# Nucleotide excision repair in yeast

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# IV Phenotypic analysis of a *rad4* mutant carrying a homologous change that in XPC leads to the XP disorder

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# 1. Abstract

XP-C patient phenotypes predominantly result from truncated proteins or lack of XPC [1]. Only two point-mutations that lead to amino acid substitutions leaving the full-length XPC protein intact have been described (P331H and W690S). Surprisingly, we find that *Saccharomyces cerevisiae* cells carrying the corresponding *rad4W496S* mutation are not UV-sensitive. However, deletion of additional Nucleotide Excision Repair (NER) genes results in additive or synergistic effects on UV resistance. More in-depth analysis of the repair capacity of these cells shows that NER is affected in the *rad4W496S* mutant and it is mainly disrupted in Global Genome Repair.

# 2. Introduction

Xeroderma pigmentosum (XP) is a rare autosomal, recessive human disorder characterized by defects in Nucleotide Excision Repair (NER). Genetic defects in human NER can also result in other disorders like Cockayne Syndrome (CS) or Trichothiodystrophy (TTD) [2]. XP causes a 2,000-fold increased incidence of sunlight induced skin cancer in patients and, depending on the complementation group, can also result in neurological degeneration [2]. The XP-C complementation group is the most common among XP patients and is defined by mutations in the *XPC* gene homologous to the core NER *RAD4* gene in yeast [3]. The XPC-hHR23A/B complex is, together with the XPC binding protein Centrin 2 involved in the initial recognition of DNA damage.

The yeast *Saccharomyces cerevisiae* is shown to be an excellent model for the study of the conserved mechanism of NER. In yeast a complex similar to that in human cells is responsible for the recognition of DNA damage. The XPC homologue in yeast, Rad4 forms a complex with Rad23, and we showed recently that also a third protein can bind to this recognition complex, Rad33 [4]. Rad33 shows no homology to Centrin2 but the binding site in Rad4 is identical to that in XPC [4]. The homology between the human and yeast recognition complexes in NER allows us to study the role of a conserved tryptophan residue, which was found to be substituted as result of a point mutation in an XPC patient. This XPC mutation is of special interest since it is one of two patient mutations that have been described to lead to an amino acid substitution, leaving the full length protein and its expression unchanged [5, 6]. One of these mutations, XPC-W690S, results in defective repair and UV sensitivity in human cells [1]. It was shown that decreased protein stability, impaired DNA binding and impaired repair all contribute the XP phenotype [9]. Furthermore, mutant XPC protein displayed reduced ssDNA binding *in vitro* while the interaction with Rad23 and Centrin 2 is still intact [10]. The W690S resides in the conserved C-terminus that is shared by Rad4-like proteins. In this region the Rad23 and TFIIH interactions are mapped [5, 7]. The affected tryptophan residue at position 690 in XPC is conserved at position 496 in yeast Rad4 (Figure 1A top panel) [1]. The information from the Rad4 protein structure allows us to pinpoint exactly where the amino acid substitution affects the protein structure [8]. The substituted residue is located in Beta Hairpin Domain 2 (BHD2) (Figure 1A lower panel) in close proximity to the bases opposite the CPD lesion [8]. The neighboring residues R494, Q495 and M498 in Rad4 are all in direct contact with the DNA backbone and bases opposite the CPD lesion [8], suggesting that the mutation might affect DNA damage binding.

To get more insight into the defect caused by this patient mutation we set out to investigate the phenotype of the corresponding *rad4W496S* mutant in *S.cerevisiae*. Surprisingly, we find that the equivalent W496S mutation in Rad4 does not result in UV sensitivity. However, repair is moderately affected in *rad4W496S* cells, with GG-NER being most severely hampered.

# 3. Material & Methods

## 3.1 Strains and UV droptest

All strains used in this study are derived from the wild-type W1588-4a strain [11]. Relevant phenotypes are described in table 1. The *rad4W496S* substitution was introduced into W1588 creating strain MGSC842. This background was made by the Pop-in Pop-out approach using a Yeast Integrating (YIp) plasmid construct, YIp-rad4CtW496S, of the Rad4 C-terminal region as indicated in figure 1A, containing the point mutation corresponding to the W496S substitution. To construct a strain with the *RAD4* point mutation and a *RAD26* deletion, disrupting TC-NER, we transformed MGSC842 with linearized *pRAD26::HIS3.* Correct introduction of the disruption construct was confirmed by PCR and Southernblot analysis. Similarly, to disrupt GG-NER we deleted *RAD16* by transforming MGSC842 with linearized *pRAD16::HUH* resulting in strain MGSC934. Liquid cultures of the relevant strains were grown overnight after which they were diluted in sterile water. For the UV droptest 1 or 2µl drops were dispensed on a 7x8 grid on a YPD plate. After UV irradiation plates were incubated in the dark at 30°C for 2-3 days.

## 3.2 Strand specific repair assay

Yeast cell cultures of 200mL YPD of $OD_{600}$=0.4 were spun down and resuspended in cold PBS to an $OD_{600}$ of 0.7. Using 254nm UV light cells were irradiated with 70J/m$^2$ at a rate of 3W/m$^2$. Next, the cells were spun down again, resuspended in YPD and aliquoted into 4 portions for the 0, 30, 60 and 120min time points. Incubation takes place at 30°C in the dark. After the indicated time-intervals cells were spun down, washed with cold water and split in two aliquots and stored at -80°C.

DNA was isolated using a combination of Yeast Cell Piercing Solution (YCPS) and Cell Lysing Solution (CLS) followed by protein precipitation using ammonium acetate and DNA precipitation using isopropanol. The cell pellets were thawed and resuspended in YCPS (0.1% SB3-14, 100mM LiCl in TE buffer) and incubated at 65°C for 30 minutes. Next, the cells were spun down and resuspended in CLS (2mM EDTA, 2% SDS) and again incubated at 65°C for 45 minutes. After placing the cells on ice for 5 minutes ammonium acetate was added to an end-concentration of 2.5M. At this stage cells were rotated for 10 minutes at room temperature before being centrifuged at 14,000rpm at 16°C for 10 minutes. The supernatant was then transferred and an equal volume of isopropanol was added. After gentle mixing and incubation at room temperature for 10 minutes, the DNA was precipitated by means of centrifugation at 14,000rpm at 16°C for 12 minutes. The DNA was then be washed with 70% Ethanol after which it was dissolved in water. *RPB2* repair was measured as described previously [4].

DNA repair was quantified by scanning the Southern Blot in a Biorad Personal Molecular Imager FX and using Biorad's Quantity One for data analysis.

# 4. Results & Discussion

## 4.1 The rad4W496S mutant is not UV sensitive

We constructed a yeast strain containing the point-mutation in *RAD4* that leads to the W496S substitution. This substitution was obtained by site-directed mutagenesis of a C-terminal *RAD4* DNA fragment. This construct was then introduced into yeast by means of pop-in pop-out methodology to generate a genomic *rad4W496S* allele (see material and methods). The UV phenotype of the resulting *rad4W496S* yeast strain was investigated in a UV droptest. Surprisingly, the *RAD4* mutant strain is as UV resistant as wild-type cells shown in figure 1B.

However, when we introduced the *rad4W496S* substitution in a *RAD23* deletion background, we observed a synergistic UV sensitivity as shown by the UV drop test of *rad23Δrad4W496S* cells in figure 1B. This strain is significantly more UV sensitive compared to either of the single mutant strains, displaying a degree of sensitivity that is similar to the NER deficient *RAD4* deletion strain. Rad23 is a direct interaction partner of Rad4 and is believed to stabilize the protein [12, 13]. Moreover, XPC-W690S was reported to be unstable [9]. Taken together the possible Rad4W496S instability might be more readily corrected by the yeast Rad23 protein, leading to UV resistance shown in figure 1B. The absence of the stabilizing effect of Rad23 might be the cause of the decrease in UV survival.

NER is characterized by two sub-pathways, GG-NER and TC-NER. In yeast, Rad16 is specific for GG-NER, while Rad26 is a TC-NER specific protein. The specificity of these proteins allows separate study of these sub-pathways by using the two different mutants. We wanted to identify whether the NER sub-pathways are both equally or differently affected by the W496S substitution. The *rad16Δrad4W496S* strain defective in GG-NER and *rad26Δrad4W496S* cells deficient in TC-NER were subjected to UV irradiation using a drop test depicted in figure 1B. TC-NER proficient *rad16Δrad4W496S* cells show a moderate UV sensitivity, however, the *rad26Δrad4W496S* strain proficient in GG-NER shows a strong synergistic effect (Figure 1B). The *rad4W496S* mutation thus genetically interacts with both TC-NER and GG-NER factors, but seems to affect GG-NER more severely.

## 4.2 The Rad4W496S mutation results in impaired NER in vivo

We found that UV survival is strongly affected by the *rad4W496S* mutation in the absence of the Rad26 TC-NER factor, suggesting a defect in GG-NER, analogous to the UV sensitivity due to the GG-NER defect resulting from the XPC-W690S protein in human cells [1]. The repair phenotype of the *rad4W496S* mutant cells were studied in more detail in different NER defective backgrounds by making use of a strand specific repair assay as described previously [14]. Repair measurements in the *rad4W496S* background show that after 2 hours of incubation, about 75% of the damages in the tran-

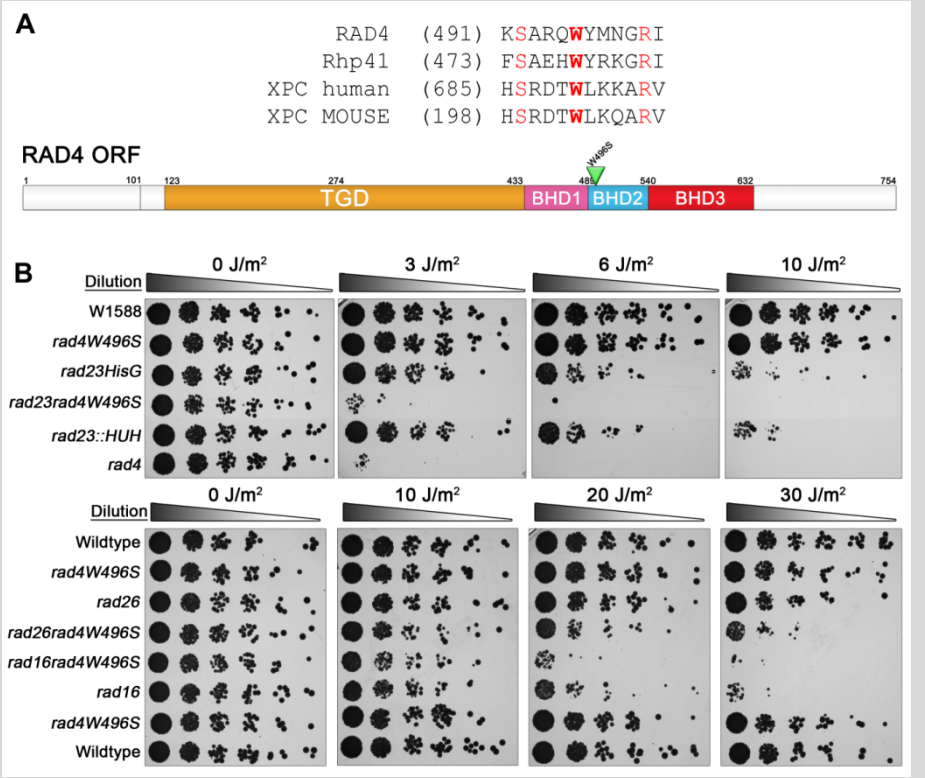


Figure 1 – *The Rad4W496S point mutation is UV resistant and displays genetic interaction with other NER genes* (A) Top panel: Sequence alignment of Rad4-like proteins depicting the conserved tryptophan at position 496 in Rad4. Bottom panel: schematic depiction of the structural domains of Rad4 [8]. (B) UV drop test indicating the genetic interaction of *rad4*W496S with several *RAD* deletions. Serial dilutions were prepared in sterile water from stationary culture starting with a one in hundred dilution. After applying the droplets, the plates were UV irradiated with the indicated doses and incubated in the dark at 30°C for 2-3 days.

scribed strand (TS) are repaired as compared to 90% in wild-type cells (Figure 2A & B). Repair of the non-transcribed strand (NTS) on the other hand, is more severely reduced to only 15% after 2 hours compared to 50-60% in wild-type cells (Figure 2). Thus the W496S mutation reduces the efficiency of TC-NER only moderately, but severely impairs GG-NER after UV irradiation.

Analogous to the survival experiments, strand specific repair was also investigated in GG-NER and TC-NER deficient backgrounds containing the *rad4W496S* mutation. GG-NER in a TC-NER deficient *rad26*Δ single mutant repairs 50-60% of the damages on both strands during the time course of 2 hours (Figure 3B & D). However, GG-NER of both strands in a *rad26*Δ*rad4W496S* double mutant is severely reduced to only 15% after 2 hours (Figure 3B & D). This again shows that GG-NER is severely impaired by the *rad4W496S* mutation.

GG-NER is completely disrupted in a *RAD16* deletion background resulting in the absence of NTS repair in *rad16*Δ*rad4W496S* cells as expected (Figure 3A & C) [15]. In this

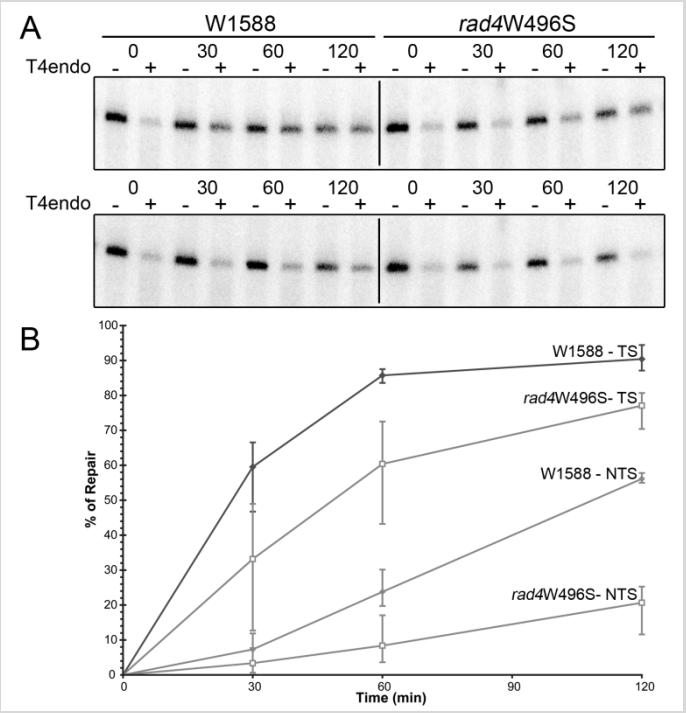


Figure 2 – *rad4W496S is disrupted both TC-NER and GG-NER*. Strand specific repair assay of *RPB2* in wild-type and *rad4W496S* mutant strains. Yeast cells were UV-irradiated with 70J/m$^2$ and allowed to recover for the indicated repair times. (A) Repair analysis on Southernblot probed for the transcribed strand (TS) or non-transcribed strand (NTS) of both wild-type and *rad4W496S* cells. (B) Graphical representation of the relative amount of repair. Data was taken from at least 3 individual experiments.

GG-NER deficient *rad16Δrad4W496S* background, TC-NER removes ~80% of the damages in the TS two hours after UV irradiation.

Taken together, our results clearly show that TC-NER is only moderately affected by the *rad4W496S* mutation, whereas GG-NER is strongly impaired. This impaired GG-NER, however, does not lead to UV sensitivity per se. Only when other NER factors, like Rad23, Rad16 or Rad26, are absent UV sensitivity is observed. UV resistance in the *rad4W496S* single mutant background indicates that NER does occur, but apparently is delayed and can therefore not be monitored in our strand-specific repair assay.

The XPC-W690S protein showed a reduced affinity for DNA, which could help to explain why the corresponding *rad4W496S* mutant displays delayed repair in yeast. GG-NER strongly depends on the DNA binding ability of the Rad4 protein and is therefore severely reduced due to the W496S substitution. A Rad4 protein that is defective in DNA binding can still function in TC-NER, because TC-NER specific factors, like Rad26 and RNA Pol II, might facilitate binding of Rad4 to the damage. In human cells TC-NER factors allow this repair pathway to function even without a Rad4-like protein. Thus, the initial steps during TC-NER that can use a mutated Rad4 protein to interact with a damage in yeast TC-NER, might circumvent the requirement for XPC in TC-NER in human cells entirely.

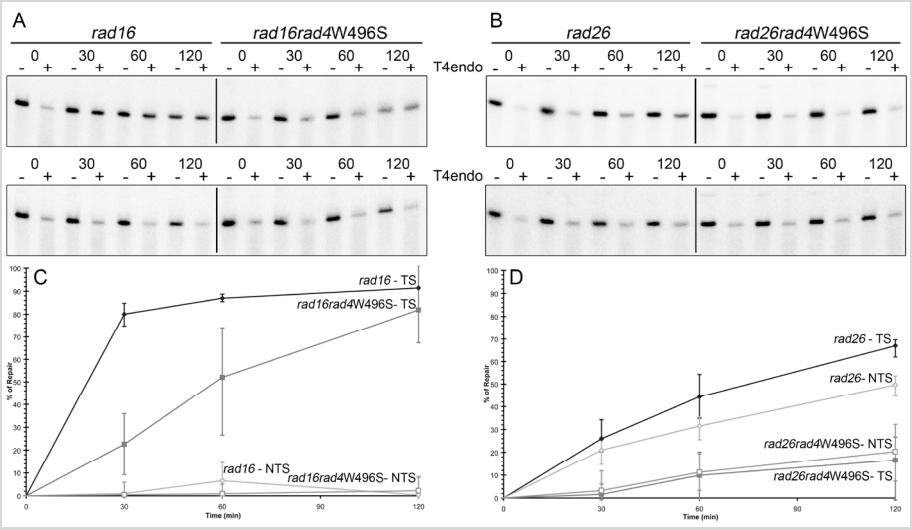


Figure 3 – *GG-NER is most significantly affected by rad4W496S*. Strand specific repair assay of *RPB2* in *rad4W496S* mutant strains deleted for *RAD16* or *RAD26*. Yeast cells were UV-irradiated with 70J/m$^2$ and allowed to recover for the indicated repair times. (A, B) Repair analysis on Southernblot probed for the transcribed strand (TS) or non-transcribed strand (NTS) of both *rad16*Δ*rad4W496S* and *rad26*Δ*rad4W496S* cells, respectively. (C, D) Graphical representation of the quantified repair data. Results were taken from at least 3 individual experiments.

Table 1 – This table lists all the strains used in this study and their respective genotype and source.

| Strain | Genotype | Source |
|---|---|---|
| *W1588-4a* | *MATα leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1* | R. Rothstein [11] |
| MGSC427 | *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 rad23::hisG* | This Lab |
| MGSC582 | *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad26Δ::HIS3* | This Lab |
| MGSC609 | *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad4Δ::HisG* | This Lab |
| MGSC621 | *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad16Δ::HUH* | This Lab |
| MGSC622 | *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad23Δ::HUH* | This Lab |
| MGSC662 | *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad33::KANMX* | This Lab |
| MGSC842 | *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad4W496S* | This Study |
| MGSC849 | *MATα leu2-3ade2-1 can1-100 his3-11,15,112 trp1-1 ura3-1 rad4W496S rad26::HIS3* | This Study |
| MGSC934 | *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad4W496S rad16Δ::HUH* | This Study |

# 5. References


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