with a a-^{32}P labeled probe directed against the TAP-tag, created by PCR with pBS1539 as target (Puig *et al.*, 2001) using primers 5' CCATGGAAAAGAGAAGATGGAAAAAG 5' and 5' GTTGACTTCCCCGCGGAATTC 3'. Blots were stripped and re-hybridized with a a-^{32}P labeled rDNA probe (Verhage *et al.*, 1996a). Slot blots were visualized using a Bio-Rad phospho-imager.

5.3 Results

Recently, we showed the involvement of the Rad4 homologue YDR314C in NER in *Saccharomyces cerevisiae* (den Dulk *et al.*, 2005). Whereas Rad4 is essential for NER in RNA pol II transcribed regions, YDR314C is specifically required for preferential repair of RNA pol I transcribed rDNA. From genome wide screens two proteins were reported to physically interact with YDR314C, Rad23 (Gavin *et al.*, 2002), the extensively studied complex partner of Rad4, and a small protein (177AA) encoded by the *YML011C* ORF (Ito *et al.*, 2001). The function of YML011C is not yet characterized and the protein contains no recognizable domains. A large scale localization experiment using GFP (Green Fluorescent Protein) tagged proteins showed that the *YML011C* gene product localizes in the nucleus (Huh *et al.*, 2003). In a genome wide competitive growth assay deletion of the *YML011C* gene was reported to confer UV sensitivity (Hanway *et al.*, 2002). These results prompted us to look further into the role of the YML011C protein.

5.3.1 Rad33 (YML011C) is involved in NER

Cells were deleted for *YML011C*, the reported UV sensitivity was confirmed (Figure 1A) and we therefore propose to designate the *YML011C* gene *RAD33*. In order to examine which, if any, DNA repair pathway is defective in *rad33* mutants, we deleted *RAD33* in various repair deficient cells and analyzed the survival after UV irradiation. The *rad33* mutation confers UV sensitivity in the post-replication repair defective *rad6* background as well as in double strand break repair defective *rad52* cells but not in cells lacking functional NER (*rad4* or *rad14* cells) (Figure 1A). This demonstrates that the UV sensitivity of *rad33* cells is specifically due to a defect in NER.

A more detailed analysis of the UV survival of NER mutants with an additional deletion of the *RAD33* gene is shown in Figure 1B,C Again we find that *rad4rad33* cells are not more sensitive than the *rad4* single mutant (Figure 1B). Deletion of *RAD33* causes UV sensitivity in both GGR (*rad16*) and TCR (*rad26*) defective backgrounds, showing that the intermediate UV phenotype of *rad33* mutants is not due to a specific GGR or TCR defect (Figure 1C). Although *rad16rad33* cells and *rad26rad33* cells are equally UV sensitive, the relative effect of the *RAD33* deletion is more pronounced in a *rad26* background, which might imply that GGR is affected more than TCR in cells deleted for *RAD33* (Figure 1C). Interestingly, the relative high survival of UV irradiated *rad23* cells is completely dependent on the presence of Rad33 since cells deleted for both *RAD23* and *RAD33* are as sensitive as complete NER deficient cells (Figure 1B).

5.3.2 Repair analysis of rad33 mutants.

To investigate whether Rad33 is actually involved in repair we analyzed the removal of CPDs in *rad33* cells. In Figure 2A,B the repair of the RNA pol II transcribed *RPB2* gene in *NER*$^+$ cells and *rad33* single mutants is shown. In *NER*$^+$ cells preferential repair of the transcribed strand (TS) due to active TCR is clearly visible. After two hours ~90% of the lesions is removed from the transcribed strand whereas ~50% of the lesions persist in the non-transcribed strand (NTS). In *rad33* cells, repair of the transcribed strand is severely reduced; no more than ~40% of the lesions is removed (Figure 2A,B). Strikingly, the effect on GGR is even more severe; repair of the non-transcribed

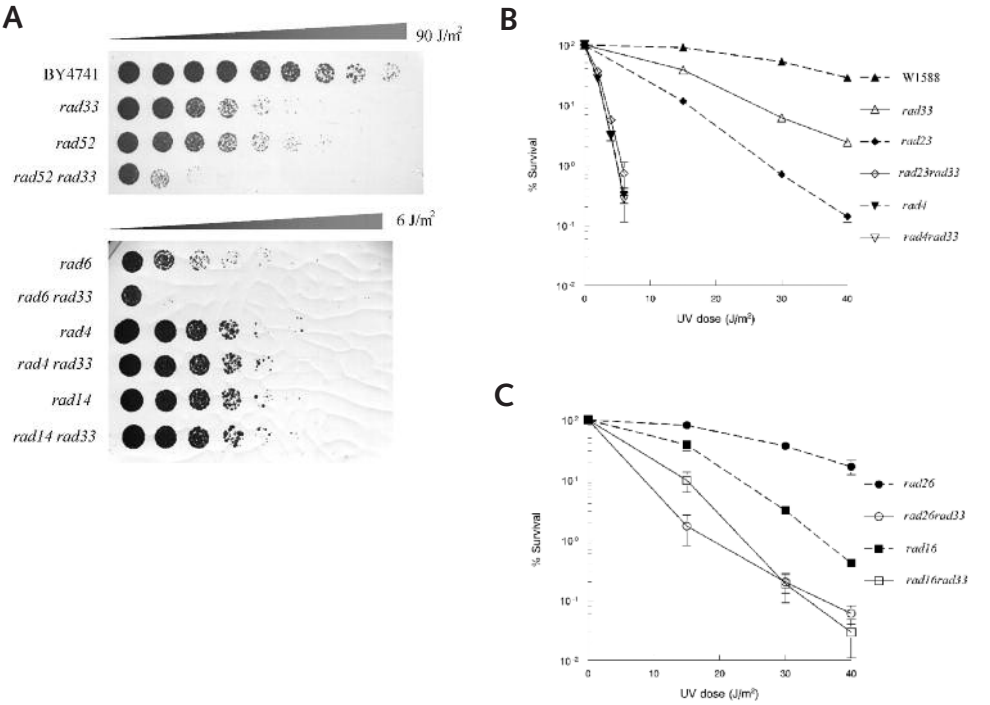

Figure 1
(A) UV droptest. Cells were grown for 3 days in YPD, diluted in water to appropriate OD_{600} values, dropped on YPD plates and irradiated with the indicated doses.
(B) UV survival curves. Cells were grown for 3 days in YPD, diluted in water to OD_{600} values that resulted in 100-200 colonies for each of the 3 administered UV doses and for the non irradiated sample. The diluted cells were plated on YPD and irradiated with the doses indicated. The irradiated cells were grown for 3 days in the dark at 30°C, colonies were counted and survival was calculated. Survival after UV was determined and plotted as a function of the applied UV dose. All strains used are isogenic to W1588 wild type. The survival of W1588 cells and *rad33* mutants (black and open triangles respectively), *rad23* and *rad23rad33* mutants (black and open diamonds respectively), *rad4* and *rad4rad33* mutants (open and black reversed triangles respectively), is depicted. The values shown in the graphs are averages of at least 3 independent experiments, error bars represent standard deviations.
(C) as (B), but for *rad16* and *rad16rad33* mutants (open and black squares respectively) and *rad26* and *rad26rad33* mutants (black and open circles respectively),

strand is absent in *rad33* cells, demonstrating that GGR is completely dependent on the Rad33 protein. Analysis of repair in *rad26* cells, in which TCR is considerably impaired (Li and Smerdon, 2002; van Gool *et al.*, 1994; Verhage *et al.*, 1996b), confirmed the role of Rad33 in GGR; deletion of *RAD33* in this background leads to a complete absence of repair in RNA pol II transcribed DNA (Figure 2C,D).

To examine the effect on TCR we measured repair in GGR deficient *rad16* mutants with an additional deletion of *RAD33*. TCR in the *RBP2* gene is slightly faster in *rad16* cells compared to *NER*$^+$ cells, consistent with our previous results (den Dulk *et al.*, 2005) (Figure 2E,F). More importantly, deletion of *RAD33* has a similar effect on TCR as observed in the *NER*$^+$ background; repair of the transcribed strand is reduced from ~90% to ~45% showing that Rad33 is required for efficient TCR.

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
MGSC 471	*rad16 ::hisG*[a]	This laboratory [b]
MGSC 479	*rad4 ::hisG*[a]	This laboratory [b]
MGSC 480	*rad23 ::hisG*[a]	This study [c]
MGSC 542	*YDR314CTAP-URA3*[a]	This study
MGSC 582	*rad26 ::HIS3*[a]	This study [d]
MGSC 623	*RAD4TAP-URA3*[a]	This study
MGSC 624	*RAD4TAP-URA3 yml011c ::KAN*[a]	This study
MGSC 625	*rad23 ::HisG RAD4TAP-URA3*[a]	This study
MGSC 626	*rad23 ::HisG yml011c::KAN RAD4TAP-URA3*[a]	This study
MGSC 627	*ydr314c ::loxLEU2lox RAD4TAP-URA3*[a]	This study
MGSC 628	*YDR314CTAP-URA3 yml011c ::KANMX*[a]	This study
MGSC 629	*rad23 ::HisG YDR314CTAP-URA3 yml011c ::KANMX*[a]	This study
MGSC 637	*RAD16TAP-URA3 yml011c ::KANMX*[a]	This study
MGSC 639	*RAD16TAP-URA3*[a]	This study
MGSC 640	*RAD14TAP-URA3*[a]	This study
MGSC 641	*RAD14TAP-URA3KL yml011c ::KANMX*[a]	This study
MGSC 650	*RAD26TAP-URA3*[a]	This study
MGSC 651	*yml011c ::KANMX RAD26TAP-URA3*[a]	This study
MGSC 653	*yml011c ::KANMX rad26::HIS3*[a]	This study
MGSC 656	*rad4 ::HisG yml011c ::KANMX*[a]	This study
MGSC 658	*rad16 ::HisG yml011c ::KANMX*[a]	This study
MGSC 660	*rad23 ::HisG yml011c ::KANMX*[a]	This study
MGSC 662	*yml011c ::KANMX*[a]	This study
MGSC 684	*YDR314CTAP-URA3 rad23 ::HisG*[a]	This study
MGSC 701	*rad4 ::HisG YDR314CTAP-URA3*[a]	This study
MGSC 702	*rad16 ::HisG YDR314CTAP-URA3*[a]	This study
MGSC 703	*RAD4TAP-URA3 rad16 ::TRP1*[a]	This study
BY4741	*MATa his3Δ leu2Δ ura3Δ met15Δ*	Euroscarf
yml011c	*MATa his3Δ leu2Δ ura3Δ met15Δ yml011c ::KANMX4*	Euroscarf
rad52	*MATa his3Δ leu2Δ ura3Δ met15Δ rad52 ::KANMX4*	Euroscarf
Y5565	*MAT his3 leu2 ura3 met15 can1 ::MFA1pr-HIS3 mfa1 ::MFa1pr-LEU2 lyp1*	A.Tong
MGST2057	Y5565 *rad4 ::URAMX*	this study
MGST2061	Y5565 *rad14 ::URAMX*	this study
MGST2059	Y5565 *rad6 ::URAMX*	this study
MGST2131	Y5565 *yml011c ::URAMX*	this study
MGST2117	*rad4 ::URAMX yml011c ::KANMX4*	this study
MGST2119	*rad6 ::URAMX yml011c ::KANMX4*	this study
MGST2139	*rad52 ::KANMX4 yml011c ::URAMX*	this study
MGST2121	*rad14 ::URAMX yml011c ::KANMX4*	this study

[a]Remainder of the genotype identical to that of W1588-4a
[b]As described previously (den Dulk *et al.*, 2005)
[c]As described by Verhage *et al.* (Verhage *et al.*, 1996c) but in W1588-4a background instead of W303
[d]Constructed as described previously (van Gool *et al.*, 1994) but in W1588-4a background instead of W303

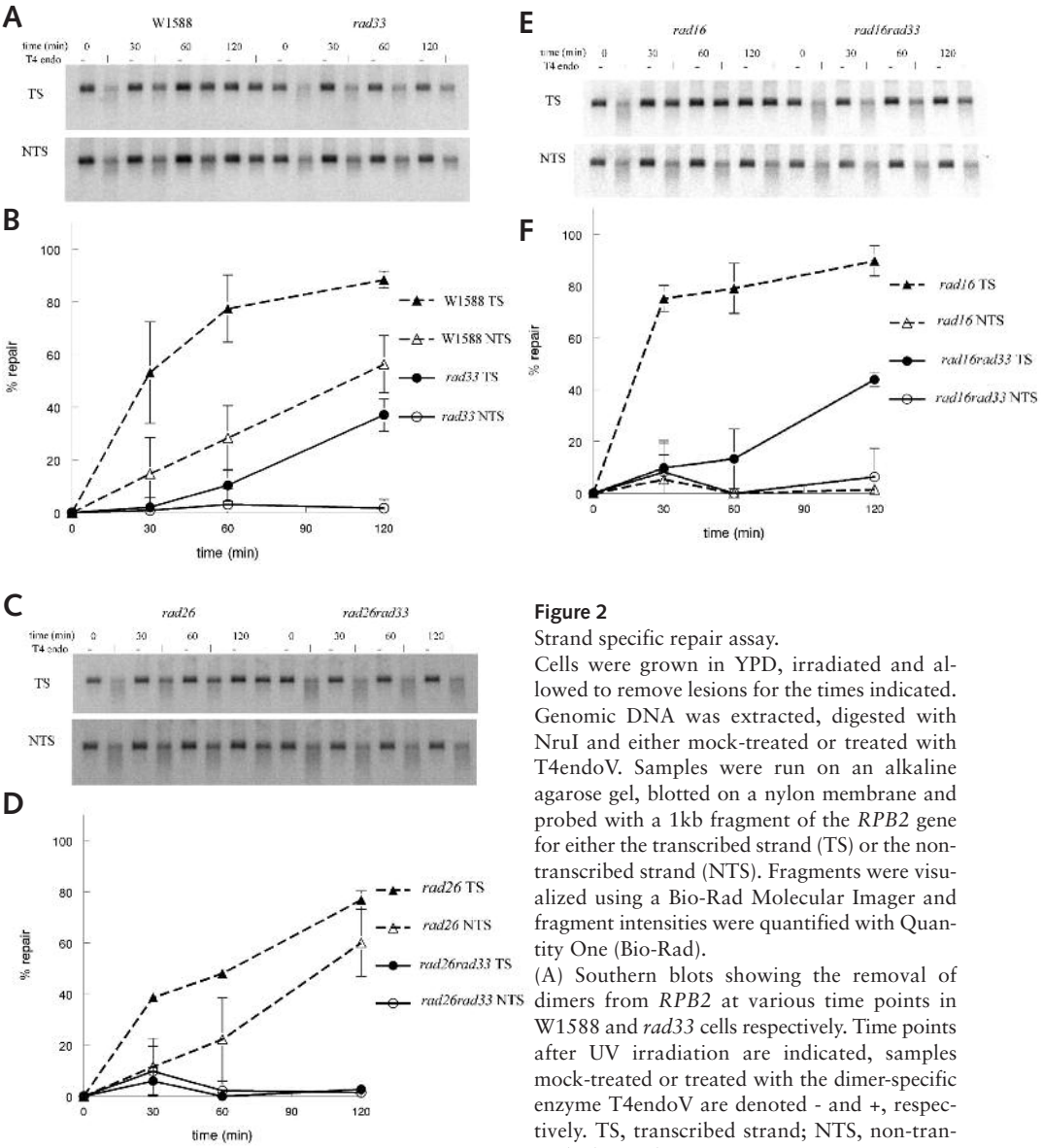

Figure 2
Strand specific repair assay.
Cells were grown in YPD, irradiated and allowed to remove lesions for the times indicated. Genomic DNA was extracted, digested with NruI and either mock-treated or treated with T4endoV. Samples were run on an alkaline agarose gel, blotted on a nylon membrane and probed with a 1kb fragment of the *RPB2* gene for either the transcribed strand (TS) or the non-transcribed strand (NTS). Fragments were visualized using a Bio-Rad Molecular Imager and fragment intensities were quantified with Quantity One (Bio-Rad).
(A) Southern blots showing the removal of dimers from *RPB2* at various time points in W1588 and *rad33* cells respectively. Time points after UV irradiation are indicated, samples mock-treated or treated with the dimer-specific enzyme T4endoV are denoted - and +, respectively. TS, transcribed strand; NTS, non-transcribed strand.
(B) Graphical representation of quantified Southern blots. The percentage removed dimers is shown as a function of time: W1588 TS and NTS; black and open triangles respectively, *rad33* TS and NTS; black and open circles respectively. Values are the mean of at least three independent experiments. Error-bars indicate standard deviations.
(C) As (A) but for *rad26* and *rad26rad33* cells.
(D) As (B) but for *rad26* and *rad26rad33* cells. (E) As (A) but for *rad16* and *rad16rad33* cells. (F) As (B) but for *rad16* and *rad16rad33* cells.

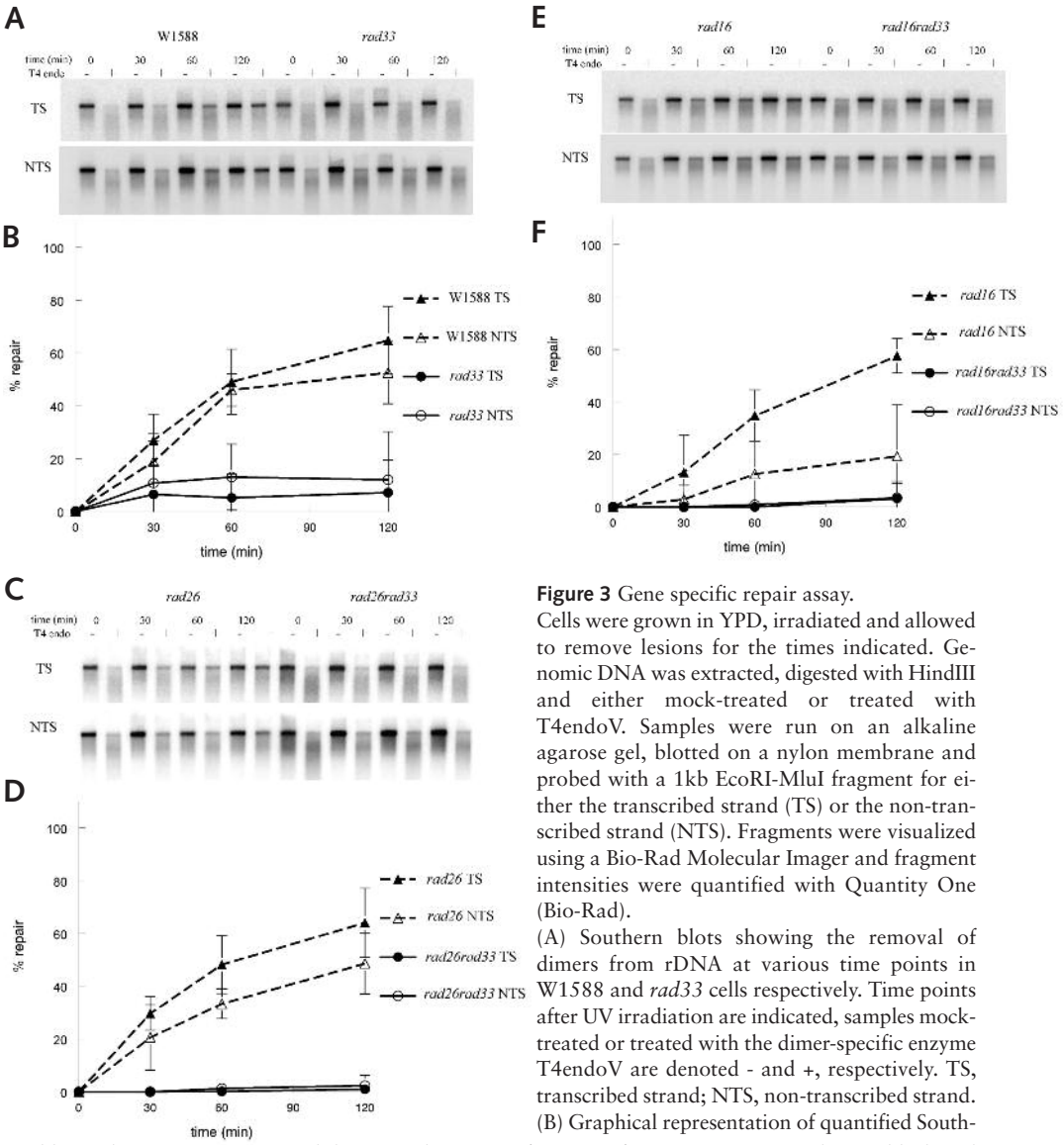

Figure 3 Gene specific repair assay.
Cells were grown in YPD, irradiated and allowed to remove lesions for the times indicated. Genomic DNA was extracted, digested with HindIII and either mock-treated or treated with T4endoV. Samples were run on an alkaline agarose gel, blotted on a nylon membrane and probed with a 1kb EcoRI-MluI fragment for either the transcribed strand (TS) or the non-transcribed strand (NTS). Fragments were visualized using a Bio-Rad Molecular Imager and fragment intensities were quantified with Quantity One (Bio-Rad).
(A) Southern blots showing the removal of dimers from rDNA at various time points in W1588 and *rad33* cells respectively. Time points after UV irradiation are indicated, samples mock-treated or treated with the dimer-specific enzyme T4endoV are denoted - and +, respectively. TS, transcribed strand; NTS, non-transcribed strand.
(B) Graphical representation of quantified Southern blots. The percentage removed dimers is shown as a function of time: W1588 TS and NTS; black and open triangles respectively, *rad33* TS and NTS; black and open circles respectively. Values are the mean of at least three independent experiments. Error-bars indicate standard deviations.
(C) As (A), but for *rad26* and *rad26rad33* cells.
(D) As (B) but for *rad26* and *rad26rad33* cells.
(E) As (A), but for *rad16* and *rad16rad33* cells. (F) As (B) but for *rad16* and *rad16rad33* cells.

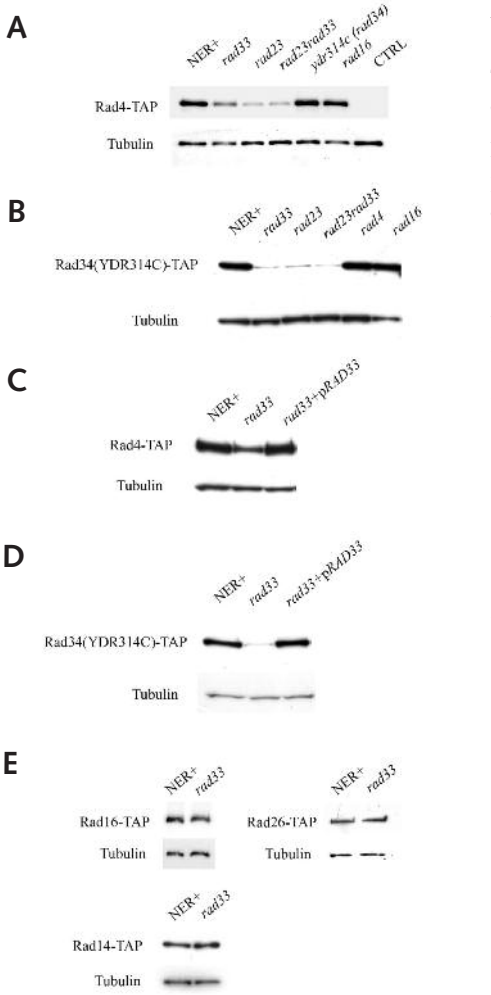

Figure 4
Western blot analysis.
Cells containing Rad4-TAP, YDR314C-TAP, Rad16-TAP, Rad26-TAP or Rad14-TAP in various NER deficient backgrounds were grown in YPD till stationary phase, proteins were extracted, run on SDS gel and transferred to western blot. Blots were hybridized with PAP or alpha tubulin antibodies and visualized using chemiluminescence and hyperfilm ECL (Amersham) or a Bio-Rad ChemiDoc system.
(A) Rad4-TAP protein levels in W1588, *rad33*, *rad23*, *rad23rad33*, *ydr314c* and *rad16* background. As control a protein extract from W1588 cells with untagged Rad4 was loaded in lane 7.
(B) YDR314C-TAP protein levels in W1588, *rad33*, *rad23*, *rad23rad33*, *rad4* and *rad16* background.
(C) Rad4-TAP protein levels in W1588 and *rad33* background and in *rad33* cells complemented with *YCpTEF2RAD33* plasmid.
(D) YDR314C-TAP protein levels in W1588 and *rad33* background and in *rad33* cells complemented with *YCpTEF2RAD33* plasmid.
(E) Rad16-TAP, Rad26-TAP and Rad14-TAP protein levels in W1588 and *rad33* background.

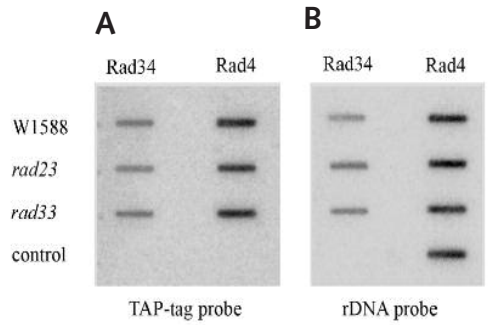

Figure 5
mRNA level analysis
Cells containing Rad4-TAP or Rad34-TAP (YDR314C-TAP) in *rad23*, *rad33* or W1588 background were grown to OD600 ~0.5; RNA was isolated and transferred to a nylon membrane. (A) Left column, total RNA isolated from cells containing Rad34-TAP in a W1588, rad23 or rad33 background. Right column, total RNA isolated from cells containing Rad4-TAP in a W1588, *rad23* or *rad33* background and, as a control for possible a-specific binding of the TAP-tag probe, RNA isolated from W1588 cells, lacking the TAP tag, is shown at the bottom of the right column. The blot was probed with a a-^{32}P labeled 550 bp TAP-tag probe. (B) As (A), but stripped and re-probed with a a-^{32}P labeled 1kb rDNA probe.

In previous experiments it was shown that there is a difference in requirement of certain factors (Rad26, Rad4) for NER between RNA pol II and RNA pol I transcribed regions (Verhage *et al.*, 1996a). The Rad4 homologue YDR314C is a clear example since it is exclusively required for strand specific repair in rDNA and has no role in repair of RNA pol II transcribed regions. The reported physical interaction between Rad33 and YDR314C could imply a prominent role of Rad33 in repair of RNA pol I transcribed rDNA. We therefore analyzed strand specific repair in the RNA pol I transcribed rDNA region. In accordance with previous studies (den Dulk *et al.*, 2005; Verhage *et al.*, 1996a) we find that repair in RNA pol I transcribed regions is slow when compared to repair of the RNA pol II transcribed *RBP2* gene. Interestingly, in *rad33* mutants repair of both the transcribed and non-transcribed strand in rDNA is almost abolished (<10%) (Figure 3A,B). Similar results were obtained when analyzing the effect of a *RAD33* deletion in *rad26* cells. In this background no repair in either strand can be detected in the absence of Rad33 (Figure 3C,D). As we reported earlier, a clear strand bias is visible in *rad16* cells due to the impaired GGR (den Dulk *et al.*, 2005; Verhage *et al.*, 1996a). In *rad16rad33* double mutants repair is completely abrogated, showing that in the absence of Rad16 NER in rDNA is dependent on Rad33 (Figure 3E,F).

We conclude that in cells lacking Rad33 both TCR and GGR are completely defective in RNA pol I transcribed rDNA (Figure 3A-F). In RNA pol II transcribed regions Rad33 is essential for GGR whereas TCR is still active although functioning with a significant lower efficiency (Figure 2A-F).

5.3.3 Deletion of RAD33 leads to a reduced level of both Rad4 and YDR314C proteins

The most remarkable effect of *RAD33* deletion is the extreme increase in UV sensitivity in the *rad23* background (Figure 1B). Several studies have shown that the NER defect in *rad23* cells is partially caused by the instability of Rad4. Suppression of the degradation of Rad4 in the absence of Rad23, by genetically crippling the proteasome or deletion of the *UBC4* gene, leads to increased survival after UV irradiation. This indicates that when Rad4 is protected from degradation it can act in NER independent of Rad23 (Lommel *et al.*, 2002; Ortolan *et al.*, 2004). The genetic interaction between *RAD23* and *RAD33* prompted us to examine the levels of Rad4 protein in *rad33* cells.

Protein extracts of *NER*$^{+}$ and *rad33* cells were analyzed on western blot. A genomically integrated TAP-tag allowed detection of Rad4 using PAP antibodies. Interestingly, deletion of *RAD33* results in a clear reduction of Rad4 protein levels (Figure 4A). Also we confirm the reported low levels of Rad4 in *rad23* mutants. Compared to *rad33* cells, the amount of Rad4 protein appears to be lower in cells deleted for *RAD23* (Figure 4A).

As YDR314C is homologous to Rad4 and was reported to interact with both Rad33 and Rad23 (Gavin *et al.*, 2002; Ito *et al.*, 2001), we tested whether deletion of *RAD23* and *RAD33* causes any effect on the protein levels of YDR314C. Strikingly, a dramatic reduction of the amount of YDR314C protein is observed in *rad33* cells (Figure 4B.). Moreover, deletion of *RAD23* causes an equally strong decrease in YDR314C protein levels (Figure 4B.). This further strengthens the homology of YDR314C with Rad4, we therefore think it is both convenient and appropriate to name the *YDR314C* gene prod-

uct Rad34. The lower levels of Rad4 and Rad34 are the specific result of the *RAD33* deletion as introduction of a plasmid bearing the *RAD33* gene leads to restoration of the wildtype level of Rad4 and Rad34 protein (Figure 4C,D). In *rad4, rad16* or *rad34* mutants no change of the Rad4 and Rad34 proteins levels is observed (Figure 4A,B).

The effect of the *RAD33* deletion seems specific for Rad4 and Rad34 since several other NER proteins (Rad14, Rad16, Rad26) are unaffected by the *rad33* deletion (Figure 4E). The *RAD4* and *RAD34* mRNA levels are similar in W1588, *rad23* and *rad33* cells showing that the reduction of Rad4 and Rad34 levels is not caused by impaired transcription (Figure 5A,B).

Importantly, cells lacking both Rad23 and Rad33 do not show an additional decrease of the Rad4 or Rad34 proteins whereas *rad23rad33* double mutants are significantly more UV sensitive than either single mutant (compare figure 4A,B and figure1B).

5.4 Discussion

In this study we examined the role of a new NER protein encoded by the *ORF YML011C*. YML011C was reported to interact with Rad34 (Ito *et al.*, 2001) and deletion of the *YML011C* gene confers UV sensitivity (Hanway *et al.*, 2002). Here we show that the *YML011C* gene product is involved in NER and we propose to designate the gene *RAD33*. Like for Rad34, no clear homologues of Rad33 are present in humans or other higher eukaryotes. However, the protein is strongly conserved in yeast species.

The UV sensitivity of cells deleted for *RAD33* is epistatic with NER. The *rad33* single mutant is moderately UV sensitive whereas deletion of *RAD33* in *rad4* or *rad14* cells does not increase the UV sensitivity. Both *rad16rad33* and *rad26rad33* mutants show increased UV sensitivity when compared to *rad16* or *rad26* cells respectively, indicating that Rad33 is involved in both GGR and TCR. Yet, the relatively more pronounced effect of the *RAD33* deletion in the *rad26* background indicates that Rad33 is more important for GGR.

Strand specific repair analysis shows that in *rad33* cells lesion removal in the transcribed strand of the *RPB2* gene is severely reduced (~50% compared to *NER*$^{+}$ cells). In *rad16rad33* double mutants a similar decrease in repair of the transcribed strand is observed, showing that Rad33 is required for efficient TCR in RNA pol II transcribed regions. Lesions in the non-transcribed strand are persistent in cells deleted for *RAD33* and repair is entirely absent in cells with both a *RAD33* and *RAD26* deletion, demonstrating that Rad33 is essential for GGR.

Since Rad33 interacts with Rad34 (Ito *et al.*, 2001), a protein shown to be involved in repair of rDNA (den Dulk *et al.*, 2005), we examined repair of RNA pol I transcribed rDNA in absence of Rad33. In *rad33* cells NER in rDNA is affected even more than repair in RNA pol II transcribed DNA. Not only GGR is completely defective, like in RNA pol II transcribed DNA, but in the rDNA region Rad33 is also essential for preferential repair of the transcribed strand.

In wildtype cells and most NER deficient mutants deletion of *RAD33* leads to intermediate UV sensitivity. However, Rad33 is essential for survival after UV irradiation in cells lacking Rad23. Multiple studies showed that the NER defect in *rad23* mutants is partially due to the instability of the Rad4 protein (Lommel *et al.*, 2002; Ortolan *et al.*, 2000; Xie *et al.*, 2004). Interestingly, in the present study we show that in *rad33* mutants both Rad4 and Rad34 protein levels are strongly reduced, whereas several other NER proteins are not affected. Furthermore, we show that the amount of Rad34 protein is also diminished in *rad23* cells, The decrease of Rad34 protein levels is the same in *rad23* and *rad33* mutants and larger than that observed for Rad4. The fact that deletion of *RAD33* affects the protein levels of Rad34 more than that of Rad4 might explain the more severe defect in repair of rDNA compared to repair of RNA pol II transcribed genes in *rad33* cells. The decrease of Rad4 protein is seen in both *rad23* and *rad33* mutants but appears to be stronger in cells deleted for *RAD23*. Analysis of mRNA levels showed that the low amount of Rad4 and Rad33 protein is not due to a defect in transcription of the genes encoding these proteins.

The fact that in the absence of Rad33 TCR in RNA pol II transcribed regions is still active whereas GGR is completely defective might indicate that the reduced amount of Rad4 is preferentially recruited to lesions recognized by TCR. This is consistent with

the observation by Lommel *et al.* (2002) that overexpression of Rad4 enhances repair of the non-transcribed strand but not of the transcribed strand. However, we show that in TCR defective *rad26rad33* cells no GGR can be measured, suggesting that in *rad33* cells Rad4 is unable to act in GGR.

Rad23 influences the protein levels of Rad4 and Rad34. It was shown that defects in the proteolysis pathway lead to increased amounts of Rad4 protein and suppresses the UV sensitivity of *rad23* cells (Lommel *et al.*, 2002; Ortolan *et al.*, 2004). This suggests that when Rad4 is protected from degradation NER can function without Rad23. Here we show that deletion of *RAD33* causes a severe defect in NER coinciding with a significant decrease of the amount of Rad4 and Rad34 protein. This could indicate that the defective NER in *rad33* mutants is, like in *rad23* cells, partially due to the low levels of Rad4 protein.

The apparent difference in Rad4 levels between *rad23* and *rad33* cells therefore might explain the difference in UV sensitivity of these mutants. Deletion of both *RAD23* and *RAD33* does not lead to a further reduction of Rad4 or Rad34 protein levels compared to a *rad23* single mutant, showing that in *rad23* cells Rad33 has no role in maintaining the amount of Rad4 protein. Intriguingly, although the Rad4 protein levels are not further reduced in *rad23rad33* cells compared to either single mutant, the *rad23rad33* double mutant is considerably more UV sensitive than *rad23* or *rad33* single mutants. The UV phenotype of *rad23rad33* cells is therefore not due to an additional decrease in Rad4 protein levels, indicating that the stabilization of Rad4 levels cannot be the sole role of Rad33 in survival after UV irradiation.

The fact that the remaining Rad4 protein in *rad33* cells can only act in TCR and not in GGR might reflect a difference in involvement of Rad4 in the two NER sub-pathways. Rad33 might not only influence the levels of Rad4, but also play a more direct role in GGR. At present it is difficult to envisage a model explaining how Rad4, Rad23, rad33 and Rad33 act in NER. As Rad23 and Rad33 are reported to interact with Rad34 and deletion of *RAD23* or *RAD33* has a similar effect on Rad4 and Rad34, it is feasible that Rad4 also interacts with Rad33. Possibly, Rad4-Rad23-Rad33 and Rad34-Rad23-Rad33 exist as two distinct complexes in the cell.

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