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

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New factors in Nucleotide Excision Repair

A study in Saccharomyces cerevisiae

Ben den Dulk

New factors in Nucleotide Excision Repair

A study in Saccharomyces cerevisiae

Proefschrift

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op gezag van Rector Magnificus Prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties

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Ben den Dulk

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Chapter 1

General introduction and scope of the thesis

General introduction and scope of the thesis

1.1 Instability of DNA

Scientists have long been puzzled by the remarkable stability of genetic traits. At the beginning of the 20th century it already was established that the hereditary units are harbored in the chromosomes. Which component of the chromosome would carry the actual genetic information was not known at that time, in fact, it could not be imagined that any molecule would be stable enough to preserve the stability exhibited by genetic traits. It would take several more decades before it dawned that DNA is the genetic carrier (Avery *et al.*, 1944), and its stability is the result of maintance by a number of different repair mechanisms.

The DNA molecule itself is certainly not stable. In the oxidative environment within the cell DNA is altered by various chemical reactions, such as hydrolysis, oxidation and base deamination (Lindahl, 1993). In addition to these endogenous threats numerous exogenous agents can potentially alter the structure of our DNA. DNA lesions interfere with essential cellular processes such as transcription and replication and can lead to cellular malfunctioning or cell death. When damages persist in the form of mutations, caused by the erroneous replication of damaged DNA, they can lead to defects such as tissue degeneration, ageing and cancer.

A major source of exogenously induced lesions in DNA is the sun. The emitted ultraviolet radiation can cause various aberrations to DNA. The most frequently occurring types are the *cis-syn* cyclobutane pyrimidine dimer (CPD) and the pyrimidine (6-4) pyrimidone photoproduct ([6-4-]PP) (Mitchell and Nairn, 1989; Pfeifer, 1997; Sage, 1993). Since the sun, and numerous other sources of DNA damage, have been present since (and essential for) the beginning of life on earth, mechanisms evolved, now known as DNA repair pathways, that protect the structural integrity of the DNA. With hindsight, the answer to the mysterious stability of genetic traits is simple; the carrier is not extraordinary stable but the information is preserved by dedicated maintenance of the carrier.

1.2 DNA repair mechanisms

As there are many different types of DNA lesions, several different kinds of DNA repair mechanisms exist. In general, repair of DNA comes in four varieties. (1) Chemical alterations can be directly reversed by photolyases or methylguanine DNA methyltransferases. (2) The ends of double strand breaks can be resealed by non-homologous end joining. (3) Double strand breaks can also be resolved via recombination with a homologous region within the same cell. (4) The damaged base can be excised, after which the DNA structure is restored by DNA synthesis using the undamaged strand as a template. Some of the key repair mechanisms are discussed below.

1.2.1 Direct reversal

Several proteins were identified that possess the ability to bind damaged nucleotides and reverse the modified nucleotide to its original state. A well known example is CPD-photolyase. This flavoprotein contains two chromophore-cofactors. The chromophore at the surface of the protein enables the protein to use energy from near-UV/blue light

as energy source (Mees *et al.*, 2004). This energy is utilized via the second chromophore in order to split the cyclobutane ring, thereby restoring the bases to their undamaged state (Sancar, 2004).

Direct reversal by photolyases is a very efficient way to remove lesions from the DNA. However, the substrate specificity of photolyases is limited to one type of injury. At present, three types of photolyases are known: CPD photolyase, (6-4)PP photolyase and cryptochrome. Whereas the CPD and (6-4)PP photolyases are clearly evolved as DNA repair factors with the sole purpose of removing CPDs or (6-4)PPs from the genome respectively, the cryptochrome proteins are not involved in DNA repair but utilize the same light harvesting mechanism to control the circadian clock and regulate growth and development in animals (Lin and Todo, 2005). Bacteria also possess this enzyme for a yet unknown purpose. Remarkably, CPD and (6-4)PP photolyase are not conserved in placental mammals.

A different type of direct reversal is employed by methylguanine DNA methyltransferase (MGMT), an enzyme that repairs methylguanines that are frequently formed by alkylating agents. MGMT transfers the methyl group from the guanine to an internal cysteine residue. An MGMT enzyme can only be used once, as the methyl group is stably attached to the cysteine, disabling the enzyme for further repair activities.

For the majority of DNA injuries a direct reversal solution is not available and repair of these lesions rely on other, generally more complex, DNA repair mechanisms.

1.2.2 Double strand break repair

Double strand breaks (DSBs) are formed frequently during cellular processes like mitotic recombination, V(D)J recombination and, in yeast, during mating type switching. Double strand breaks can also be induced by exogenous sources, such as ionizing irradiation and cytotoxins like bleomycin. DSBs are obviously hazardous to the genetic integrity and can lead to a wide range of genetic alterations including loss of heterozygosity, translocations, deletions and even chromosome loss (Jackson, 2002). DSBs are dealt with by DSB repair, which is a collective term for two different mechanisms that mend the broken DNA molecule.

Firstly, the sub-pathway responsible for the repair of DSB in the absence of a homologous donor is termed Non Homologous End Joining (NHEJ), a system that directly joins the disconnected DNA ends by ligation. In yeast, the Ku70/Ku80 and MRX complexes stabilize the ends of the DSB, after which the DNA is sealed by DNA ligase (Lewis and Resnick, 2000). The simplest mode of NHEJ involves DSBs with complementary overhangs including 5' phosphate and 3' hydroxyl groups, which can be re-ligated error free. Yet, the sealing of most breaks requires processing of the loose ends prior to ligation, resulting in deletions or insertions of basepairs. NHEJ is therefore associated with error prone repair of breaks. Despite the error proneness, the NHEJ pathway contributes significantly to the genome stability and suppression of tumorgenesis (Ferguson *et al.*, 2000; Karanjawala *et al.*, 1999).

In the presence of a homologous donor sequence within the same cell, a DSB can be restored via a second sub-pathway, Homologous Recombination (HR). This is a complex procedure, requiring a set of genes in the *RAD52* epistasis group. Repair is established by DNA synthesis using the homologous sequence as template. After the

induction of a DSB, the ends of the DSB are resected 5' to 3'. Once a homologous sequence is detected by means of the Rad51-ssDNA nucleoprotein filament, strand invasion of the 3' single strand tails with a homologous DNA molecule, allowing DNA synthesis using the 3' tail as a priming sequence. The D loop, formed as a consequence of the strand invasion is able to pair with the other side of the DSB resulting in a double Holliday junction. The non-invading strand can now be extended and subsequent filling of the gaps, ligation and resolution of the holliday junction re-establishes the double stranded DNA (Heyer, 2004; Krogh and Symington, 2004). The two DSB-repair systems share the same substrates but the relative activity of the two pathways varies between organisms, cell type and cell stage (Shrivastav *et al.*, 2008).

1.2.3 Nucleotide excision repair

Substrate versatility is a hallmark of the NER system, as it recognizes and removes many different lesions that are mainly generated by exogenous sources. NER substrates include UV induced CPDs and (6-4)PPs, intrastrand crosslinks and various bulky DNA adducts. The *in vitro* reconstituted NER reaction requires at least 16 proteins, each performing a specific step in the reaction leading to the removal of the lesion. The damaged DNA is identified by the NER damage sensors, after which a region of DNA surrounding the lesion is unwound to create a single strand bubble of ~30nt. At the junctions of this bubble, single strand incisions are made and the oligonucleotide containing the lesion is removed. The resulting single stranded gap is then filled by DNA polymerase and sealed by DNA ligase. Given the broad range of substrates it is assumed that NER senses a common feature in the damaged DNA. The NER mechanism is the focus of this thesis and will be discussed further in the following chapters. The question how such a diversity of chemically unrelated lesions is recognized by NER is addressed in chapter 3.

1.2.4 Base excision repair

The base excision repair (BER) pathway deals with the majority of base modifications, inappropriate bases and base losses which are endogenously formed with a high frequency (Holmquist, 1998). Substrates for the BER system are numerous and include the apurinic/apyrimidinic (AP) sites (Boiteux and Guillet, 2004) and the 7,8-dihydro-8-oxoguanine (8-oxoguanine) sites (Fortini *et al.*, 2003), which are both the result from injury to DNA via reactive oxygen species. In contrast to NER, BER does not employ the same proteins for each type of substrate. In fact, the BER pathway refers to a large collection of individually operating glycosylases, each capable of removing only one or a few different types of lesions. The glycosylases remove the damaged base by hydrolysis of the N-glycosylic bond that links the base to the deoxyribose-phosphate backbone. The phosphate backbone of the remaining apurinic/apyrimidinic site is then incised by an AP-endonuclease (Barzilay and Hickson, 1995) and DNA polymerase and DNA ligase subsequently complete the restoration of the DNA.

1.3 DNA damage tolerance

Some DNA lesions will inevitably escape detection by the various damage surveillance proteins and persist into the S phase. Additionally, DNA damage will also be induced during the replication itself. Without assistance, the replication fork can not proceed through damaged DNA and will arrest at the site of the lesion, posing a threat to the viability of the cell. In this case the cell diverts to an alternative means to cope with these lesions. Several mechanisms, collectively referred to as 'DNA damage tolerance', have evolved to resolve the arrested replication machinery on the DNA, some at the cost of inducing mutations. These pathways are also known as 'Post Replication Repair', which is not entirely accurate as the lesion is not removed, but rather bypassed.

Post replication recombination repair involves homologous recombination (HR) using the undamaged sister chromatid as template. This system might be of especial value to solve specific mishaps that can occur following the collision between replication fork and lesion (Li and Heyer, 2008). When a lesion blocks the DNA polymerase but not the helicase unit, the helicase will generate an excess of single stranded DNA, which can result in a DSB after endonucleolytic activity. DSBs are also generated when single stranded DNA breaks induced by reactive oxygen species are encountered by replicating DNA polymerases (Li and Heyer, 2008). In these situations the replication machinery will be displaced to allow repair via HR. Once the generated DSB is resolved DNA synthesis can resume past the site of the lesion. HR is also responsible for filling of the gaps generated in daughter strands opposite base damage (Morimatsu and Kowalczykowski, 2003; Sogo *et al.*, 2002).

Replication fork regression is an alternative mechanism to circumvent the damaged template. Here synthesis switches from the damaged template to the newly synthesized daughter strand. The mechanism is yet poorly understood, however, recent evidence supports a role of Rad5, the caretaker of the error-free branch of damage tolerance, in Replication Fork Regression in yeast (Blastyak *et al.*, 2007).

The best characterized form of replication associated damage tolerance is Translesion Synthesis (TLS). The TLS system consists of various alternative DNA polymerases, all characterized by a compromised fidelity due to a larger active site and/or the absence of exonucleolytic proofreading (For review, see Friedberg (2005)). These polymerases are thereby able to incorporate bases opposite templates containing damaged nucleotides that do not meet the requirement of recognition by replicative DNA polymerases α, δ or ε. TLS is therefore error prone, however, a certain degree of fidelity is realized in this pathway by deploying specific DNA polymerase for specific types of lesions. For example, thymine dimers can not be processed by DNA polymerase a, δ or ε, but DNA polymerase ŋ can effectively pass through these lesions, mainly by correctly inserting two Adenine's opposite the dimer (Washington *et al.*, 2001). Specific usage of DNA polymerase to resolve stalled replication at a CPD linked thymine lesion hence is error free in the great majority of cases. The precise mechanism behind when and how TLS polymerases are recruited is far from fully understood. It is shown however that the proliferating nuclear antigen (PCNA) sliding clamp, which acts as a processivity factor for replicative DNA polymerases, is a key player in the TLS pathway, as it is responsible for the recruitment of certain TLS polymerases (Hoege *et al.*, 2002).

It is not known in detail what feature of the stalled replication fork determines which

of the above described damage tolerance systems is applied. Different post translational modifications of PCNA were shown to act as molecular switches that determine whether the lesion stalling the replication fork will be bypassed via TLS or repaired via post replication repair (Haracska *et al.*, 2004; Hoege *et al.*, 2002; Ulrich *et al.*, 2005; Watts, 2006).

1.4 The scope of this thesis

The research described in this thesis focuses on the role of the Rad4-Rad23 complex, an essential factor in damage recognition of eukaryotic Nucleotide Excision Repair (NER).

After a general introduction in chapter 1, chapter 2 introduces the mechanism of the basic NER reaction and discusses the two sub-pathways of NER, Global Genome Repair (GGR) and Transcription Coupled Repair (TCR).

In chapter 3 an introduction is given on one of the most intriguing aspects of the NER system: its ability to detect many different types of lesions within a huge number of undamaged bases. A possible model of eukaryotic damage recognition is presented, based on the prokaryotic system in which damage recognition is elucidated in considerable detail.

Chapter 4 describes the identification of a homologue of Rad4 in *Saccharomyces cerevisiae,* Rad34. This protein is specifically involved in NER in the relatively small rDNA region. Like their human homologue XPC, Rad4 and Rad34 form a complex with Rad23. Interestingly, in yeast both Rad4 and Rad34 also bind to another small (20kDa) protein that we have identified as a new NER factor, designated Rad33. A study of the NER defect of *rad33* cells is presented in Chapter 5.

Chapter 6 discusses possible analogous roles of Centrin2 in human cells and Rad33 in yeast cells. Although the proteins do not share clear sequence homology, the predicted structures of Rad33 shows resemblance with that of Centrin2. Furthermore, we show that Centrin2 and Rad33 interact with XPC and Rad4, respectively, via the same conserved motif.

Chapter 7 contains a summary of the presented work in this thesis and concluding remarks.

Chapter 2

Nucleotide excision repair

Nucleotide excision repair

Nucleotide Excision Repair (NER)

Nucleotide excision repair is different from other repair mechanisms in its ability to recognize and remove a broad spectrum of structurally unrelated lesions, including platinum adducts, polycyclic aromatic hydrocarbons, aromatic amines, cholesterol adducts and psoralen adducts. In humans, NER is of particular importance in the prevention of skin cancer as it is the sole pathway for repair of lesions induced by UV irradiation, like cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts ((6-4)PPs).

The basic NER mechanism is highly conserved from bacteria to mammals. In general, three steps can be discerned: (1) damage recognition, (2) excision of the damaged oligonucleotide and (3) DNA synthesis (**figure 1**). Among eukaryotes the homology is extended further; most of the proteins carrying out the basic NER reaction are structurally and functionally conserved from yeast to man. *In vitro* reconstitution of the human and yeast NER reactions greatly contributed to our present understanding of the mechanism (Guzder *et al.*, 1995b; He *et al.*, 1996; Mu *et al.*, 1995). The eukaryotic NER reaction is schematically depicted in **figure 2**. The proteins involved in NER in *S. cerevisiae* and their human counterparts are summarized in **Table 1.**

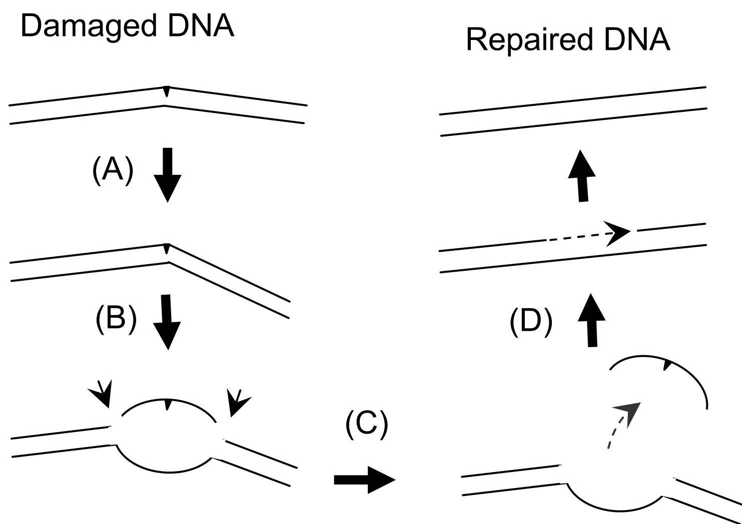

Figure 1
Schematic representation of the NER reaction. (A) Damaged DNA is recognized, possibly leading to a conformational change in the DNA (discussed in chapter 3). (B) Incisions are made on both sides of the lesion. (C) A ~30nt oligonucleotide containing the lesion is removed. (D) DNA is re-synthesized using the undamaged strand as template. The DNA is restored when the remaining nick is sealed by DNA ligase.

Table 1: NER factors

Yeast	Human	Role
Core-NER factors		
Rad4-Rad23	XPC-hHR23B*	damage recognition
TFIIH		open complex formation
Rad3	XPD	
Rad25	XPB	
Tfb1	p62	
Tfb2	p55	
Ssl1	p44	
Tfb4	p34	
Tfb5	p8	
Rad14	XPA	damage verification, coordination
RPA		stabilization of pre-incision complex
Rfa1	RPA1	
Rfa2	RPA2	
Rfa3	RPA3	
Rad2	XPG (ERCC5)	3' incision
Rad1-Rad10	XPF-ERCC1	5' incision
GGR factors		
Rad7-Rad16	-	
-	UV-DDB	
TCR factors		
Rad26	CSB	
-	CSA	

* In human cells XPC-hHR23B is not required for the TCR pathway, see 2.6

2.1 General mechanism

Although it has previously been suggested that the NER proteins reside in one complex, referred to as 'the repairosome' ((Feaver et al., 1993), it is now firmly established that the NER factors operate in a sequential manner (Guzder *et al.*, 1996b; Park and Choi, 2006; Riedl *et al.*, 2003; Volker *et al.*, 2001). The reaction outlined in this paragraph describes the NER system as it functions *in vitro*. This basic NER reaction, stripped down to the essential components only, is often referred to as the 'core NER reaction' and the proteins involved as 'core NER proteins'. The NER reaction up to the point of DNA synthesis requires six factors, most of which consist of multiple subunits. With the exception of Rad23, all the NER proteins in the reconstituted reaction are essential and sufficient for the incision to occur. The actual NER reaction *in vivo* involves several additional factors, including proteins that facilitate the coupling of the NER pathway to transcription (Transcription-coupled Repair (TCR), discussed in section 2.6) and proteins that specifically allow repair of non-transcribed regions (Global Genome Repair (GGR), discussed in section 2.5).

Rad4-Rad23/XPC-hHR23B (the yeast/human complexes respectively) initiates the reaction by binding to the damaged DNA. This crucial step in the NER reaction is not yet fully elucidated and is discussed in further detail in chapter 3. Once bound to the lesion, the Rad4-Rad23/XPC-hHR23B complex recruits transcription factor IIH (TFIIH). The helicase activity of TFIIH is required to initiate the unwinding of the helix surrounding the lesion. TFIIH also triggers the recruitment of Rad14/XPA, Rad2/XPG and RPA to the site of the lesion, which further stimulates the demarcation of the DNA. The collaborative action of the proteins present at this point results in the formation of the so called 'open complex', a single stranded bubble region of ~30nt that is the substrate for the endonucleases that eventually remove the lesion.

Given its affinity for damaged DNA and interactions with almost all other core-NER factors, the Rad14/XPA protein is presumed to be a damage verification factor and to be of central importance for the correct positioning of the other NER factors in relation to the lesion. Due to its strong affinity for ssDNA Replication protein A (RPA) is thought to stabilize the open pre-incision complex.

Once the pre-incision complex is properly in place, incisions are made at both sides of the lesion, 5' by the Rad1-Rad10 (XPF-ERCC1) complex and 3' by the Rad2 (XPG) protein. The oligomer containing the lesion (24-30 nucleotides long) is then released. The reaction is completed when the new DNA, synthesized using the undamaged strand as a template, is ligated. For reviews on the (core) NER mechanism see Prakash and Prakash (2000), de Laat *et al.* (1999), Gillet and Scharer (2006) and Park and Choi (2006).

2.2 The order of assembly

Of the six NER factors that are essential for the *in vitro* incision reaction, four (Rad4-Rad23/XPC-hHR23B, Rad14/XPA, RPA and TFIIH) have been shown to possess affinity for damaged DNA. It has long been unclear which of these factors acts before the others in the detection of DNA damage or whether damaged DNA has to be simultaneously bound by two or more factors in order to be processed by NER.

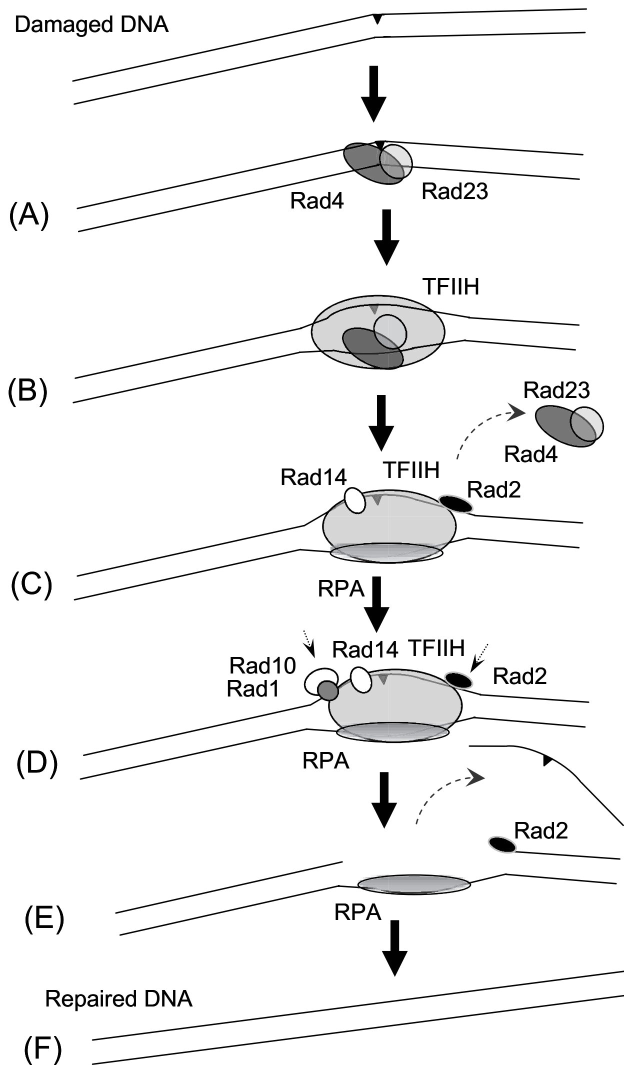

Figure 2
Schematic outline of the steps in the eukaryotic core-NER reaction. The names of the proteins refer to the Saccharomyces cerevisiae proteins. The names of the human homologues can be found in table 1. (A) Damaged DNA is bound by the Rad4-Rad23 complex (the Rad33 protein, not shown here, is expected to form a heterotrimeric complex with Rad4-Rad23, see chapters 5 and 6). (B) TFIIH is recruited by Rad4-Rad23. The helicase activity of TFIIH facilitates strand separation. (C) Rad4, RPA and Rad2 are subsequently recruited, which trigger further separation of the strands leading to the formation of the so called 'pre-incision complex'. In this step Rad4-Rad23 is thought to leave the NER complex. (D) Upon arrival of the Rad1-Rad10 complex, incisions are made both 5' and 3' to the lesion by Rad1-Rad10 and Rad2 respectively. (E) The oligonucleotide containing the lesion is removed. (F) DNA polymerase replicates the undamaged strand; the remaining nick is sealed by DNA ligase. RPA and Rad2 are implicated in the recruitment of the replication machinery to the site of the excised oligonucleotide.

Rad14/XPA was shown to bind UV damaged DNA with a preference over undamaged DNA and was therefore implicated in the first step of the NER mechanism (Guzder *et al.*, 1993; Robins *et al.*, 1991). However, later experiments demonstrated that the Rad4-Rad23/XPC-hHR23B complex also has affinity for damaged DNA (Jansen *et al.*, 1998; Sugasawa *et al.*, 1998) and, by using a damage recognition competition assay, XPC was shown to act before XPA in the NER process. Pre-incubation of damaged DNA with XPC enhanced the *in vitro* NER reaction, whereas pre-incubation with XPA had no effect (Sugasawa *et al.*, 1998). The initiating role of XPC was substantiated by *in situ* immuno-fluorescence experiments in cultured mammalian cells in which the translocation of NER proteins to locally UV-irradiated sites was monitored (Volker *et al.*, 2001). The authors demonstrated that migration of XPA and TFIIH to the site of damage is dependent on the XPC protein, whereas both XPC and TFIIH are recruited to the UV irradiated zone in the absence of XPA (Volker *et al.*, 2001). Mathematical modeling of kinetic experiments in living cells, using GFP-tagged NER proteins, predicted that a sequential assembly of NER factors as indicated by the experiments from Volker *et al.* (2001) is consistent with the actual rate of lesion removal by NER (Politi *et al.*, 2005).

Elegant *in vitro* studies using an immobilized DNA fragment containing a single cisplatin lesion also confirmed the initiating role of XPC-hHR23B (Riedl *et al.*, 2003). The DNA fragment was incubated with either a cell extract or a mix of purified NER enzymes, then washed, and finally analyzed for the associated NER factors. These factors were subsequently assayed for activity in a NER complementation assay. In the absence of ATP, only the XPC-hHR23B and TFIIH complexes were bound to the damaged fragment. In reactions lacking TFIIH the XPC-hHR23B complex could still bind, but in the reverse experiment TFIIH did not interact with damaged DNA. Interestingly, only in the presence of ATP all the core-NER factors were found associated with the DNA, indicating that ATP driven strand separation by TFIIH is essential for recruitment of the other NER proteins.

In further tests this system was used to evaluate the sequential assembly and disassembly by adding combinations of NER proteins to the initiation complex (XPC-hHR23B-TFIIH). This experiment determined that the assembly of the pre-incision complex occurs in 5 steps: (1) XPC-hHR23B (2) TFIIH (3) XPA (4) RPA, XPG, release of XPC-hHR23B (5) ERCC1-XPF (Riedl *et al.*, 2003). The early departure of the XPC-hHR23B complex (in step 4) is consistent with observations by others (Wakasugi and Sancar, 1998; You *et al.*, 2003). This event will contribute to the damage recognition efficiency, as it is likely that the released XPC-hHR23B can continue searching for other lesions.

These results clearly support the initiating role of Rad4-Rad23/XPC-hHR23B in NER. Nevertheless, the order of assembly is still under debate. For example, specific lesions within the substrate range of NER may require improvisation of the NER reaction. For example, psoralen adducts are reported to be bound by RPA and not by XPA or XPC, whereas all three factors are required for the incision of this lesion (Reardon and Sancar, 2002). Since the order of assembly to this type of lesions has not been studied *in vivo* it cannot be excluded that Rad4/XPC is not the initiator for all lesions. The repair of CPDs, the most common UV induced lesion and arguably most relevant NER substrate, appears not to be initiated by Rad4-Rad23/XPC-hHR23B alone. CPDs are

efficiently removed *in vivo*, but the Rad4-Rad23/XPC-hH23B complex is unable to bind these dimers *in vitro*. This lack of affinity for CPD lesions led to reluctance to accept the model in which Rad4/XPC is the first protein at the site of the lesion. Since removal of CPDs was detected in the reconstituted NER system whereas none of the included factors were able to bind CPDs, it was proposed that XPC-hHR23B, XPA and RPA cooperatively act in recognition of these lesions (Kesseler *et al.*, 2007; Reardon and Sancar, 2003). Yet, other groups reported that CPDs are not repaired at all *in vitro* (Sugasawa *et al.*, 2001; Szymkowski *et al.*, 1993), indicating that *in vivo* an additional factor may be involved in repair of these dimers. Indeed, in human cells GGR of CPDs is fully dependent on the GGR specific factor UV-DDB. UV-DDB is also involved in the repair of (6-4)PPs, but these lesions can still be repaired in the absence of UV-DDB (Hwang *et al.*, 1999; Moser *et al.*, 2005). Moreover, as UV-DDB was found to be required for the localization of XPC to CPDs, but not to (6-4)PPs (Fitch *et al.*, 2003), it seems that *in vivo* Rad4-Rad23/XPC-hHR23B is not the initiator of NER for all types of lesions.

UV induced post-translational modification of NER proteins might play a role in the assembly of the NER complex. It has been shown that XPC is ubiquitylated in response to UV irradiation, a modification that enhances the affinity of XPC for DNA (for review, see Sugasawa (2006) and Bergink *et al.* (2007)). It is interesting to note that the UV induced ubiquitylation of XPC appears to be independent of the other core-NER factors, but requires the GGR factor UV-DDB, supporting the notion that XPC-hHR23B is involved in an early stage of the NER process.

In the following paragraphs the individual NER factors are briefly discussed. The role of the Rad4-Rad23/XPC-hHR23B complex is described in more detail, as this factor is central in the research presented in chapters 4-6.

2.3 Rad4-Rad23/XPC-hHR23B acts as damage sensor

In vivo, Rad4/XPC is always found in association with Rad23/hHR23. The purified yeast Rad4-Rad23 complex shows preferential binding to UV induced lesions as well as to N-acetoxy-2-acetylaminofluorene (AAF) adducts (Guzder *et al.*, 1998b; Jansen *et al.*, 1998). Rad23 has no affinity for DNA (Guzder *et al.*, 1998b; Xie *et al.*, 2004), but appears to stimulate the binding of Rad4 to (damaged) DNA (Xie *et al.*, 2004).

Like Rad4-Rad23, the human orthologous complex XPC-hHR23B also possesses affinity for damaged DNA (Batty *et al.*, 2000; Reardon *et al.*, 1996; Sugasawa *et al.*, 1998; Sugasawa *et al.*, 2001; Wakasugi and Sancar, 1999). Pre-steady-state kinetics analysis of the interaction between XPC-hHR23B and DNA indicated that the affinity for damaged DNA is determined by faster association of XPC-hHR23B, whereas the dissociation of the complex is similar for damaged and undamaged DNA (Trego and Turchi, 2006).

The DNA binding assays mentioned above were conducted using naked DNA, whereas *in vivo* nucleosomal DNA is the substrate for NER. It has been reported that the absolute affinity of XPC for both undamaged DNA and DNA fragments containing a (6-4)PP lesion is decreased in the presence of nucleosomes (Yasuda *et al.*, 2005). The reduction in affinity is more prominent for undamaged DNA fragments and as a result the specific affinity of XPC for damaged DNA is increased (Yasuda *et al.*, 2005).

These findings imply that the damage specificity of XPC *in vivo* is higher than that observed in assays studying the binding to nucleosome free DNA.

The overall affinity of Rad4/XPC for damaged DNA depends on the type of lesion. The observed increase in binding of the Rad4-Rad23 complex to DNA fragments containing an AAF is limited since only ~3 fold preference for the damaged fragment was observed (Jansen *et al.*, 1998). However, the presence of ~4,5 CPDs and ~1,5 (6-4)PPs in an 130bp DNA fragment enhances the Rad4-Rad23 binding by a factor ~6000 (Guzder *et al.*, 1998b). Interestingly, pre-treatment of the irradiated DNA with *E. coli* photolyase, which specifically removes CPDs from the DNA (Sancar *et al.*, 1985), did not alter the affinity of Rad4-Rad23 for the fragment, showing that the binding of Rad4-Rad23 to UV-irradiated DNA is largely determined by the presence of (6-4)PPs. Consistent with these findings, the human XPC-hHR23B complex is not able to distinguish DNA fragments containing a CPD from undamaged DNA (Batty *et al.*, 2000; Hey *et al.*, 2002; Kusumoto *et al.*, 2001; Sugasawa *et al.*, 2001).

Recently the crystal structure of a truncated Rad4 protein in complex with a Rad23 peptide, was solved, as well as the structure of the same complex bound to an oligonucleotide containing a CPD lesion (placed within a stretch of three mismatched nucleotides to facilitate binding) (Min and Pavletich, 2007). This study provided more insight into the interaction of Rad4-Rad23 with (damaged) DNA. Four distinct domains were identified on the Rad4 protein, a catalytically inactive, amino-terminal transglutaminase domain (TGD) and three β-hairpin domains (BHD1-3) located in the carboxy-terminal region (Min and Pavletich, 2007). The DNA fragment is contacted by two distinct regions of the Rad4 protein. A C-clamp like structure formed by the TGD and BHD1 domains binds to an 11 base-pair undamaged region 3' of the lesion. The affinity of this C-clamp structure for intact dsDNA may explain the considerable binding of Rad4/XPC to undamaged DNA (Batty and Wood, 2000; Thoma and Vasquez, 2003). The BDH2 and BDH3 domains cooperate in binding to the DNA containing the CPD. Aromatic residues in Rad4 facilitate the crucial contacts made with the nucleotides on the undamaged strand opposite the lesion. Interestingly, the BHD3 is inserted through the DNA duplex, resulting in displacement of the two linked thymines that constitute the CPD, as well as their undamaged adenine counterparts. Rad4 contacts the undamaged adenines with both the BHD2 and BHD3 while the CPD is exposed to the solvent. Several residues of the TGD and BDH1-3 domains that are involved in structure stabilization or DNA binding are conserved between Rad4 and XPC. This is particularly the case for the BDH3 domain that is essential for the interaction between Rad4 and the nucleotides opposite the lesion, suggesting that the homologues use the same approach to perceive damaged DNA.

Modeling of the structures of free and DNA-bound Rad4 revealed that Rad4 undergoes a conformational change when bound to the DNA. The boundaries of the four separate Rad4 domains were suggested to function as hinges, each hinge bending 6°-12° in the Rad4-DNA complex. In the predicted structure of Rad4 bound to undamaged dsDNA, only the free Rad4 structure could be fitted and not the CPD-bound, hinged, form (Min and Pavletich, 2007). This might indicate that the presence of a lesion enables Rad4, along with the DNA, to change to a conformation that will be recognized by downstream NER factors. Bound to undamaged DNA, Rad4/XPC may not be able to induce the bending of the DNA, or alternatively, the conformational change

will be energetically less favorable. Based on atomic force microscopy studies with XPC-hHR23B the latter possibility seems more likely, as non damaged DNA is curved ~50° when bound by XPC-hHR23B. The presence of a cholesterol moiety led to a ~40° XPC-hHR23B induced bend which 'trapped' the complex, indicating that the bend conformation of the XPC-hHR23B-DNA complex is energetically favorable at the site of a lesion (Janicijevic *et al.*, 2003).

2.3.1 The role of Rad23/hHR23B in the Rad4-Rad23/XPC-hHR23B complex

Of all NER factors, the role of Rad23 in the NER process is the most enigmatic. Rad23 forms a complex with Rad4 (Guzder *et al.*, 1998b), but is present in ~10 fold excess over Rad4 (Ghaemmaghami *et al.*, 2003), which might indicate that Rad23 has additional activities beyond NER. Indeed, Rad23 has been shown to function as an escort to shuttle ubiquitylated proteins to the proteasome (Chen and Madura, 2002; Rao and Sastry, 2002) and appears to be involved in centriole duplication (Biggins *et al.*, 1996).

Cells deprived of Rad23 show intermediate UV sensitivity, comparable to that of *rad16* cells, in which nearly 50% of all lesions are removed (Verhage *et al.*, 1996c). Remarkably, no repair is detected in *rad23* cells (Gillette *et al.*, 2001; Verhage *et al.*, 1996c). The reason for the high survival rate of *rad23* cells in comparison to the virtual absence of NER in these mutants remains to be elucidated. The most obvious explanation is that Rad4 alone can still initiate the NER reaction with a very low efficiency. The hardly detectable removal of lesions in *rad23* cells somehow greatly contributes to cellular survival after damage induction. It might be speculated that the few NER events activated by Rad4 are still enough to activate the signaling cascade that leads to cell cycle arrest (reviewed by Carr (2002)), allowing more time to deal with the lesions present. It has been shown that functional NER is required to activate the UV induced cell cycle arrest in yeast (Giannattasio *et al.*, 2004). The presence of Rad4, Rad14 and Rad2 is essential to initiate cell cycle arrest. On the other hand, partial NER deficient cells lacking either *RAD16* or *RAD26* do still arrest upon UV irradiation (Giannattasio *et al.*, 2004). Cell cycle arrest in *rad23* mutants was not examined, but as these cells do possess residual NER activity (Mueller and Smerdon, 1996) it may be possible that Rad23 is not essential for DNA damage induced cell cycle arrest, therefore allowing *rad23* cells more time to cope with the lesions via other ways.

An additional explanation for the relative high survival of *rad23* cells after UV irradiation may be NER activity that occurs after the time during which repair is monitored in most NER assays. However, the presence of this possible 'late repair' in *rad23* cells remains unclear. Our own data and that of Gillette *et al.* (2001) do not show any repair, even after 3 or 4 hours following UV irradiation whereas other reports show ~40% repair in *rad23* cells at similar times after damage induction (Gillette *et al.*, 2006; Mueller and Smerdon, 1996).

In human cells two homologues of Rad23 are present, hHR23A and hHR23B (human homologue of Rad23), which are functionally interchangeable in NER (Sugasawa *et al.*, 1997). Due to the relative abundance of hHR23B compared to hHR23A, XPC is found predominantly in complex with hHR23B (Okuda *et al.*, 2004). Mice lacking mHR23B show severe developmental abnormalities whereas mHR23A knockout mice have no clear phenotype. Deletion of both Rad23 homologues is incompati-

ble with life, showing that the function of Rad23 in mammals is clearly not confined to NER. However, stable cell lines could be derived from mHR23A/B double knockout mouse embryos. Analysis of these embryonic fibroblasts show that the absence of both Rad23 homologues causes a similar defect in NER as that of XP-C cells (Ng *et al.*, 2003), indicating that XPC cannot function without either hHR23A or hHR23B.

The function of Rad23 in the Rad4-Rad23 complex is far from being elucidated. Addition of Rad23/hHR23B stimulates the affinity of Rad4/XPC for damaged DNA (Batty *et al.*, 2000; Bunick *et al.*, 2006; Xie *et al.*, 2004) and increases the efficiency of an *in vitro* reconstituted NER reaction (Masutani *et al.*, 1997; Sugasawa *et al.*, 1996). The domain in hHR23B responsible for the interaction with XPC was pinned down to a 56 amino acid sequence. Addition of this small polypeptide to a cell free NER reaction stimulates XPC dependent NER activity to near wildtype levels (Masutani *et al.*, 1997), indicating that, *in vitro,* binding of the 56 amino acid sequence of hHR23B to XPC is enough to induce a conformational change which enhances the activity of XPC in NER. However, analysis of the situation *in vivo* reveals that the role of Rad23 is more complex.

Does Rad23 regulate Rad4 levels?

An extensively discussed role of Rad23 is its possible involvement in the regulation of Rad4 levels. Based on the observation that introduction of the *RAD4* gene in *E.coli* confers lethality (Siede and Eckardt-Schupp, 1986), it was assumed that the Rad4 protein interferes with cellular metabolism, presumably due to its affinity for (damaged) DNA.

The observed decrease of Rad4/XPC levels in cells devoid of Rad23/hHR23B led to the suggestion that one function, or even the primary function, of Rad23 in NER is to stabilize Rad4 (Lommel *et al.*, 2002; Ng *et al.*, 2003; Ortolan *et al.*, 2004; Xie *et al.*, 2004). The toxic effect of Rad4 in *E. coli* prompted a model in which Rad23 is involved in the regulation (i.e. stabilization) of Rad4, inducing the Rad4 levels only in the presence of DNA damage. This model thus assumes that (part of) the NER defect in *rad23* cells is caused by the permanently reduced levels of Rad4.

Yet, over-expression of Rad4 in yeast *rad23* cells does not significantly enhance the UV survival and addition of purified Rad4 to *rad23* cell extracts does not complement the defective incision reaction (Lommel *et al.*, 2002; Xie *et al.*, 2004). Moreover, reduced Rad4 levels were also observed in cells expressing a Rad23 mutant that lost its interaction with Rad4, but these cells are only mildly UV sensitive (Ortolan *et al.*, 2004).

These observations strongly suggest that the repair defect in *rad23* cells is not, or only partially, related to the reduced quantity of Rad4 proteins. Moreover, constitutive over-expression of Rad4, or of both Rad4 and Rad23 simultaneously, has no harmful consequence for cellular survival (Lommel *et al.,* 2002; Xie *et al.,* 2004 and our own unpublished observations) and therefore does not indicate interference of Rad4 with DNA metabolism.

In mammalian cells the instability of XPC is a partial cause of the NER defect in cells devoid of mHR23A and mHR23B (Ng *et al.*, 2003). The reduction of XPC levels in mouse embryonic mHR23A/B double-knockout fibroblasts is more pronounced than that of Rad4 in yeast *rad23* cells. In contrast to yeast cells however, in this system the NER defect can be partially alleviated by either over-expression of XPC or microinjec-

tion of XPC cDNA (Ng *et al.*, 2003). Interestingly, when mHR23A/B knockout cells were injected with a cocktail of XPC and hHR23B cDNA, a toxic effect was observed. These results were in agreement with the toxicity of Rad4 in *E. coli* and interpreted as indicative for a toxic effect of high levels of hHR23B-stabilized XPC (Ng *et al.*, 2003). However, the observation that microinjection of XPC cDNA is toxic only in combination with hHR23B cDNA injection might also indicate that it is the hHR23B cDNA that confers toxicity, as exclusive injection of the latter was not tested (Ng *et al.*, 2003). Until the role of Rad23/hHR23B in Rad4/XPC regulation is fully clarified, it may also be considered that the observed instability of Rad4/XPC in cells devoid of Rad23/hHR23B is the result of artificially forcing Rad4/XPC out of its natural conformation. In the case of NER in yeast *rad23* cells, the instability of Rad4 seems not the main cause of the NER defect (Ortolan *et al.*, 2004; Xie *et al.*, 2004). In mammalian cells, the reduction of XPC does constitute part of the NER defect in mHR23A/B knockout cells (Ng *et al.*, 2003), but this effect does not necessarily mean that the role of Rad23/hHR23B is to regulate Rad4/XPC via altering its stability.

The model on the Rad4-regulating role of Rad23 was recently given a new twist. Most of the reports showing that the Rad4 protein is prone to degradation in *rad23* cells make use of epitope-tagged Rad4/XPC for visualization of the proteins. However, based on experiments in which untagged Rad4 levels were monitored using an antibody raised against yeast Rad4, the stabilizing effect of Rad23 on Rad4 was challenged (Gillette *et al.*, 2006). Although the steady state levels of Rad4 were found lower in *rad23* cells, no significant instability was observed, leading to the suggestion that the previously observed instability was caused by the presence of epitope-tags. Strikingly, the authors reported that *RAD4* mRNA levels are reduced in *rad23* cells and suggested that Rad23 is involved in transcription regulation of the Rad4 protein (Gillette *et al.*, 2006). However, we could not confirm these results and found no reduction of Rad4 mRNA levels in *rad23* cells (chapter 5). Nevertheless, in human cells hHR23B was recently also implicated in transcriptional upregulation of XPC. DNA damage induction leads to an increase of XPC levels in a p53 dependent manner (Adimoolam and Ford, 2002). As hHR23B was reported to be involved in genotoxic dependent stabilization of p53 (Kaur *et al.*, 2007), it may be indirectly responsible for the DNA damage induced upregulation of XPC.

The involvement of the 19S proteasome subunit in NER

In addition to the Rad4/XPC interacting domain (R4B), Rad23/hHR23B contains three other domains: an amino-terminal ubiquitin-like domain (UbL) and two ubiquitin associating domains (UBA), one at the carboxy terminus and one in between the UbL and R4B domain.

The UBA domains interact with ubiquitin and can inhibit the formation of polyubiquitin chains (Bertolaet *et al.*, 2001; Chen *et al.*, 2001). Cells expressing a mutant Rad23 protein that does no longer interact with ubiquitin via its UBA domains are not UV sensitive, indicating that these domains are not required for NER (Bertolaet *et al.*, 2001; Ortolan *et al.*, 2004) and are probably involved in the role of Rad23 in shuttling proteins to the proteasome.

In contrast, the UbL domain of Rad23 is involved in NER. The amino acid compo-

sition of the UbL domain is highly similar to that of ubiquitin. In fact, the role of Rad23 in NER is retained when the UbL domain of Rad23 is replaced by genuine ubiquitin (Watkins *et al.*, 1993). Via this UbL domain Rad23 interacts with the 26S proteasome (Schauber *et al.*, 1998) and deletion of the UbL domain (Rad23UbLΔ) confers weak UV sensitivity, suggesting that the interaction of Rad23 with the proteasome is required for efficient NER (Schauber *et al.*, 1998). Indeed, results from *in vitro* NER assays demonstrated that the proteasome has a stimulatory effect on repair. Interestingly, not the proteolytic 20S component, but the 19S regulatory subunit is responsible for the NER enhancement (Russell *et al.*, 1999). Yeast cells carrying mutations in the 19S proteasome subunit display UV sensitivity epistatic with that of *rad23UbLΔ* cells, confirming that the interaction between the 19S subunit and Rad23 facilitates optimal NER activity and is mediated via the UbL domain of Rad23 (Gillette *et al.*, 2001).

Interesting results were obtained in studies using a Rad23 mutant that lacks the Rad4-binding domain (Rad23R4BΔ). In cells expressing the Rad23R4BD protein the interaction between Rad4 and Rad23 is abolished, and consequently the level of Rad4 protein is reduced. The *rad23R4BΔ* cells are only mildly UV sensitive compared to *rad23* cells (Ortolan *et al.*, 2004), implying that Rad23, even when not in complex with Rad4, does contribute to survival after UV irradiation. Despite Rad23 lost its interaction with Rad4, the effect of Rad23 on UV survival is somehow still dependent on the presence of functional Rad4. Interestingly, *rad23* cells in which Rad23R4BΔ is co-expressed with Rad23UblΔ exhibit a fully NER proficient UV phenotype (Ortolan *et al.*, 2004). This indicates that independently operating Rad23 proteins carry out two distinct roles in the NER process. One role requires the interaction with the proteasome, the other requires the interaction with Rad4.

As binding partner of Rad4, the most obvious role of Rad23 is to enhance or regulate the activity of Rad4, conceivably by inducing a conformational change of the Rad4 protein. Additionally, Rad23/hHR23B might contribute to the NER process downstream of the Rad4-Rad23/XPC-hHR23B damage binding. The displacement of XPC from DNA is enhanced in the presence of hHR23B (You *et al.*, 2003). By stimulating this release, hHR23B will increase the average number of XPC-hHR23B complexes available for damage sensing.

There is yet no explanation how the fraction of Rad23 proteins that interacts with the proteasome, possibly physically separated from the other NER proteins (Ortolan *et al.*, 2004), plays a role in the NER process. Whereas the proteasome stimulates NER in wildtype cells, the UV sensitivity of *rad23* cells can be partially alleviated by the introduction of *sug1* or *sug2* point-mutations that destabilize the 19S subunit. In *rad23* cells the effect of the 19S subunit thus seems inhibitory rather than stimulatory (Gillette *et al.*, 2001). This could indicate that NER requires the regulatory subunit of the proteasome for optimal efficiency, but needs Rad23 to protect certain NER proteins from an inhibitory effect of the proteasome.

2.3.2 Other proteins binding the Rad4-Rad23/XPC-hHR23B complex

Centrin2

The calmodulin-like protein Centrin2 was previously known as part of the centrosome and required for centriole separation during centrosome duplication (Lutz *et al.*, 2001;

Salisbury *et al.*, 2002). Since the majority of the Centrin proteins is not associated with the centrosome it was expected that Centrin2 is involved in other processes as well (Paoletti *et al.*, 1996). An additional role of Centrin2 transpired when it was identified as part of the XPC-hHR23B complex (Araki *et al.*, 2001). The heterotrimeric XPC-hHR23B-Centrin2 complex was found to be stable, even in the presence of high salt concentration. The Centrin2 protein, together with hHR23B, stimulates the *in vitro* NER reaction, possibly due to its stabilizing effect on XPC (Araki et al., 2001). Through binding assays using truncated XPC proteins the region responsible for the interaction with Centrin2 was determined and further analysis led to the identification of three conserved residues that are essential for the interaction between XPC and Centrin2 (Nishi *et al.*, 2005). Cells expressing a XPC protein in which these residues are mutated to alanines (XPC-AAA mutant) are impaired in the overall removal of (6-4)PPs. Since Centrin2 is part of the XPC-hHR23B complex that is required for GGR, it was assumed that the reduced repair caused by the disrupted interaction between XPC and Centrin2 reflects a specific defect in the GGR pathway (Nishi *et al.*, 2005). Addition of XPC-AAA to an in vitro NER assay that includes Centrin2 has only a small effect on the NER reaction compared to the addition of authentic XPC, which results in a markedly enhanced NER efficiency (Nishi *et al.*, 2005). The role of Centrin2 in the XPC-hHR23B complex is yet unknown. Based on *in vitro* assays it appears that one role of Centrin2 is the enhancement of the stability and DNA binding activity of XPC. However, like the other XPC binding partner hHR23B, the Centrin2 protein may have additional value for NER *in vivo*.

Rad33

We recently identified a new protein involved in NER of *S. cerevisiae,* Rad33 (chapters 5 and 6 of this thesis). Interaction studies show that Rad33 is part of the Rad4-Rad23 complex. Cells deleted for *RAD33* are UV sensitive and defective in the GGR sub-pathway. TCR is still active in cells lacking the Rad33 protein, but with a significant reduced efficiency (chapter 5). In cells deprived of both Rad26 and Rad33 no removal of CPDs from the *RPB2* gene is detected, however, with regard to UV survival, *rad33rad26* mutants do not show a complete NER deficient phenotype (chapter 5). Further UV-survival tests indicate that the residual UV survival of *rad33rad26* mutants is caused by GGR and not by Rad26-independent TCR, as *rad33rad26rad16* triple mutants exhibit UV sensitivity associated with a complete NER defect (unpublished observations). This shows that there is some remaining GGR activity in cells lacking Rad33. The fact that in *rad33* cells no residual GGR activity is detected in our repair assays, in which we measure the CPD removal in the *RPB2* gene, could indicate that GGR is still active in other regions of the genome. Alternatively, other types of UV induced lesions might be (partially) removed in the absence of Rad33. This latter option is not inconceivable, since CPDs represent one of the most challenging lesions for Rad4/XPC damage recognition. In cells lacking Rad33 a slight alteration in the conformation of Rad4 can possibly affect CPD recognition more severely than (6-4)PP binding.

Interestingly, the predicted structure of Rad33 resembles that of Cdc31, the only yeast homologue of the human Centrin proteins. The calcium binding EF hand domains (Lewit-Bentley and Rety, 2000) characteristic for the calmodulin-like proteins

can not be recognized in Rad33 however (unpublished observations). We have shown that Rad33 binds to Rad4 via the same three residues that connect XPC to Centrin2. Mutation of these amino acids to alanines abolishes the interaction between Rad4 and Rad33 and leads to a NER defect similar to that of *rad33* cells (chapter 6). These findings indicate that the role of Rad33 in NER may be similar to that of Centrin2 in human cells. However, as for Centrin2, the precise role of Rad33 remains elusive. One possible hint emerged from protein-protein interaction screens, that report a relative high number of Rad33 interacting proteins that are implicated in the organization of the cytoskeleton (5 out of 8 interactions Rvs167, Rvs161, Mlc1, Crn1, Lsb3, (Krogan *et al.*, 2006)). These interactions could possibly indicate a role for Rad33 in localizing the NER process on the nuclear matrix. Yet, we did not find a UV survival defect in cells deleted for any of these genes (unpublished observations).

Cdc31

Whether Rad33 is a functional homologue of Centrin2 is uncertain, as the authentic *S. cerevisiae* sequence homologue of Centrin2, Cdc31, was recently also detected in the Rad4-Rad23 complex (Chen and Madura, 2008). This study established a role of Cdc31 in the regulation of protein stability via interaction with the proteasome (independent of Rad23) and ubiquitylated proteins. The role of Cdc31 in NER was not thoroughly investigated; cells expressing a Cdc31 mutant, that is impaired in the interaction with Rad4, were found slightly sensitive towards UV irradiation but it was not examined whether this increased UV sensitivity was due to a defect in NER and actual repair activity in these cells has not been analyzed (Chen and Madura, 2008). The fraction of Cdc31 associated with Rad4-Rad23 is dependent on the growth phase. Compared to stationary cells significantly lower amounts of Cdc31 are present in the Rad4-Rad23 complexes in actively growing cells. The authors suggested that Cdc31 may play a role in cell cycle regulation upon damage induction. It is conceivable that Rad33 and Cdc31 bind Rad4 via the same site and that the alternating interaction of Rad4 with these proteins is dependent on the growth phase of the cells, possibly constituting a means of regulating the activity of Rad4. However, we have observed no significant differences between *rad33* deletion mutants and cells in which the Rad33 interaction site on Rad4 was disabled (chapter 6), implying that Cdc31 either binds Rad4 via other residues, or has a limited contribution to NER.

2.3.3 The Rad4 homologue Rad34 in yeast

In *S. cerevisiae* we identified a previously unknown NER protein, Rad34. This NER factor shares sequence homology with Rad4, mainly in the (conserved) carboxy terminal region. Like Rad4, Rad34 is involved in NER, but its role is confined to the RNA polymerase I (RNA pol I) transcribed rDNA locus (den Dulk *et al.*, 2005). In this region NER is organized slightly different compared to in RNA pol II transcribed DNA. UV induced lesions are preferentially removed from the RNA pol I transcribed strand, similar to NER in RNA pol II transcribed DNA (Conconi *et al.*, 2002; Verhage *et al.*, 1996a). However, in contrast to TCR in RNA pol II transcribed DNA, RNA pol I transcription-coupled repair functions independently of the Rad26 protein (Verhage *et al.*,

1996a). We showed that the preferential repair of the RNA pol I transcribed strand is dependent on Rad34 (chapter 4). Rad4 cannot substitute for Rad34 in this mode of repair and, similarly, Rad34 can not replace Rad4 in NER of RNA pol II transcribed DNA nor in GGR in the rDNA locus.

Like Rad4, Rad34 directly interacts with both Rad23 and Rad33, suggesting it resides in a similar complex as the Rad4 protein. In human cells no homologue of Rad34 has been identified, which might be the reason that TCR of RNA pol I transcribed DNA is absent altogether in the human system (Christians and Hanawalt, 1993). The role of the yeast Rad34 protein (in chapter 4 also referred to as YDR314C) is further discussed in chapter 4.

2.3.4 TFIIH

Binding of Rad4-Rad23-Rad33/XPC-hHR23B-Centrin2 is followed by the recruitment of TFIIH (Transcription Factor IIH) (Yokoi *et al.*, 2000). TFIIH consists of 10 proteins: Rad25, Rad3, Tbf1, Tfb2, Ssl1, Tfb4, the CAK (CDK-activating kinase) subunits Tbf3, Kin28 and Ccl1 and the recently identified 10th subunit Tfb5, which is the only non-essential component of TFIIH (Giglia-Mari *et al.*, 2004; Ranish *et al.*, 2004) (for the names of the human homologues, see table 1). TFIIH is involved in the initiation of both RNA pol I and II transcription (Hoogstraten *et al.*, 2002; Iben *et al.*, 2002; Lu *et al.*, 1992), cell cycle progression (Jona *et al.*, 2002) and in NER (Feaver *et al.*, 1993; Schaeffer *et al.*, 1993). For NER *in vitro* the core complex, lacking CAK, is sufficient for the incision to occur (Araujo *et al.*, 2000; Guzder *et al.*, 1995b; Mu *et al.*, 1996). Addition of the CAK complex does not stimulate NER and might even be inhibitory to the NER activity (Araujo *et al.*, 2000; Coin *et al.*, 2006). Recent studies show that CAK is released from the TFIIH core complex upon DNA damage induction. This dissociation stimulates the NER reaction and is dependent on the XPA protein (Coin *et al.*, 2008). Tfb5/p8 significantly contributes to the efficiency of NER, presumably by conferring structural stability to the TFIIH core complex (Zhou *et al.*, 2007) and stimulation of the ATPase activity of XPB/Rad25 (Coin *et al.*, 2006).

The key components of TFIIH are the helicases Rad25/XPB and Rad3/XPD. Rad3/XPD exhibits ATPase activity and acts as a 5' > 3' helicase on partially duplex substrates (Sung *et al.*, 1987). Rad25/XPB harbors similar biochemical activities, but its helicase activity is of opposite polarity (Guzder *et al.*, 1994). In the traditional NER models, the helicase activity of these TFIIH components facilitates the partial unwinding of the DNA bound by Rad4-Rad23/XPC-hHR23B in order to physically separate the damaged from the undamaged strand (de Laat *et al.*, 1999; Prakash and Prakash, 2000). ATP dependent lesion demarcation by TFIIH in NER comprises a 10-20bp region (Evans *et al.*, 1997a) which is similar to the size of the promoter opening by TFIIH involved in transcription (Holstege *et al.*, 1996), indicating that the same biochemical actions of TFIIH are utilized for distinct purposes in NER and transcription. Consistent with this observation, TFIIH was found to shuttle between transcription and NER (Hoogstraten *et al.*, 2002; Riedl *et al.*, 2003). However, some subunits of TFIIH are specifically involved in either transcription or repair. The helicase activity of XPD/Rad3 is essential for NER but not required for transcription (Winkler *et al.*, 2000). This observation suggests that the collaborative actions of Rad3 and Rad25 helicases create the

unwound DNA structure, often referred to as 'bubble' or 'open complex' (Deschavanne and Harosh, 1993; Guzder *et al.*, 1995a; Sung *et al.*, 1987). However, it was recently shown that whereas the ATPase activity of XPB (Rad25) is essential for NER, inhibition of the helicase activity did not affect the formation of an open complex (Coin *et al.*, 2006; Coin *et al.*, 2007). This observation led the authors to suggest a model in which DNA wrapping around XPB will induce local melting of the double stranded DNA to create an anchor point for the XPD helicase activity (Coin *et al.*, 2007). The observations above suggest that the helicase activities of Rad3/XPD and Rad25/XPB are specifically involved in NER and transcription respectively.

In addition to creating accessibility for the downstream NER factors, eventually providing a platform that allows excision of the damaged oligonucleotide, the strand separation activity of TFIIH is also implicated in the localization/verification of the lesion. The observation that the Rad3 helicase activity is inhibited by the presence of DNA damage suggested that this block might serve the purpose of damage verification (Naegeli *et al.*, 1992) and prompted a model in which Rad3 helicase activity embodies a strand-discriminating mechanism for NER (Naegeli *et al.*, 1993a; Naegeli *et al.*, 1993b). Possibly, NER will only proceed when TFIIH helicase activity is inhibited by a lesion. In this case, the damaged base will always be present in the strand bound by Rad3/XPD, and in the direct vicinity of this protein. As adducts that do not generate considerable distortion to the secondary structure of the DNA helix still pose a block for the Rad3 helicase activity (Naegeli *et al.*, 1993a), the blockage may allow verification of lesions (e.g. CPDs) that are weakly recognized by the upstream damage binding factors. An experiment in which the contacts of the NER proteins engaged in repair of a psoralen adduct was examined also implicated Rad3/XPD in damage recognition. This study revealed that XPD, and not the conventional damage recognition/verification factors XPC or XPA, is in direct contact with the lesion (Reardon and Sancar, 2002).

2.3.5 Rad14/XPA

Rad14/XPA enters the NER complex after TFIIH has partially separated the DNA strands surrounding the lesion (de Laat *et al.*, 1999; Gillet and Scharer, 2006; Riedl *et al.*, 2003). Binding of both XPC-hHR23B and XPA stimulates the ATPase activity of TFIIH (Winkler *et al.*, 2001) and in absence of XPA only intermediate separation of the DNA strands is observed (Evans *et al.*, 1997b; Mu *et al.*, 1997b), showing that the formation of the complete open complex requires Rad14/XPA.

Rad14/XPA exhibits affinity for damaged DNA (Asahina *et al.*, 1994; Guzder *et al.*, 1993; Robins *et al.*, 1991). Given that Rad14/XPA acts after binding of XPC-hHR23B and TFIIH, the damage recognition role of Rad14/XPA is considered to be a verifying one. The observation that cells expressing a mutant Rad14 protein are unable to repair CPDs, but can still remove thymine hydrates (Jones *et al.*, 1997) indeed suggests that Rad14/XPA is somehow involved in assessment of the lesion. However, the way in which Rad14/XPA contributes to damage verification is unclear.

The DNA binding domain of XPA is positioned in a central 122 residue fragment (Kuraoka *et al.*, 1996). The solved NMR structure of this domain revealed that XPA contains a cleft containing a cluster of conserved, positively charged side chains, shaped

such that it theoretically can accommodate a single or double stranded DNA fragment (Ikegami *et al.*, 1998). Systemic site directed mutagenesis confirmed that the positively charged residues are indeed essential for the XPA-DNA interaction (Camenisch *et al.*, 2007). It was therefore predicted that XPA binds to DNA backbone regions where the negative electrostatic potential is locally increased due to the concentration of phosphate residues, i.e., XPA preferentially binds to DNA that is bend or distorted. Indeed, it was reported that the binding of XPA to damaged DNA can be solely ascribed to its affinity for DNA distortions (Camenisch *et al.*, 2006; Missura *et al.*, 2001; Yang *et al.*, 2006).

2.3.6 RPA

The heterotrimeric Replication Protein A complex (RPA) has strong affinity for ssDNA and apart from in NER, it uses this quality in several processes, including DNA replication, recombination, mismatch repair and the DNA damage checkpoint (Cortez, 2005; Fanning *et al.*, 2006; Li, 2008). In NER RPA has a dual role, as it is essential for incision (Guzder *et al.*, 1995b; Mu *et al.*, 1995) as well as for DNA synthesis after the excision of the damaged oligonucleotide (Coverley *et al.*, 1991).

The binding of RPA to ssDNA is thought to stabilize the pre-incision complex. RPA can bind ssDNA in two modes, it binds to patches of 8-10nt but has a more stable interaction with ssDNA stretches of ~30nt (Blackwell and Borowiec, 1994). This may suggests that a transition from the former mode to the latter assists in the extension of the bubble structure initiated by TFIIH.

RPA is also implicated in damage recognition/verification, as it preferentially binds DNA containing UV or cisplatin induced lesions (Burns *et al.*, 1996; Clugston *et al.*, 1992; Patrick and Turchi, 1998). The interaction between RPA with XPA is reported to synergistically enhance the affinity of both the proteins for DNA (He *et al.*, 1995; Li *et al.*, 1995). In more recent studies however no effect of RPA on the damage binding of XPA was observed (Liu *et al.*, 2005). The synergistic effect on damage binding might only be utilized after both proteins have individually entered the pre-incision complex, as the diffusion rate of free XPA *in vivo* does not reveal an interaction of XPA with RPA and, furthermore, RPA binds the NER complex in the absence of XPA (Rademakers *et al.*, 2003).

The affinity of RPA for damaged DNA is largely dependent on the presence of ssDNA stretches, which are formed as a result of the lesion (Maltseva *et al.*, 2008; Patrick and Turchi, 1999). It therefore seems that specific affinity of RPA for the lesion will be lost in the context of the ssDNA bubble which is the substrate for RPA *in vivo*. The observation that RPA binds the undamaged strand of the bubble structure, and also interacts with the nuclease that performs the incision, may indicate that RPA coordinates the incision reaction, ensuring that the damaged strand is excised (de Laat *et al.*, 1998; Hermanson-Miller and Turchi, 2002).

2.3.7 Rad2/XPG and Rad1-Rad10/XPF-ERCC1

Once the pre-incision complex is properly constructed, incisions are made by means of hydrolysis of the phosphodiester bonds. The nicks are placed 2-8nt from the 3' side

and 15-24nt from 5' side of the lesions, dependent on, and corresponding to, the size of the open complex (Evans *et al.*, 1997b; Huang *et al.*, 1992). The 3' nick is made by the Rad2/XPG protein at the junction of single stranded and double stranded DNA (Guzder *et al.*, 1995b; Matsunaga *et al.*, 1995; O'Donovan *et al.*, 1994). XPG contains two highly conserved nuclease motifs separated by a spacer region that is required for substrate specificity and interaction with TFIIH (Dunand-Sauthier *et al.*, 2005). It was recently shown that XPG is required for PCNA recruitment and suggested that it may counteract the inhibition of PCNA and DNA pol δ by p21 (Mocquet *et al.*, 2008).

The 5' incision shortly follows the incision by Rad2/XPG and is applied by the Rad1-Rad10/XPF-ERCC1 complex (Mu *et al.*, 1996). Like Rad2/XPG, the Rad1-Rad10/XPF-ERCC1 endonuclease complex nicks the DNA at the transition from double to single stranded DNA, but in the opposite orientation. The recruitment of XPF-ERCC1 to sites of local UV irradiation is dependent on XPA (Volker *et al.*, 2001). In agreement with this observation it was recently demonstrated that yeast cells expressing a mutant Rad1 protein that lost its interaction with Rad14 are completely NER defective, underlining the importance of Rad14/XPA for the correct positioning of the nuclease (Guzder *et al.*, 2006). The 5' incision by XPF-ERCC1 triggers the recruitment of PCNA and Replication factor C (RFC) (Mocquet *et al.*, 2008). Besides their role in the excision step, XPG/Rad2 and XPF-ERCC1/Rad1-Rad10 are thus both involved in mediation of the DNA synthesis after the excision of the damaged oligonucleotide

2.4 NER sub-pathways

The NER mechanism depicted in figure 2 is based on repair experiments using naked DNA substrates. NER *in vivo* has to deal with DNA that is wrapped around nucleosomes or even further compacted in heterochromatin structures. In addition the DNA is subjected to various other processes like transcription and replication. As a result NER *in vivo* is more complicated and the core-NER factors alone are not enough to achieve removal of lesions within the cell. Additional proteins are required for NER *in vivo* and the requirement of these proteins is the basis for the division of the NER system into two sub-pathways. Global Genome Repair (**GGR**) is the sub-pathway involved in genome-wide damage removal, essential for NER in non-transcribed or silenced DNA. Transcription Coupled Repair (**TCR**) is specifically involved in repair of lesions in the template strand of actively transcribed DNA. Deletion of genes that are specifically involved in one of the two sub-pathways leads to a partial NER defect. Obviously, deletion of one of the genes encoding a core-NER factor will abolish both sub-pathways.

Besides the core NER proteins and the essential GGR and TCR factors, more proteins contribute to the NER system. Cells lacking these proteins might only exhibit a subtle NER defect and the role of such proteins is frequently not clarified at this point in time. Possibly, most of these 'auxiliary NER factors' have yet to be discovered.

2.5 Global Genome Repair

Yeast GGR factors

Global Genome Repair (GGR) is the NER sub-pathway specifically required for repair of lesions in non-transcribed DNA. In yeast the Rad7-Rad16 complex is essential for GGR; cells deleted for either *RAD7* or *RAD16* are completely NER defective with the exception of repair of the template strand of actively transcribed genes (Tijsterman *et al.*, 1996; Verhage *et al.*, 1994). The role of this GGR specific complex in repair of non-transcribed DNA is still obscure however.

Rad7 contains a leucine rich repeat (LRR), which covers the main part of the protein, and a SOCS box motif which will be discussed later in this chapter (Gillette *et al.*, 2006; Perozzi and Prakash, 1986). The Rad7 protein does possess DNA binding activity, but has no preference for damaged DNA (Guzder *et al.*, 1999). Cloning of the *RAD16* gene and subsequent analysis of the protein (Bang *et al.*, 1992) revealed that Rad16 is a member of the Swi2/Snf2 type putative helicases, which are implicated in the ATP dependent local alteration of the DNA-histone interactions (Richmond and Peterson, 1996).

The group of Prakash showed that the Rad7-Rad16 complex specifically binds UV-damaged DNA (Guzder *et al.*, 1998a). ATP hydrolysis is not essential for the DNA binding of Rad7-Rad16, but the substitution of ATP for non-hydrolysable ATP slightly reduces the affinity for damaged DNA. The observation that ATP hydrolysis by Rad7-Rad16 is dependent on the presence of DNA, but inhibited when the DNA is pre-treated with UV, prompted speculation on a model in which the Rad7-Rad16 complex translocates along the DNA until a lesion is encountered (Guzder *et al.*, 1998a). Rad7-Rad16 was reported to interact with Rad14 (Rodriguez *et al.*, 1998) and with the Rad4-Rad23 complex. The latter interaction synergistically enhances the damage specificity of both Rad4-Rad23 and Rad7-Rad16 (Guzder *et al.*, 1999).

It is generally presumed that Rad7-Rad16 functions as damage sensor in the context of chromatin. It is proposed that the DNA dependent ATPase activity of the Rad7-Rad16 complex represents helicase activity that remodels the chromatin, thereby allowing access for the downstream NER factors. Consistent with this model, the Rad7-Rad16 complex is dispensable for *in vitro* reconstituted NER reactions which employ naked DNA as substrate (Guzder *et al.*, 1995b). Although there is yet no evidence that the ATPase activity of Rad16 is directly involved in chromatin remodeling, Rad7 and Rad16 were recently shown to be involved in this process via histone acetylation. In yeast cells, UV irradiation induces acetylation of lys-9 and lys-14 of histone H3 by the histone acetyl transferase Gnc5 which coincides with a global increased accessibility of the chromatin (Yu *et al.*, 2005). This modification was observed in wild-type cells, but also in *rad4* or *rad14* mutants, showing that the core-NER reaction is not required for the acetylation and the associated chromatin remodeling. This particular histone modification appears important for the NER reaction, since repair of CPDs is impaired in *gnc5* mutants (Teng *et al.*, 2002). It was recently demonstrated that the acetylation of histone H3 is strongly reduced in *rad7* or *rad16* mutants (Teng *et al.*, 2008). The link between NER and histone acetylation was studied further in the mating type specific *MFA2* gene, which is active in a-mating type cells and silenced in α-mating type cells. When the level of histone H3 acetylation was brought to constitutively higher levels by deletion of the *TUP1* gene, which is responsible for the

repression of the *MFA2* gene, lesions in *MFA2* and other Tup1 regulated genes could be removed in the absence of the Rad7-Rad16 complex (Teng *et al.*, 2008). These observations fit a model in which Rad7-Rad16 is involved in UV irradiation induced alteration of chromatin, likely enabling the core NER factors to reach lesions that would be unexposed in repressed chromatin. Interestingly, whereas Rad4 and Rad14 are not required to trigger chromatin remodeling at the *MFA2* gene, these proteins are necessary to allow the chromatin to return to its original state (Yu *et al.*, 2005), suggesting that the chromatin is kept in an 'open' conformation until repair has been completed. These are the first studies providing evidence for the actual involvement of the Rad7-Rad16 complex in chromatin remodeling. Nevertheless, chromatin remodeling is not the sole role of Rad7-Rad16, as it is also required for NER of non-transcribed regions that are free of nucleosomes (Lettieri *et al.*, 2008). Conceivably, the affinity of Rad7-Rad16 for damaged DNA may be required in these regions but this putative requirement for damage recognition contradicts the fact that the Rad7-Rad16 complex is redundant for NER *in vitro*.

Rad4 might be involved in the remodeling of chromatin as well. The Rad4-Rad23 complex transiently associates with Snf6 and Snf5, two subunits of the Swi2/Snf2 chromatin remodeling complex (Gong *et al.*, 2006). The interaction is stimulated by UV irradiation and strains deleted for *SNF6* show ~50% reduced repair of the silent *HML* locus, indicating that Snf6 is involved in GGR. Moreover, UV induced chromatin remodeling of the *HML* locus was reduced ~5 fold. No evidence for an interaction between Snf6 and the GGR factor Rad16 could be obtained, suggesting that the Snf5/Snf6 dependent chromatin remodeling operates independently from the Rad7-Rad16 complex. Nevertheless, the Snf5/Snf6 proteins are not fully required for, and committed to, GGR, as compared to *rad16* cells *snf6* mutants exhibit only mild UV sensitivity that is not epistatic with the *rad16* deletion, suggesting that the Snf6 is also involved in TCR.

Some reports link the role of the Rad7-Rad16 complex to later steps of the NER reaction. Rad16 was shown to alter the superhelical density of DNA *in vitro* (Yu *et al.*, 2004). The generation of superhelicity is enhanced in the presence of Rad7 and Abf1 (a protein previously shown to bind the Rad7-Rad16 complex (Reed *et al.*, 1999)) and dependent on hydrolysable ATP. This activity was found to stimulate the excision of the damaged oligonucleotide. This result led the authors to suggest that the role of Rad7-Rad16 is to actively excise the damaged oligonucleotide after incisions have taken place (Yu *et al.*, 2004). There is however no explanation why Rad7 and Rad16 are not needed to excise the oligonucleotide in the TCR pathway. Another confusing aspect of the proposed post-incision role of Rad7-Rad16 is that pyrimidine hydrates, which represent a small fraction of the total of UV induced lesions, are repaired by NER but independently of the Rad7-Rad16 complex. Although it cannot be excluded that oligonucleotides containing this specific lesion somehow do not require the superhelical torsion generated by Rad7-Rad16 to be removed from the post-incision complex, it seems more probable that a difference in damage recognition, or other pre-incision events, explains why Rad7-Rad16 are not required for NER of this type of lesion. Even though the report on NER of pyrimidine hydrates is from the same authors that proposed the post-excision model, the issue of Rad7-Rad16 independent repair of these lesions is not discussed by Yu *et al.* (Yu *et al.*, 2004). *In sum,* a post-incision role of the Rad7-Rad16 complex remains controversial.

Human GGR factors

The factors specifically involved in the GGR pathway differ between human and yeast cells. A main difference is that Rad4 in yeast is essential for all NER; both GGR and TCR sub-pathways are completely disabled in *rad4* cells. The human homologue XPC however is specifically involved in GGR and not needed for TCR. But in analogy with the yeast system, XPC is not enough to perform GGR and an additional factor, termed UV-DDB (UV Damaged DNA Binding protein), is required. UV-DDB is a heterodimeric complex consisting of the 127kDa DDB1 protein and the 48kDa DDB2 protein, which both share no homology with the yeast GGR proteins Rad7 or Rad16. Via the DDB2 subunit UV-DDB preferentially interacts with damaged DNA, binding particularly with a high preference to (6-4)PPs (Treiber *et al.*, 1992) and only modestly to CPDs (Fujiwara *et al.*, 1999; Reardon *et al.*, 1993). UV-DDB localizes on chromatin in response to UV irradiation (Otrin *et al.*, 1997), independently from both XPA and XPC (Wakasugi *et al.*, 2002), indicating that UV-DDB rather than XPC is the initiator of the GGR pathway.

The cooperation of XPC and UV-DDB is elucidated in some detail. UV-DDB was found to associate with Cul4A and Roc1 (Li *et al.*, 2006a; Shiyanov *et al.*, 1999) , thereby forming a four protein complex that fits the requirements for a Cullin RING ubiquitin ligase, namely: a Cullin (CUL4A), a RING finger protein that facilitates the E3 ligase activity (Roc1), an adapter protein (DDB1) and a substrate binding protein (DDB2). For a review on the architecture of Cullin RING ubiquitin ligases (including the UV-DDB E3 ligase) see Petroski and Deshaies (2005). The UV-DDB complex includes the COP9 signalosome (CSN) and the ubiquitin-like modifier NEDD8. The conjugation of NEDD8 to the Cullin unit is reported to enhance the E3 ligase activity (Read *et al.*, 2000; Wu *et al.*, 2000). CSN is proposed to act as a negative regulator of E3 ligase activity by de-conjugating the NEDD8 from Cul4A, as well as by its de-ubiquitylation activity (Groisman *et al.*, 2003).

In non-irradiated cells the UV-DDB E3 ligase complex is associated with CSN, devoid of NEDD8 and thus presumably in an inactive form. Upon UV irradiation, the UV-DDB E3 ligase is translocated to the chromatin. In this fraction NEDD8 is associated with UV-DDB whereas CSN is not detected, suggesting that the UV-DDB E3 ligase is activated by UV irradiation (Groisman *et al.*, 2003). Interestingly, it was found that UV irradiation triggers the poly-ubiquitylation of both XPC and DDB2 by the UV-DDB E3 ligase complex (Sugasawa *et al.*, 2005; Wang *et al.*, 2005; Wang *et al.*, 2007). The ubiquitylation alters the *in vitro* DNA binding properties of XPC as well as that of UV-DDB; the affinity of UV-DDB for both damaged and undamaged DNA is completely abolished whereas the general DNA binding of XPC is enhanced. However, the specificity of XPC for damaged DNA is not enhanced (Sugasawa *et al.*, 2005). The different fates of the ubiquitylated proteins *in vivo* is even more pronounced, as ubiquitylated DDB2 is targeted for degradation and XPC is not (El-Mahdy *et al.*, 2006; Sugasawa *et al.*, 2005).

The UV-DDB dependent ubiquitylation, combined with the identified interaction between XPC and DDB2 and the earlier reported UV-DDB dependent recruitment of XPC to CPDs (Fitch *et al.*, 2003) and (6-4)PPs (Moser *et al.*, 2005), led to a model in which UV-DDB (via its superior affinity for UV-induced lesions) binds the lesion prior to XPC and subsequently recruits the XPC-hHR23B complex. The joining of XPC-hHR23B

and the UV-DDB E3 complex triggers the ubiquitylation of XPC and DDB2. The inverse effects of ubiquitylation on DNA binding allows XPC to replace UV-DDB at the site of the lesion (Sugasawa *et al.*, 2005; Sugasawa, 2006). Considering the fact that the DNA bound XPC-hHR23B is labeled with ubiquitin moieties only when it is 'loaded' by the more specific UV-DDB factor, it can be speculated that the ubiquitin-tagged XPC-hHR23B is the preferred target of the downstream NER factors as the chance that an actual lesion is present is higher than at sites bound by a unmodified XPC-hHR23B complex. The question that is still unanswered is how UV-DDB distinguishes damaged from undamaged DNA. Detailed structural analysis has not yet been performed, however, using a technique called 'circular permutation analysis' (Wu and Crothers, 1984) it was determined that the binding of UV-DDB to a (6-4)PP or an abasic site induces a bend of 54° or 57° respectively and that the centre of the bend co-localizes with the position of the lesion (Mizukoshi *et al.*, 1999). This finding may indicate that UV-DDB scans for increased local flexibility, a feature that is suggested to be the common determinant of all NER lesions (Isaacs and Spielmann, 2004).

Parallels between yeast and human GGR

The human DDB1 and DDB2 proteins that make up the UV-DDB complex show no structural homology to the yeast Rad7 and Rad16 proteins, which might indicate that the GGR pathways in yeast and humans apply different approaches in order to allow NER of non-transcribed DNA. However, in recent years several similarities between the two GGR systems were identified.

As described above UV-DDB is part of a Cullin-RING ubiquitin ligase. The Cullin based ubiquitin ligases recruit the target protein using a substrate specificity protein that is linked to the amino-terminal domain of the Cullin via an adapter protein. The substrate specificity protein is characterized by the presence of a F-box or SOCS box motif to bind the adapter protein (Kile *et al.*, 2002), and by a substrate-interaction-motif, such as WD-40 repeats or LRR domains, required to recruit the substrate. The carboxy terminal domain of the Cullin interacts with the RING finger protein which is the catalytic core of the E3 ligase.

Interestingly, the yeast GGR protein Rad7 was reported to bind Elc1 in a large scale interaction screen (Ho *et al.*, 2002). Elc1 is the yeast homologue of Elongin C, a commonly used adapter protein in the Cullin-RING ubiquitin ligases (Petroski and Deshaies, 2005). Rad7 contains a Leucine Rich Repeat (LRR) that might be used as a substrate recruiting motif. Further sequence analysis of the Rad7 protein also revealed the presence of a SOCS box. Cul3 was found to co-precipitate with the Rad7-Rad16 complex (Gillette *et al.*, 2006). Since Rad16 contains a RING finger, all elements of a Cullin-RING ubiquitin ligase are at hand in the yeast GGR complex. Indeed, a Cul3-Elc1-Rad7-Rad16 could be isolated and was shown to possess E3 ligase activity *in vitro,* using Rad4 as a substrate (Gillette *et al.*, 2006). The authors also showed UV induced mono-ubiquitylation of Rad4 *in vivo* that is dependent on the Rad7 protein. These findings are in clear analogy with the situation in human cells, where UV-DDB is shown to ubiquitylate XPC in response to UV irradiation (Sugasawa *et al.*, 2005). However, whereas in human cells XPC is polyubiquitylated, the Rad4 modification shown by Gillette *et al.* is monoubiquitylation. In addition, the biological relevance of the ubiquitylation of Rad4 (and other possible targets) by the Cul3-Elc1-Rad7-Rad16

complex for the NER system is doubtful since mutation of the SOCS box of Rad7 abolishes the E3 ligase activity but does not confer UV sensitivity (Gillette *et al.*, 2006).

Nevertheless, the data above show that yeast and human GGR factors share some functional homology. In this light it is remarkable that the proteins that are specifically involved in GGR are not conserved. Why is the Rad7-Rad16 complex not conserved while the substitute of this complex in human cells seems to operate in a similar fashion? The evolution of a different GGR factor in human cells might be related to the vastly larger genome compared to that in yeast. As the human genome consists largely of non-transcribed DNA it is therefore mainly dependent on GGR for repair. Other differences in chromatin structure might also have contributed to the different architecture of the GGR complexes in yeast and human cells.

2.6 Transcription Coupled Repair (TCR)

Transcription Coupled Repair (TCR) is confined to the transcribed strand of actively transcribed genes. NER is coupled to transcription by both RNA pol I and II, but transcription by RNA pol III does not lead to preferential repair (Aboussekhra and Thoma, 1998; Dammann *et al.*, 1993). The majority of DNA is transcribed by RNA pol II and therefore almost all research on TCR has been focused on RNA pol II transcribed regions.

It is generally assumed that the basis of TCR lies in the blockage of RNA pol II (and probably also RNA pol I) once it runs into a damaged nucleotide (Mei Kwei *et al.*, 2004; Tornaletti *et al.*, 1999; Tornaletti, 2005). In this model RNA pol is considered to function as a damage sensor for the NER system. Additional TCR specific factors are implicated in the recruitment of the core-NER proteins to the site of the arrested RNA pol. Several studies hinted towards a physical coupling between RNA pol and NER, but the only conclusive evidence for a ‘TCR factor’ dependent recruitment of NER proteins to stalled RNA pol came from recent chromatin immuno-precipitation experiments (Fousteri *et al.*, 2006). This study shows that the human TCR factor CSB is required for the co-immuno-precipitation of NER proteins with UV-stalled transcription elongation complexes, strongly suggesting that NER proteins are recruited by CSB to the site of stalled RNA polymerase.

It is unclear if, and how, TCR would distinguish between damage-arrested RNA pol complexes and transcription that is blocked by natural occurring hindrances. Nevertheless, it is well described that transcription is arrested on NER substrates (Tornaletti, 2005). Solved structures of RNA pol II stalled on several different CPD containing DNA fragments revealed that the CPD enters the active site of RNA pol II (Brueckner *et al.*, 2007). A uridine is then misincorporated opposite to the 5’-thymine of the CPD. Interestingly, blockage of RNA pol II is not observed when the uridine is artificially replaced by adenosine, showing that the misincorporated uridine is the cause of the stalled RNA pol (Brueckner *et al.*, 2007).

There is no clear model that explains the subsequent steps that lead to the removal of the lesion. Also the fate of the RNA pol after or during eukaryotic TCR is, despite extensive research, still not clear. The prokaryotic TCR mechanism is unraveled in considerable detail and in *E. coli* the blocked RNA pol is shown to be dissociated from the DNA (Deaconescu *et al.*, 2007; Roberts and Park, 2004). RNA pol may also be dis-

placed in eukaryotic TCR, however, since the genes are longer and transcription is slower (up to 16 hours for the largest human gene, dystrophin (Tennyson *et al.*, 1995)) abortion of the transcription seems not a favorable event. Results from *in vitro* experiments suggested that TFIIS dependent transcript cleavage might permit transcription to resume after blockage by, and subsequent removal of, an obstructing CPD or cisplatin lesion (Donahue *et al.*, 1994; Tornaletti *et al.*, 2003). In addition, immuno-precipitation experiments show that TFIIS is specifically enriched in the chromatin fraction of UV irradiated cells (Fousteri *et al.*, 2006). However, yeast cells lacking functional TFIIS are not impaired in TCR (Verhage *et al.*, 1997). Possibly TFIIS dependent pausing of transcription occurs only in higher eukaryotes. In human cells resumption of transcription is expected to be more important than in yeast, since yeast cells contain generally shorter transcripts.

Human TCR factors

In the human system the CSA and CSB proteins are essential for the TCR pathway (Troelstra *et al.*, 1992). Cells deprived of CSB lack preferential repair of the transcribed DNA. The CSB protein is a member of the family of Swi2/Snf2 type putative helicases, but no helicase activity of CSB has been detected (Selby and Sancar, 1997b). However, the CSB protein hydrolyses ATP *in vitro* in the presence of single or double stranded DNA (Selby and Sancar, 1997b) by which it is able to remodel the chromatin structure, a feature that might improve the accessibility of the lesion to other NER factors (Citterio *et al.*, 2000). The DNA conformation may be additionally or alternatively altered by the wrapping of the DNA around the CSB proteins, which was shown to occur in an ATP binding – not hydrolysis – dependent manner (Beerens *et al.*, 2005). CSB binds DNA and interacts with the NER factors TFIIH and XPA (Selby and Sancar, 1997b) and also binds elongating RNA pol II (van Gool *et al.*, 1997), suggesting that CSB recruits the NER machinery to the site of a stalled RNA pol.

Compared to CSB the role of CSA is less well characterized, the *CSA* gene encodes a protein containing WD40 repeats (Henning *et al.*, 1995), which can be involved in various processes such as transcription regulation or signal transduction. WD40 repeats are also used as substrate-interaction motifs in Cullin RING ubiquitin ligases. The underlying common function of all WD-repeat proteins is coordinating the assembly of multi-protein complexes, suggesting that CSA may mediate the interactions between transcription and the NER machinery. Indeed, CSA is reported to be involved in the UV-induced recruitment of the XPA binding protein XAB2, the nucleosomal binding protein HMGN1 and TFIIS (Fousteri *et al.*, 2006). Interestingly, CSA and CSB do not reside in complex but upon DNA damage induction CSA is translocated to the nuclear matrix and co-localizes with elongating RNA pol II (Kamiuchi *et al.*, 2002). The translocation is dependent on CSB, TFIIH, elongative transcription and chromatin structure (Saijo *et al.*, 2007), suggesting that CSB and TFIIH dependent UV induced chromatin alterations at the site of a stalled RNA pol II trigger the translocation of CSA to the nuclear matrix. Interestingly, CSA resides in a Cullin based E3 ligase complex, very similar to the UV-DDB Cullin Ring ubiquitin ligase complex (Groisman *et al.*, 2003). As part of this complex, CSA is required for UV induced ubiquitylation of CSB in the later stages of repair. The ubiquitylated CSB is prone to proteosomal degradation, which stimulates transcription recovery once TCR is completed (Groisman *et al.*, 2006).

Yeast TCR factors

In yeast the main TCR factor is Rad26, the homologue of CSB. Like CSB, Rad26 also displays DNA dependent ATPase activity (Guzder *et al.*, 1996) and interacts with TFIIH (Guzder *et al.*, 1996a; Ho *et al.*, 2002). No functional homologue of CSA has yet been identified in yeast cells. The Rad28 protein, which is similar to CSA in terms of sequence homology, does not contribute to strand specific repair and deletion of *RAD28* does not confer UV sensitivity even in the absence of both GGR and Rad26 (Bhatia *et al.*, 1996).

Yeast cells deleted for the *RAD26* gene are severely defective in preferential repair of the transcribed strand (van Gool *et al.*, 1994; Verhage *et al.*, 1996b). In contrast to CSB cells however, *rad26* cells are not sensitive towards UV irradiation since the impaired removal of lesions from the transcribed strand is masked by the very efficient GGR pathway in yeast. Indeed, in the absence of GGR the *rad26* deletion confers severe UV sensitivity (Verhage *et al.*, 1996b).

Yeast cells lacking the Rad26 protein are not completely TCR defective however (Verhage *et al.*, 1996b). The activity of Rad26 independent TCR varies from gene to gene (Tijsterman *et al.*, 1997; Verhage *et al.*, 1996b), from strain to strain (Gregory and Sweder, 2001) and depends on the carbon source utilized (Bucheli *et al.*, 2001). Even within a gene the requirement of Rad26 for TCR is not uniform. Regions directly downstream from the transcription start site (position +1 to ~ +40) can be repaired via Rad26-independent TCR (Tijsterman *et al.*, 1997). It seems that some forms of transcription are 'TCR competent' whereas other forms do require Rad26 in order to perform NER (Jansen, 2002). The precise reason why certain modes of transcription do or do not require Rad26 for NER is still unknown. Interestingly, TCR is fully functional in cells lacking both Rad26 and the transcription elongation factor Spt4 (Jansen *et al.*, 2000), showing that genetic crippling of elongative transcription leads to a general 'TCR competent' transcription mode and possibly implies that the presence of Spt4 obstructs the NER machinery from reaching the site of the lesion.

Studies from the group of Michael Smerdon demonstrated that the Rad26 independent TCR is dependent on the RNA pol II subunit Rpb9, as *rad16rad26rpb9* triple mutants are completely NER deficient (Li and Smerdon, 2002). The authors proposed that that TCR is comprised of two independent sub-pathways that are regulated by another subunit of RNA pol II, Rpb4. The presence of Rpb4 in RNA pol II channels the TCR pathway through the Rad26 dependent mode, possibly by physically recruiting the Rad26 protein. This would be in agreement with the fact that Rad26 is superfluous for the coupling of repair to transcription by RNA pol I, which does not contain Rpb4. In the absence of Rpb4, TCR is mediated by Rpb9. How Rpb9 facilitates TCR is yet unknown. Rpb9 is required for UV-induced degradation of RNA pol II but, surprisingly, a truncated Rpb9 protein that is deficient in promoting RNA pol II degradation is still proficient in TCR (Li *et al.*, 2006b).

In human cells the TCR factors CSA and CSB are, like Rad26, also not essential for all TCR, as preferential repair of the transcribed strand is fully efficient around the transcription initiation site in CSA or CSB cells (Tu *et al.*, 1997, , 1998). It has not yet been tested whether the human homologue of Rpb9, which is functionally interchangeable with yeast Rpb9 with regard to their role in transcription (McKune *et al.*, 1995), is responsible for the CSA/CSB independent repair. However, since the tenth

subunit of TFIIH, Tfb5 (p8/TTD-A in humans) is, apart from essential for GGR, specifically required for Rpb9 mediated TCR (Li *et al.*, 2007), it is interesting to investigate whether in human cells p8/TTD-A is involved in CSA/CSB independent TCR.

Requirement of Rad4-Rad23/XPC-hHR23B in TCR

In human cells TCR involves RNA pol II and the TCR factors CSA and CSB, but XPC is dispensable for this process. This indicates that RNA pol II, in cooperation with CSA and CSB, substitute for the initial damage recognition role XPC has in GGR. In contrast however, TCR in yeast cells is dependent on the Rad4 protein. Thus although sequence comparison and biochemical data show that XPC and Rad4 are homologues, the activity of XPC is not required in TCR whereas that of Rad4 is indispensable.

XPC, like Rad4, is essential for *in vitro* reconstituted NER. However, XPC is not required for the removal of a CPD lesion placed at the 3' end of a 10nt stretch of non-complementary bases, a construct suggested to mimic a transcription bubble structure at a stalled RNA pol (Mu and Sancar, 1997). Possibly, this NER activity might constitute the basis for XPC independent TCR in human cells. However, this substrate has not been tested in a yeast NER system that lacks Rad4. It thus remains unclear whether yeast and human NER factors operating downstream of Rad4/XPC differ in their ability to recognize a substrate presented by a stalled RNA pol II, or whether the difference between the requirement of Rad4/XPC is determined by a distinctive structure of the stalled RNA pol in yeast and humans. It may be expected that the answer to the long standing question what determines the difference in the requirement of XPC or Rad4 in TCR will greatly contribute to our knowledge on the TCR pathway as well as on the role of Rad4 and XPC in NER.

2.7 Genetic disorders associated with defects in NER

Three different human genetic disorders are associated with inherited defects in the NER system: Xerderma Pigmentosum (XP), Cockayne Syndrome (CS) and Trichothiodystrophy (TTD). These diseases underscore the biological relevance of the pathway. Xeroderma Pigmentosum (XP) patients bear a mutation in one of the XP genes and suffer from extreme UV sensitivity, a dry, parchment-like skin and multiple skin cancers. Severe cases also include neurological abnormalities (Lehmann, 2003; Zghal *et al.*, 2005). Seven different complementation groups were identified (XP-A to XP-G). Cell lines derived from XP-C and XP-E patients are completely and partially defective in the GGR sub-pathway respectively whereas the other complementation groups exhibit a complete NER defect. The partial GGR defect probably explains the relatively mild clinical phenotype of XP-E patients. XP-C patients exhibit more classical XP features, but do not have neurological problems.

Cockayne Syndrome (**CS**) is the result of a TCR defect due to a mutation in one of the CS genes, CSA or CSB. Although most XP groups lack both GGR and TCR, the clinical features of CS are more severe and markedly different from those of XP. CS patients exhibit developmental defects like physical and mental retardation and a bird-like face, (Nance and Berry, 1992). The fact that patients carrying a mutation in one of the CS genes have clinical defects that are beyond those of XP patients indicates that the CSA and CSB proteins are not only specific TCR factors but are also involved in

transcription. Indeed, CSB appears to be involved in general transcription efficiency as CSB cells exhibit a ~50% reduction in mRNA synthesis compared to *NER*$^{+}$ cell lines (Balajee *et al.*, 1997) and addition of the CSB protein to an *in vitro* transcription reaction leads to a ~3 fold increase in mRNA synthesis (Selby and Sancar, 1997a). Although CS patients are sensitive towards sunlight they exhibit no predisposition to skin cancer, which might be explained by the functional GGR pathway in these cells or, alternatively, by the early age of death of CS patients (~12 years).

Patients with the disorder Trichothiodystrophy (**TTD**) exhibit clinical features similar to those of CS patients with the addition of brittle hair and nails and ichthyosis (Bergmann and Egly, 2001; de Boer *et al.*, 1998; Itin *et al.*, 2001). Most photosensitive patients carry a mutation in the XPD (yeast *RAD3*) gene whereas only a small number of patients are mutated in the *XPB (*yeast *RAD25*) (Weeda *et al.*, 1997) or the *TFB5* gene (Giglia-Mari *et al.*, 2004). These genes all encode subunits of the TFIIH complex and indeed TTD is accompied by a reduced level of TFIIH (Vermeulen *et al.*, 2000). Like for CS, the clinical phenotype of TTD patients therefore is likely linked to defects in both repair and transcription.

Chapter 3

Damage recognition by NER

Damage recognition by NER

Damage recognition by NER

Obviously, a crucial step in NER is the decision where in the genome it is appropriate to incise the DNA. Although the damage recognition step is extensively studied it is still unknown how NER is able to make this decision and how it detects the damaged nucleotides in the genome. Damage recognition involves several aspects. The basis of recognition is to distinguish a damaged nucleotide from the undamaged ones. Enzymes involved in this step must somehow be able to sense certain features of the DNA that reveal whether it is damaged or not. This is especially a puzzling ability since NER is able to recognize a large number of chemically and structurally unrelated lesions. Apart from this feature, damage recognition entails additional aspects. Before a specific DNA fragment is examined by the NER damage sensors, the location of the lesion must be determined; enzymes have to search the genome for damaged nucleotides. How the NER factors search the genome for lesions is currently not known. The recognition factors may be constantly binding and dissociating, or may scan along the DNA until an injury is detected. Furthermore, the NER damage sensors may continuously probe for lesions, or alternatively, the search might only be started in the case lesions are present. How damage recognition is regulated is not known in detail, but various regulating mechanisms appear to be present that might activate, or increase the activity of, NER proteins once the presence of DNA damage is detected. Of these three different phases of recognition the final step, the actual detection of a lesion, is decisive for the incision. The topic of this chapter is the mechanism by which the GGR sub-pathway is able to determine the presence of a lesion in the DNA.

An important challenge faced by the NER damage recognition factors is the relative tiny number of lesions compared to the amount of undamaged DNA. Amidst the more than 12 million base pairs in yeast, or an overwhelming 3.3 billion base pairs in humans, NER must be able to discriminate a damaged from an undamaged base. Several NER proteins exhibit specific affinity for DNA lesions, but the preference for damaged DNA over undamaged DNA of these factors is typically only around a 1000 fold. None of the proposed damage sensors possess the extraordinary specificity required to accomplish lesion-detection in the context of the genome. In fact, it is hard to imagine that such a protein exists at all. This suggests that NER factors must cooperate to accomplish efficient recognition of lesions. One possibility is that a pre-assembled damage sensor, consisting of multiple NER proteins with affinity for damaged DNA, provides higher specificity. However, such a complex could only possess enhanced specificity when its individual components would recognize different aspects of the damaged DNA. It is doubtfull whether such complexes exist, as analysis of the diffusion rate of NER proteins indicates that the NER damage recognition proteins operate separately before engaging the DNA (Houtsmuller *et al.*, 1999; Rademakers *et al.*, 2003).

Alternatively, the incision reaction may only be initiated when two or more independently probing damage sensors are bound to the same DNA region. In this case, the chance that the bound region actually does contain a lesion will be synergistically higher compared to DNA bound by a single factor. However, as NER factors operate in a predetermined order (see 2.2) it seems more likely that the factor binding initially will recruit proteins that verify whether the bound DNA actually contains a lesion. When this double check method will be applied, the first encounter with a lesion is still depend-

ent on the search by the first factor. Considering the low specificity of the single NER factors, this factor should be present in very large quantities and verification by additional factor(s) should occur fast to achieve efficient recognition. The NER damage sensor(s) may also apply a different approach. A NER factor may 'slide' along the DNA in search for damaged nucleotides, as recently demonstrated for damage binding factors of the mismatch repair system (Gorman *et al.*, 2007) and previously for photolyase (van Noort *et al.*, 1998). In theory this can be an efficient method to locate the lesion, searching the DNA in a more systematic manner rather than random binding and dissociation. Yet, such a scanning mechanism will almost certainly require the remodeling of the chromatin structure. Indeed, chromatin remodeling is thought to be an important part of GGR, but it has yet to be explained how NER copes with chromatin during the search for DNA lesions.

3.1 The composition of NER substrates

It is hard to imagine that there is a common feature shared by all the different lesions that are removed by NER. It is therefore conceivable that the NER damage recognition factor(s) are able to detect a deviation in one or more of the characteristics of undamaged DNA brought about by the presence of the lesion. All NER substrates invoke changes to the standard Watson-Crick geometry of the DNA (Dip *et al.*, 2004), alterations commonly referred to as 'helix distortion'. The efficiency by which the different NER substrates are repaired via the GGR pathway increases proportionately with the degree of helix distortion imposed by the lesions (Gunz *et al.*, 1996). For example, the deformation of the DNA helix induced by (6-4)PPs is more severe than that caused by CPDs (Kim *et al.*, 1995; McAteer *et al.*, 1998), accordingly, both Rad4 and XPC bind to (6-4)PPs with a strong preference over CPDs (Batty *et al.*, 2000; Guzder *et al.*, 1998b; Kusumoto *et al.*, 2001).

Intra-strand crosslinks caused by *cis*-diamminedichloroplatinum (cisplatin) induce helix distortion in a variable degree, dependent on the type of crosslink (Bellon *et al.*, 1991). Consistent with the notion described above, a 1,3 GTG cisplatin crosslink is removed more efficiently than the less helix distorting 1,2-GG and 1,2-AG variants (Moggs *et al.*, 1997). Furthermore, the positioning of one or two non-complementary bases opposite a 1,2-GG-cisplatin crosslink or a CPD also improves the repair of these lesions (Moggs *et al.*, 1997; Mu *et al.*, 1997a; Sugasawa *et al.*, 2001). The observations above suggest that deviation from the Watson-Crick geometry is the determining factor that allows recognition of the various lesions by the NER damage recognition proteins. Indeed, lesions that do not perturb the DNA helix, like C4' backbone modifications, are not detected by NER (Hess *et al.*, 1997b). Since mismatches and small DNA loops however are extremely poor NER substrates it seems that disturbances of the DNA helix alone are also not sufficient to meet the criteria of NER recognition (Hess *et al.*, 1997a; Hess *et al.*, 1997b; Moggs *et al.*, 1997; Mu *et al.*, 1997a). Work from the group of Hanspeter Naegeli demonstrated that sites exhibiting disturbed base pairing are only repaired in the presence of a modified nucleotide (Hess *et al.*, 1997b), even when these two features are positioned 15 nucleotides apart (Buschta-Hedayat *et al.*, 1999). These observations show that NER recognizes two aspects of damaged DNA, and may possibly indicate that the recognition of these two features occurs by

separate subunits of the NER machinery.

In later experiments from the same group the excision of a non-distorting pivaloyl adduct was monitored strand specifically. By insertion of additional nucleotides the effect of a one-sided DNA bulge on the incision reaction was examined (Buterin *et al.*, 2005). In agreement with earlier results, a pivaloyl adduct that does not interfere with normal base pairing was only recognized by the NER machinery in the presence of the DNA bulge in the undamaged strand (Buterin *et al.*, 2005). Interestingly, the DNA was not incised when this bulge was located in the same strand as the adduct. Moreover, DNA fragments in which the inserted nucleotides in the opposite strand also contained a pivaloyl adduct were also not incised (Buterin *et al.*, 2005), suggesting that NER senses DNA damage via deformations in the undamaged strand of the damaged DNA. Consistent with this assumption, XPC-hHR23B binding to photoreactive damages was inhibited when a modified base was positioned in the opposite strand (Maltseva *et al.*, 2008) and the affinity of XPC for UV treated ssDNA is lower than that for undamaged ssDNA (Maillard *et al.*, 2007a).

The data above show that NER senses DNA that exhibits helical distortion and contains a chemically modified nucleotide. Of these two, helical distortion appears the more conspicuous feature and hence a better target for the initial search for DNA damage. Indeed, binding of the initiator of NER, Rad4-Rad23/XPC-hHR23B, is not dependent on the presence of a chemical modification (Sugasawa *et al.*, 2001), suggesting that NER initially recognizes helical distortion. As mentioned earlier, helix distortion refers to the deviation from standard, undamaged DNA. But what constitutes the deviation recognized by NER? One model assumes that thermodynamic destabilization of the damaged helix facilitates binding of the NER damage sensors (Geacintov *et al.*, 2002; Gunz *et al.*, 1996). Thermodynamic destabilization is associated with the lowering of the melting temperature of damaged DNA compared to that of undamaged molecules. The presence of certain lesions however, such as psoralen crosslinks, increase the thermodynamic stability of the DNA rather than destabilize it, and still are recognized by NER (Shi and Hearst, 1986; Thoma *et al.*, 2005). Moreover, a perfectly normal TAT/ATA trimer is thermodynamically even less stable than a GGC/CGG mismatch. Therefore it seems that thermodynamic destabilization alone can not explain how NER initially identifies damages within the DNA. In view of this, Isaac and Spielmann (2004) proposed an alternative model, in which an increase in local flexibility in damaged DNA is a determining factor in damage recognition. This model is based on the observation that conformational alterations in the DNA caused by covalent modifications decrease the energy required to bend the DNA. It was proposed that the NER damage sensors search for DNA that exhibit increased flexibility. While probing the DNA for lesions, the NER damage sensors will attempt to force the DNA into a deformed conformation. The energy required to induce bending of the damaged DNA must be sufficiently small to (temporarily) trap the sensor protein as it scans the DNA (Isaacs and Spielmann, 2004). The observation that binding of XPC-hHR23B induces a strong bend in the DNA, which is fixed at the position of a lesion supports this hypothesis (Janicijevic *et al.*, 2003).

An alternative, though not intrinsically different, view on damage recognition arose from mathematical analysis of the double helix (Blagoev *et al.*, 2006; Maillard *et al.*, 2007a). Mathematical models describing the dynamics of double stranded DNA show

that thermal fluctuations constantly cause the DNA strands to oscillate with respect to each other, creating short lived bubble structures (Alexandrov *et al.*, 2006). In undamaged DNA the oscillations are assumed to be too fast (on the pico to nanosecond scale) to allow detection by the NER machinery. However, even the presence of a relatively non-distorting CPD dimer leads to a 3 fold increase of the average distance between the two strands and 25 times increased occurrence of longer lived, larger bubble structures (Blagoev *et al.*, 2006). This model postulates that NER detects the single stranded nature of the DNA in the close vicinity of the lesion. An important aspect of this model is that the oscillations are most pronounced in the undamaged strand, which is in concord which several damage binding features of Rad4-Rad23/XPC-hHR23B (Maillard *et al.*, 2007a; Maltseva *et al.*, 2008; Min and Pavletich, 2007).

Summarizing, the exact nature of the 'helix distortion' that is required to catch the attention of the NER machinery is not known, but it is clear that NER recognizes DNA that deviates from standard B-DNA. It seems that the NER damage sensors force the DNA into a different conformation, a transaction that is only possible, or more stable, in the presence of DNA damage. This identification of an aberration in the structure of the DNA helix might also represent a method by which NER searches the genome. A NER factor may scan along the DNA, searching for regions that are susceptible to the conformational change it is trying to inflict. Once the DNA can be forced into a certain changed conformation, the NER factor traps itself, thereby forming a signal for downstream NER factors to further inspect the bend region. The receptiveness of the DNA to this transaction signifies that a lesion may be present, but does not yet confirm the presence or precise location of a chemically modified nucleotide. It is likely that the initial damage sensor, stably in complex with the conformationally changed DNA, is bound by subsequent NER factors that verify the presence of an adducted nucleotide.

3.2 The prokaryotic damage recognition model

To get a better understanding of eukaryotic damage recognition the mechanism in the prokaryotic system, which is elucidated in considerably detail, is discussed here. The number of proteins involved in the NER reaction in prokaryotes is limited; just three proteins are required for the basic incision reaction whereas eukaryotic NER employs at least 16 proteins. The three prokaryotic players UvrA, UvrB and UvrC (collectively known as the UvrABC system) can nevertheless cope with a similar diversity of substrates and the lesions are removed in a similar fashion.

Two of the three proteins required for the incision reaction are involved in damage recognition, while the third, UvrC, is required for the incision. Damage recognition is not separated from 'downstream' NER events but is in fact intertwined with the construction of the pre-incision complex. In line with the 'bipartite recognition model' (Hess *et al.*, 1997a) two steps can be discerned in prokaryotic damage recognition, initial detection of helical distortion (by $UvrA_2$ and UvrB) and subsequent recognition of the base modification (by UvrB). For detailed reviews on prokaryotic NER see Truglio *et al.* (2006a) and Van Houten *et al.* (2005). A summary of the damage recognition mechanism is described below.

UvrA and UvrB reside in a $UvrA_2UvrB_2$ complex (Malta *et al.*, 2007). The initial contact with (damaged) DNA is made by the $UvrA_2$ subunit, which exhibits roughly

1000 fold preference for binding to damaged DNA over undamaged DNA (Seeberg and Steinum, 1982). The affinity of $UvrA_2$ for (damaged) DNA appears to be largely based on electrostatic interactions (Pakotiprapha *et al.*, 2008) and the $UvrA_2$ subunit is therefore expected to bind helical distorted DNA in general and to be insufficient for determining the true nature of the deformed DNA region.

The decision whether to abort or continue the NER reaction is made by UvrB, the protein that can be considered to be the central damage recognition factor of the UvrABC system. The UvrB protein contains several domains required for interactions with $UvrA_2$ and UvrC, but the key features in UvrB that enable damage recognition are a ß-hairpin and six helicase motifs that are dispersed throughout the protein. The crystal structure confirms that UvrB meets the requirements of a *bona fide* helicase, suggesting that UvrB functions in the separation of the DNA strands, similar to the role of TFIIH in eukaryotic NER.

After the $UvrA_2$-DNA interaction positioned the $UvrA_2$-$UvrB_2$ complex at a potential lesion, the DNA is transferred from the UvrA dimer to $UvrB_2$, initiating the actual recognition of the lesion. Once the DNA is bound by UvrB, the ß-hairpin is inserted in between the DNA strands, clamping one of the two strand behind the ß-hairpin. Several aromatic residues at the base of the ß-hairpin interact with the DNA via hydrophobic interactions. Two specific residues (Tyr92 and Tyr93) are thought to force the base out of the helix, a mechanism referred to as 'base flipping' (Malta *et al.*, 2006; Moolenaar *et al.*, 2001). The insertion of the ß-hairpin and the subsequent flipping of bases is presumed to be only possible when base stacking interactions are loosened, a property that is shared by all the lesions repaired by NER (Van Houten and Snowden, 1993).

Based on the crystal structure of UvrB bound to a ssDNA loop it was suggested that, once one of the strands is clamped behind the ß-hairpin, ATPase driven 3' > 5' translocation of a few nucleotides facilitates a mechanism to pinpoint the precise location of the lesion (Truglio *et al.*, 2006b). During the translocation the nucleotides are proposed to be flipped out one by one into a hydrophobic pocket of UvrB. When a damaged nucleotide is encountered, the translocation will be arrested as it will not fit in the hydrophobic pocket (Truglio *et al.*, 2006b). In this model, the damaged base will always be located directly 5' of the flipped-out nucleotide. These results are supported by the notion of Malta *et al.* that flipping of the base 3' adjacent to the lesion may be the general mechanism for damage recognition (Malta *et al.*, 2006).

Summarizing, in prokaryotic NER high specificity is achieved by combining a general scanning for sites that display helical distortion followed by a more detailed detection of the damaged nucleotide. The search for DNA exhibiting helical distortion is initially performed by UvrA but also involves UvrB, which uses its ß-hairpin to detect regions of disturbed basepairing or basestacking. In both the $UvrA_2$-UvrB-DNA and UvrB-DNA complexes the DNA is wrapped around the UvrB protein, causing a sharp kink in the DNA (Shi *et al.*, 1992; Verhoeven *et al.*, 2001). In line with the general mechanism to probe for helix distortion suggested in the previous paragraph, the $UvrA_2$-$UvrB_2$ complex may probe for DNA regions that are stable in this forced conformation. However, it appears more likely that the wrapping of DNA facilitates the possibility to place the ß-hairpin in between the strands when destabilized DNA is encountered. Successful insertion will trap the complex and trigger ATP driven translo-

cation to localize the damaged nucleotide. UvrB can only examine one strand and at this stage UvrB does not 'know' which strand contains the lesion. However, the presence of two UvrB molecules in the $UvrA_2$-$UvrB_2$ complex might enable the inspection of both strands of the DNA; when no damage is detected in the first search, the DNA might be transferred to the second UvrB protein to inspect the other strand (Verhoeven *et al.*, 2002).

3.3 Damage recognition in eukaryotic NER

Sensing helical distortion

Assuming that the principle of damage recognition in eukaryotes is comparable with that in the prokaryotic system, a two step mechanism will also be applied in eukaryotes. In the core-NER reaction Rad4-Rad23/XPC-hHR23B is the initial damage recognition factor and hence the primary candidate to function as a sensor for helical aberrations. Rad4/XPC indeed displays a general affinity for distorted DNA. XPC-hHR23B binds mismatch bubble structures regardless of the presence of a modified base (Sugasawa *et al.*, 2001). The crystal structure of CPD-bound Rad4 (discussed in section 2.3) implicates that Rad4 has affinity for DNA that is destabilized such that it allows insertion of the Rad4 ß-hairpin in between the strands of the DNA. In addition, a predicted conformational change of the DNA bound Rad4 is postulated to introduce a kink in the DNA (Min and Pavletich, 2007). Binding of XPC-hHR23B is observed to kink DNA fragments regardless of the presence of a lesion. When the DNA fragment contains a lesion, XPC-hHR23B is fixed at the site of the injury (Janicijevic *et al.*, 2003). Similar to what has been suggested above for $UvrA_2$-$UvrB_2$, the kinking of the DNA might be required to allow inspection of basepairing by the ß-hairpin. Possibly, Rad4-Rad23/XPC-hHR23B may actively scan along the DNA for regions that are susceptible to insertion of the ß-hairpin. The domain in Rad4 that binds undamaged DNA adjacent to the lesion (Min and Pavletich, 2007) might function to anchor the DNA while attempting to place the hairpin between the strands of the DNA. The crystal structure shows no direct contact between Rad4 and the damaged nucleotides (Min and Pavletich, 2007), indicating that a chemical modification is not required in order to facilitate the Rad4-DNA interaction. This strongly suggests that Rad4-Rad23/XPC-hHR23B recognizes the consequences of the presence of the lesion and that the lesion itself has to be detected by a different factor.

Recognition of the damaged nucleotide

In prokaryotes, ATP driven helicase activity of UvrB confirms the presence and the precise location of the damaged nucleotide, utilizing the ß-hairpin to flip out nucleotides one by one, until it arrests when the damaged nucleotide is encountered (see 3.2). In eukaryotes a similar mechanism may be applied. Here, a ß-hairpin is already inserted through the DNA by Rad4. Rad4 lacks helicase or ATPase activity and can therefore not employ an UvrB-like damage-localization mechanism by itself. Yet, a second NER factor might translocate the DNA while the Rad4 ß-hairpin is kept in place in between the DNA strands. The only core-NER factor possessing helicase activity is TFIIH, which is indeed the first factor that is recruited after Rad4-Rad23/XPC-hHR23B binding (Volker *et al.*, 2001; Yokoi *et al.*, 2000). Rad4-Rad23/XPC-hHR23B and TFIIH could

cooperate to locate the damaged nucleotide, and consequently confirm that the DNA bound by Rad4/XPC is actually damaged. As Rad4 binds the undamaged strand and the flipped out thymine dimer is exposed towards the solvent (Min and Pavletich, 2007), it must be assumed that TFIIH not only provides the helicase activity, but also the equivalent of the UvrB hydrophobic pocket that arrests the translocation once a damaged nucleotide will not fit into the pocket. Very recently, analysis of the crystal structure of an archeal XPD protein (a helicase subunit of TFIIH) showed the presence of a narrow pocket that could hold non-adducted bases but would reject damaged substrates, prompting the suggestion that this pocket may enable XPD to verify the presence of damaged nucleotides (Wolski *et al.*, 2008).

Alternatively, the TFIIH helicase activity may be involved in the verification of the lesion separately from base flipping by the Rad4/XPC *ß*-hairpin. Since the Rad3 helicase activity is inhibited in the presence of DNA damage, it has previously been proposed that Rad3/XPD will arrest when processing a damaged nucleotide (Naegeli *et al.*, 1992, , 1993a) and that by this feature TFIIH might determine the presence and location of a chemical modification to the nucleotide (Wood, 1999).

Rad3/XPD will inspect one strand of the potentially damaged DNA, but it is not clear how NER proceeds when no lesion is identified in the examined strand. Will the complex disassemble or does TFIIH somehow check the second strand? Possibly, initial binding of Rad4/XPC already notifies in which strand the lesion is present. Although Rad4 also binds DNA regions that do not contain a damaged nucleotide, when a lesion *is* present, binding of Rad4 may confer strand specificity. The Rad4-CPD crystal structure shows that the CPD is approached from the side of the undamaged strand and in fact predicts that Rad4 will be unable to approach the DNA from the strand that contains the CPD (Min and Pavletich, 2007). In concord, It has been shown that binding of XPC-hHR23B to ssDNA is inhibited by the presence of DNA damage (Maillard *et al.*, 2007b; Trego and Turchi, 2006) and experiments by Maltseva *et al.* (2008) show that XPC-hHR23B requires an undamaged strand opposite the adducted strand in order to bind. These results strongly suggest that Rad4/XPC recognizes deviations in the undamaged strand of damaged DNA, in support of the model postulating that the presence of a NER substrate leads to an increase in oscillation of primarily the undamaged DNA strand (Blagoev *et al.*, 2006; Maillard *et al.*, 2007a).

In eukaryotes an additional protein, Rad14/XPA, is implicated in damage recognition. The role of Rad14/XPA is not clarified, but since the protein acts after TFIIH and preferentially binds to damaged DNA *in vitro* it was assumed that Rad14/XPA functions as a damage verification factor. Nevertheless, Rad14/XPA does not appear to examine the damaged DNA in more detail than Rad4/XPC does in the initial probing. A detailed examination of the DNA binding characteristics of XPA demonstrated that the affinity of XPA for damaged DNA is entirely based on the presence of deformations in the DNA helix and does not require any chemical modification of nucleotides (Missura *et al.*, 2001). Also the binding of the XPA-RPA complex, which was reported to possess superior damage specificity compared to XPA or RPA alone, is dependent on helix distortion only (Missura *et al.*, 2001). Based on the affinity of XPA for certain DNA structures the authors concluded that rigid bending of the deoxyribose-phosphate backbone is the predominant factor that determines the high affinity interaction of XPA with DNA (Missura *et al.*, 2001). These data implicate Rad14/XPA in the recognition

of specific structural features of the developing open complex, not in the direct sensing of the lesion. As the inclusion of XPA in the XPC-hHR23B-TFIIH-DNA complex is reported to stimulate strand separation by TFIIH (Coin *et al.*, 2006; Winkler *et al.*, 2001) it seems that lesion-verification by TFIIH is initiated only after recruitment of XPA, implicating the protein in indirect verification of the lesion. In general XPA appears mainly involved in the architecture of the pre-incision complex. The DNA binding properties of Rad14/XPA and its interactions with most of the core-NER proteins likely enables the coordination of the NER complex in relation to the damaged DNA.

The data discussed here strongly suggest that Rad4-Rad23/XPC-hHR23B and TFIIH are the key players in damage sensing in eukaryotes (Figure 3). The heart of the damage recognition is performed by the TFIIH helicase subunit Rad3/XPD. Arrest of helicase activity serves as a signal to proceed with the reaction; when TFIIH is not obstructed it may dissociate from the substrate along with Rad4-Rad23/XPC-hHR23B. The true nature of the helicase-arrest is not essentially relevant for this model of damage recognition; it may require 'active' base-flipping by the *ß*-hairpin (as suggested for prokaryotic NER), but may also be an intrinsic characteristic of the Rad3/XPD (or perhaps all) helicase(s). Importantly, the central enzyme in damage recognition, TFIIH, does not require affinity for damaged DNA, a property that was the basis to implicate certain NER proteins in the damage recognition process.

The principle of damage recognition in prokaryotes and eukaryotes might be comparable since similar tools are applied (the *ß*-hairpin and helicase activity); however, the events that lead to recognition are organized differently in these two systems. In prokaryotes the two recognition proteins (UvrA and UvrB) exist in one complex whereas in eukaryotes Rad4/XPC and TFIIH operate as separate units, although some reports show interaction between Rad4-Rad23/XPC-hHR23B and TFIIH in absence of DNA damage (Drapkin *et al.*, 1994; Mu *et al.*, 1995). In prokaryotes, UvrB is involved both in the detection of helix distortion and in the precise localization of the lesion, i.e., it first inserts its *ß*-hairpin through the DNA strands and then applies helicase activity. Rad4/XPC senses helical disrupted DNA and sets the stage for the localization of the lesion in the process. However, the required ATPase/helicase activity for the damage-verification is provided by the Rad3/XPD subunit of the consequently recruited factor TFIIH.

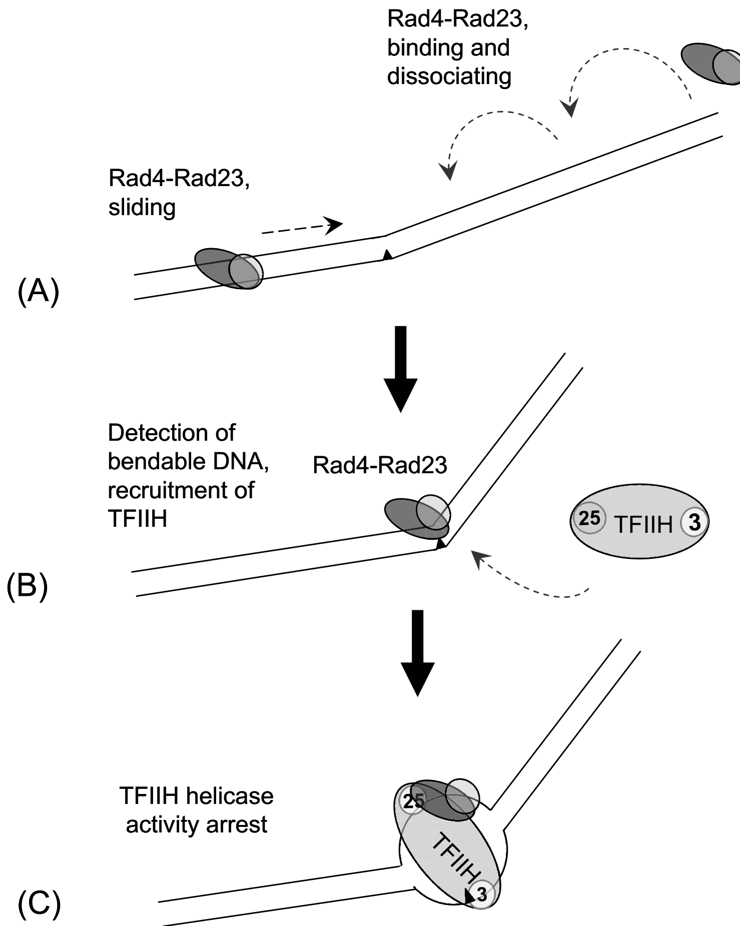

Figure 3
Possible model for damage recognition. (A) Damaged DNA is scanned by Rad4-Rad23, probing for regions that are destabilized such that the Rad4-β-hairpin can be inserted in between the DNA strands and/or DNA that can be forced into a curved conformation. How this scanning is performed is not precisely known. Rad4-Rad23 could slide along the DNA until a lesion is encountered or the Rad4-Rad23 complex may continuously bind and dissociate until a damaged region is encountered. (B) Once Rad4-Rad23 is bound to a DNA region that is bendable and/or susceptible to the insertion of the β-hairpin, Rad4-Rad23 is (temporarily) trapped, allowing the recruitment of TFIIH. (C) The helicase activity of TFIIH separates the two strands. The presence of a modified nucleotide will block the Rad3 helicase, triggering the further formation of the NER pre-incision complex. Absence of a modified nucleotide will lead to disassembly of the DNA-Rad4-Rad23-TFIIH complex.

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Chapter

4

The Rad4 homologue YDR314C is essential for strand-specific repair of RNA polymerase I-transcribed rDNA in *Saccharomyces cerevisiae*

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The Rad4 homologue YDR314C is essential for strand-specific repair of RNA polymerase I-transcribed rDNA in *Saccharomyces cerevisiae*

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Summary

The *Saccharomyces cerevisiae* protein Rad4 is involved in damage recognition in Nucleotide Excision Repair (NER). In RNA polymerase II transcribed regions Rad4 is essential for both NER subpathways Global Genome Repair (GGR) and Transcription Coupled Repair (TCR). In ribosomal DNA (rDNA), however, the RNA polymerase I transcribed strand can be repaired in the absence of Rad4. In *Saccharomyces cerevisiae* the YDR314C protein shows homology to Rad4. The possible involvement of YDR314C in NER was studied by analyzing strand specific CPD removal in both RNA pol I and RNA pol II transcribed genes. Here we show that the Rad4-independent repair of rDNA is dependent on YDR314C. Moreover, in Rad4 proficient cells preferential repair of the transcribed strand of RNA pol I transcribed genes was lost after deletion of *YDR314C,* demonstrating that Rad4 cannot replace YDR314C. CPD removal from the RNA pol II transcribed *RPB2* gene was unaffected in *ydr314c* mutants. We conclude that the two homologous proteins Rad4 and YDR314C are both involved in NER and probably have a similar function, but operate at different loci in the genome and are unable to replace each other.

1 Introduction

Nucleotide Excision Repair (NER) is a DNA repair process capable of recognizing and removing a wide variety of helix distorting lesions, like the UV induced 6-4 photoproducts (6-4PP) and cyclobutane pyrimidine dimers (CPD). After recognition of the damage, a single strand DNA fragment containing the lesion is excised, allowing DNA synthesis using the undamaged strand as a template (de Laat *et al.*, 1999; Prakash and Prakash, 2000). The basic mechanism of NER is present in organisms ranging from *Escherichia coli* to man. The core NER proteins have been identified using an *in vitro* reconstituted system with purified proteins (Guzder *et al.*, 1995; He *et al.*, 1996; Mu *et al.*, 1996). One of the essential components of the NER reaction in S*accharomyces cerevisiae* is the damage recognition protein Rad4. Binding of the Rad4-Rad23 complex to the damaged site initiates the recruitment of the other NER proteins that cooperatively complete the repair of the damaged DNA (Guzder *et al.*, 1998; Jansen *et al.*, 1998).

In vivo, additional proteins are required to facilitate efficient removal of lesions. Extensive studies in various organisms revealed that certain NER proteins are specifically involved in preferential repair of the transcribed strand of transcriptionally active DNA. This process is designated Transcription Coupled Repair (TCR) and, in yeast, requires Rad26, Rpb4 and Rpb9 (van Gool *et al.*, 1994; Li and Smerdon, 2002). Other proteins, like Rad7 and Rad16, are specifically involved in removal of lesions throughout the entire genome, a process referred to as Global Genome Repair (GGR). The core NER proteins, like Rad4, are essential for both GGR and TCR (Bang *et al.*, 1992; Verhage *et al.*, 1994). Previously, however, we showed that Rad4 is not essential for strand specific repair of RNA pol I transcribed rDNA, whereas all other core NER proteins, including Rad23, are indispensable (Verhage *et al.*, 1996a).

In human cells the XPC-hHR23B complex is homologous to the Rad4-Rad23 complex in *Saccharomyces cerevisiae* (Legerski and Peterson, 1992; Masutani *et al.*, 1994). In contrast to *rad4* mutants, cells devoid of XPC are completely defective in repair of RNA pol I transcribed rDNA (Christians and Hanawalt, 1994). Moreover, Rad4 and XPC differ in their contributions to GGR and TCR in RNA pol II transcribed genes. XPC cells are only defective in GGR (Venema *et al.*, 1991) whereas *rad4* cells lack both GGR and TCR (Verhage *et al.*, 1994).

The yet uncharacterized *Saccharomyces cerevisiae* protein YDR314C displays homology with established Rad4 homologues (Anantharaman *et al.*, 2001; Marti *et al.*, 2003). Moreover, analogous to Rad4, YDR314C is reported to co-immunoprecipitate with Rad23 in a large scale interaction study (Gavin *et al.*, 2002). These similarities suggest that the *YDR314C* gene product could be a functional Rad4 homologue.

In the fission yeast *Schizosaccharomyces pombe* two Rad4 sequence homologues were identified as well. Both homologues, designated Rhp41 and Rhp42, have been to shown to be involved in NER (Fukumoto *et al.*, 2002; Marti *et al.*, 2003). Strand specific repair analysis indicated that Rhp42 is involved in GGR whereas Rhp41 has a role in both TCR and GGR (Fukumoto *et al.*, 2002). Epistasis studies confirmed the role of Rhp41 in both NER subpathways (Marti *et al.*, 2003). However, deletion of *rhp42*$^{+}$ in cells lacking GGR due to a mutation in the *rhp7* gene, resulted in increased UV sensitivity, whereas deletion of *rhp42*$^{+}$ in TCR deficient *rhp26* mutants did not,

suggesting that Rhp42 is involved in TCR rather than GGR. On the other hand, transcription recovery, indicative for the efficiency of repair in transcribed DNA, was affected in *rhp41* cells but not in *rhp42* cells, contradicting the results from the epistasis analysis. Rhp41 and Rhp42 are apparently both involved in NER, but their relative contribution to GGR and TCR is not yet clear.

In *Saccharomyces cerevisiae* no function has yet been assigned to the *YDR314C* gene product. In this paper the involvement of YDR314C in NER is described. We show that YDR314C cannot substitute for Rad4 in RNA pol II transcribed regions but is essential for preferential repair of RNA pol I transcribed rDNA.

2 Results

A Rad4 homologue in Saccharomyces cerevisiae

Recently, an open reading frame in *Saccharomyces cerevisiae* was identified that shows substantial resemblance to Rad4 (Anantharaman *et al.*, 2001; Marti *et al.*, 2003). The homology between all functional Rad4 proteins is limited to the carboxyl terminal region referred to as a Rad4 protein family A (Rad4pfam-A) domain (Bateman *et al.*, 2004) (Fig. 1A). The exclusive conservation of the carboxyl terminal region suggests that the characteristics essential for NER are embedded within this domain. Indeed, for the human Rad4 homologue it was shown that the carboxyl terminal region is essential for the interactions with TFIIH, hHR23B and damaged DNA (Uchida *et al.*, 2002). The carboxyl terminal region of the yeast Rad4 homologues contains, partially overlapping the pfam-A domain, an ancient transglutaminase fold (Anantharaman *et al.*, 2001), which is also present in peptide-N-glycanases. In the Rad4 family members, however, the predicted catalytic residue is absent, suggesting that the transglutaminase fold is inactive. In contrast to the carboxyl termini, considerable diversity exists among the amino terminal regions of the Rad4 homologues. This indicates that apart from the shared function, additional functions might be present.

Interestingly, in *Saccharomyces cerevisiae* the yet uncharacterized ORF *YDR314C* encodes a protein containing a carboxyl terminal Rad4pfam-A domain (Marti *et al.*, 2003) (Fig. 1A,B). In addition to the sequence homology, the *YDR314C* gene product was, like Rad4, found to co-immunoprecipitate with Rad23 in a large-scale tandem-affinity purification (TAP) experiment (Gavin *et al.*, 2002). The sequence homology and the interaction with Rad23 indicate that YDR314C could be a genuine Rad4 homologue and consequently may have a similar function in NER. On the other hand, the UV sensitivity of *rad4* mutants is comparable to that of the other core NER mutants. Indeed, deletion of *YDR314C*, even in *rad4* and *rad16* mutants, does not affect sensitivity towards UV irradiation (Fig. 2A,B) or other DNA damaging and stress inducing agents (data not shown).

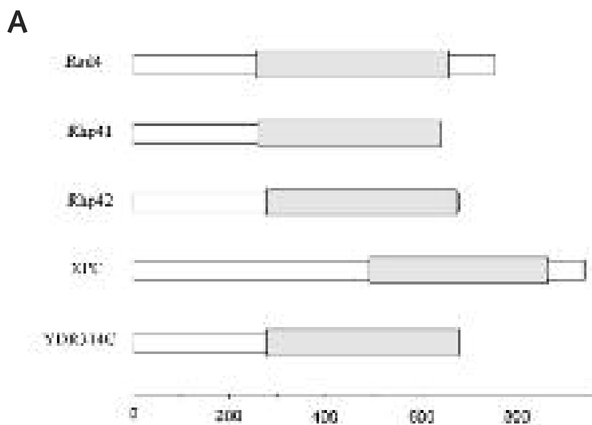

Figure 1
(A) Schematic representation of Rad4 homologues. The gray shaded boxes represent the conserved region that is categorized as a Rad4pfamA domain (Bateman *et al.*, 2004). The amino acid position is represented at the bottom of the figure.

B

Figure 1

(B) Alignment of the Rad4pfam-A domains of Rad4, Rhp41, Rhp42, XPC and YDR314C. Protein sequences were aligned with the clustalW program version 1.82. Similar and identical residues are boxed light and dark gray respectively.

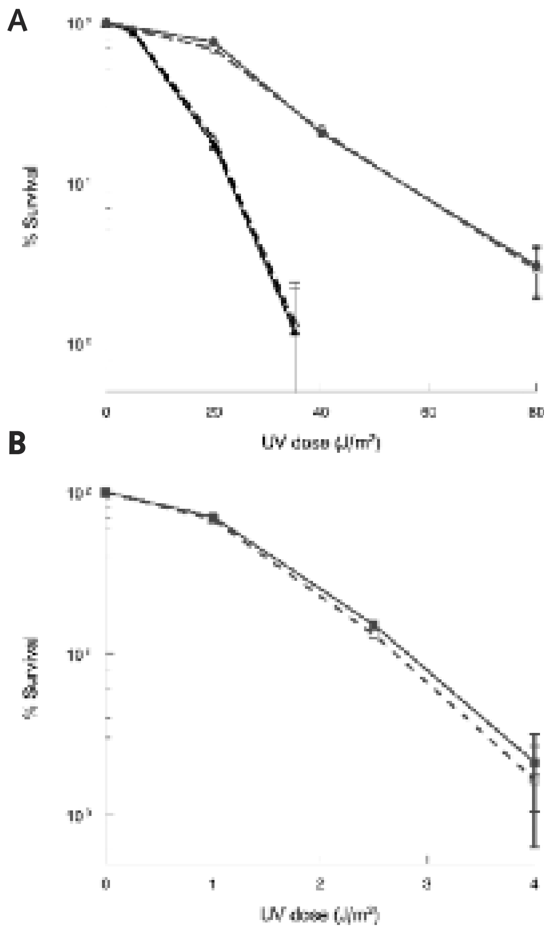

Figure 2
UV survival test. Cells were grown for 3 days in YPD, diluted in water to OD_{600} values that resulted in 100-200 colonies for each of the 3 administered UV doses and for the non irradiated sample. The diluted cells were plated on YPD and irradiated with the doses indicated. The irradiated 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.
(A) UV survival of W1588 and *ydr314c* mutants (black and open circles respectively) and of *rad16* and *rad16ydr314c* mutants (black and open triangles respectively).
(B) Survival of *rad4* and *rad4ydr314c* mutants (black and open squares respectively). The values depicted in the graphs are averages of at least 3 independent experiments, error bars represent standard deviations.

CPD removal in RNA pol I transcribed rDNA

Previously we showed that the ribosomal DNA (rDNA) locus can be repaired in the absence of Rad4 (Verhage *et al.*, 1996a). The rRNA genes are present in ~150 tandemly repeated units of 9.1 kb. The densely packed rDNA is localized in the nucleolus, a membrane-free intranuclear compartment. The rRNA genes are highly transcribed, yet, depending on the growth rate, no more than 40% to 60% of the repeats is transcriptionally active (Dammann *et al.*, 1993). Each repeat consists of a 5S and 35S unit that is transcribed by pol III or pol I respectively. UV induced lesions in the rDNA locus are repaired by NER and it was shown that preferential repair of the transcribed strand occurs (Verhage *et al.*, 1996a; Conconi *et al.*, 2002; Meier *et al.*, 2002). Cells deleted for *RAD4* are still capable of repairing the RNA pol I transcribed strand of rDNA whereas repair is completely abrogated in cells lacking one of the other core NER proteins.

A plausible explanation for the Rad4-independent repair in rDNA could be that another protein fulfils the damage recognition role in NER in the RNA pol I transcribed regions. Considering the similarities of YDR314C and Rad4, we investigated the role of YDR314C in Rad4-independent repair. CPD removal from RNA pol I transcribed

rDNA was analyzed in *rad4* and *rad4ydr314c* mutants using strand specific probes.

Cells lacking Rad4 are defective in CPD removal except for lesions in the RNA pol I transcribed strand, which can be repaired to approximately 50% (Fig. 3A,B) (Verhage *et al.*, 1996a). Interestingly, the Rad4-independent repair is completely abrogated when *YDR314C* is deleted (Fig. 3A,B), demonstrating that YDR314C is indeed responsible for the repair of RNA pol I transcribed rDNA in *rad4* mutants.

We subsequently examined the role of YDR314C in rDNA repair in cells containing functional Rad4. Single *ydr314c* mutants were analyzed for CPD removal in RNA pol I transcribed rDNA. Figures 3C and 3D show that in NER^+ cells the non-transcribed strand is repaired slightly slower than the transcribed strand and that the overall repair of both strands is significantly lower compared to CPD removal in RNA pol II transcribed regions (compare Fig. 3C,D and 4A,B). After two hours, 70% of the lesions is removed from the transcribed strand and 65% from the non-transcribed strand, corresponding to our results reported earlier (Verhage *et al.*, 1996a).

In *ydr314c* mutants the percentage of removed lesions after two hours is reduced to 55% in the non-transcribed strand and 50% in the transcribed strand (Fig. 3C,D). Thus, in the absence of YDR314C a substantial amount of lesions can still be removed, albeit with lower efficiency. The slight decrease in dimer removal observed in the non-transcribed strand of rDNA might indicate that YDR314C is involved in GGR. However, the fact that GGR is completely defective in *rad4* mutants shows that YDR314C can not replace Rad4 in GGR, implying that YDR314C is not directly involved in GGR of pol I transcribed rDNA.

To investigate a possible role of YDR314C in strand specific repair, we measured the effect of a *YDR314C* deletion in GGR defective *rad16* cells. Due to the impaired GGR, the difference in repair-efficiency between the transcribed and non-transcribed strand is more pronounced in a *rad16* background (Verhage *et al.*, 1996b). For RNA pol I transcribed rDNA, deletion of *RAD16* does not lead to a complete defect in GGR like in RNA pol II transcribed genes, but lesion removal from the non-transcribed strand is reduced to 30%. A clear strand bias can be observed since the transcribed strand is repaired to 70% (Fig. 3E,F) (Verhage *et al.*, 1996a). Interestingly, preferential repair of the transcribed strand is completely absent after deletion of *YDR314C* in *rad16* mutants (Fig. 3E,F), even when lesion removal was analyzed after 4 hours of incubation (Fig. 3G,H). These results demonstrate that YDR314C is essential for the preferential repair of the RNA pol I transcribed strand in rDNA.

CPD removal in RNA pol II transcribed DNA

The experiments above show that Rad4 is unable to function in strand specific repair of RNA pol I transcribed rDNA, whereas YDR314C is essential for this mode of repair. Thus, Rad4 cannot replace YDR314C in rDNA repair. In RNA pol II transcribed genes on the other hand, NER is dependent on Rad4. To examine whether YDR314C can substitute for Rad4 in NER of RNA pol II transcribed genes, CPD removal from both strands of the *RPB2* gene was measured in *ydr314c* mutants. We show that the *YDR314C* deletion has no effect on the repair-efficiency (Fig. 4A,B), even when *YDR314C* is deleted in a *rad16* mutant, in which TCR is the sole mode of repair (Fig. 4C,D). These results demonstrate that YDR314C has no role in NER of the *RPB2* gene, suggesting that YDR314C is not involved in repair of RNA pol II transcribed genes in general.

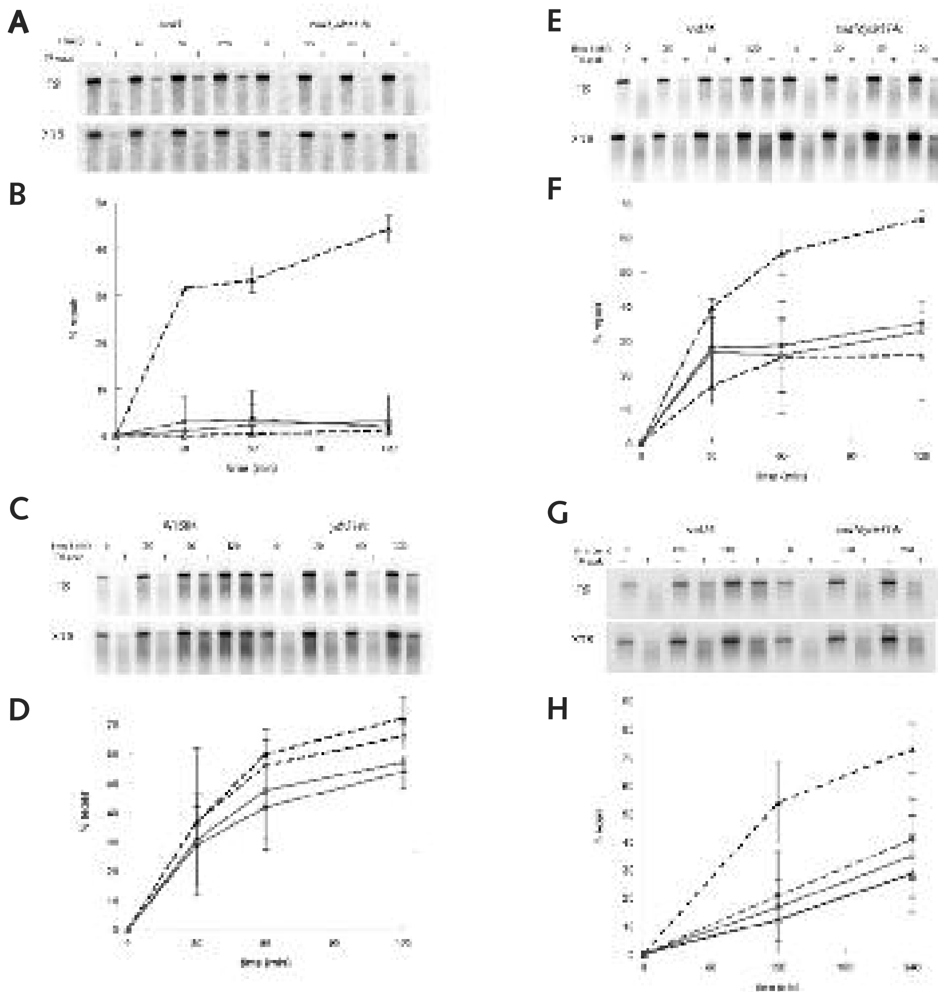

Figure 3
Gene specific repair assay. Cells were grown in YPD, irradiated and allowed to remove lesions for the times indicated. Genomic DNA was extracted, digested with HindIII and either mock-treated or treated with T4endoV. Samples were run on an alkaline agarose gel, blotted on a nylon membrane and probed with an EcoRI-MruI rDNA 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 *rad4* and *rad4ydr314c* mutants 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 as a function of time. *rad4* TS and NTS (black and open triangles respectively) and *rad4ydr314c* 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 W1588 and *ydr314c* cells. (D) As (B) but for W1588 and *ydr314c* cells. (E) As (A), but for *rad16* and *rad16ydr314c* mutants. (F) As (B) but for *rad16* and *rad16ydr314c* mutants. (G) As (E) but samples taken after 0, 120 and 240 minutes respectively. (H) As (F) but samples taken after 0, 120 and 240 minutes respectively.

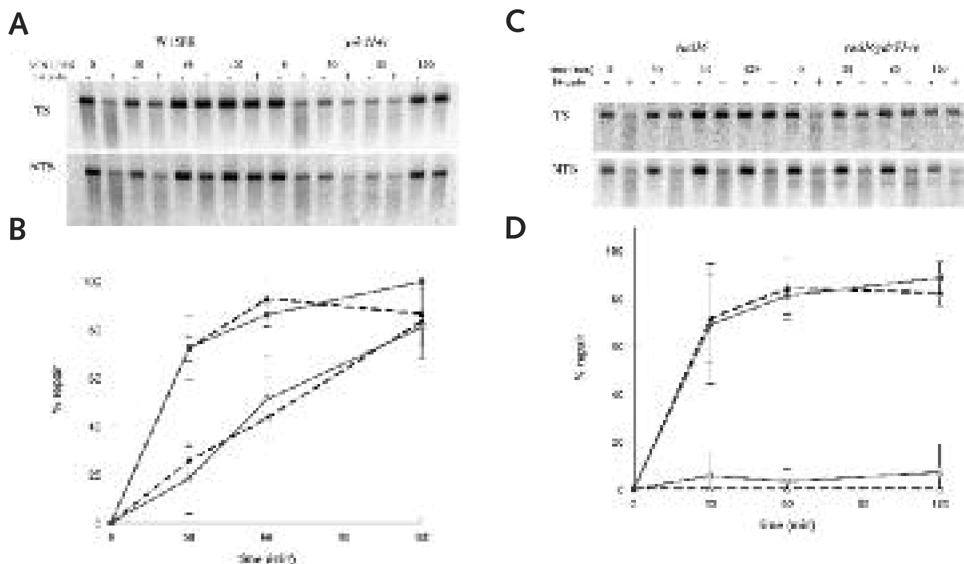

Figure 4
Gene specific repair assay. Cells were grown in YPD, irradiated and allowed to remove lesions for the times indicated. Genomic DNA was extracted, digested with HindIII and either mock-treated or treated with T4endoV. Samples were run on an alkaline agarose gel, blotted on a nylon membrane and probed with an EcoRI-MruI rDNA 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 wildtype cells (W1588) and the *ydr314c* mutant. 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 as a function of time. W1588 TS and NTS (black and open triangles respectively) and *ydr314c* 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 *rad16* and *rad16ydr314c* mutants.
(D) As (B) but for *rad16* and *rad16ydr314c* mutants.

3 Discussion

The *YDR314C* gene product shows homology to the members of the Rad4 family (Anantharaman *et al.*, 2001; Marti *et al.*, 2003) and interaction with Rad23 has been reported (Gavin *et al.*, 2002), suggesting a role for YDR314C in NER. In genome wide screens *ydr314c* mutants exhibit poor growth in medium containing nystatin or sorbitol (Giaever *et al.*, 2002). Furthermore, a synthetic lethal interaction of *YDR314C* and *CHS1* was reported *(Tong et al., 2004)*. These phenotypes might indicate involvement in processes like amino acid synthesis, osmoregulation and cell wall maintenance.

Here we show that the *YDR314C* gene product is responsible for Rad4-independent repair in the RNA pol I transcribed rDNA locus. Moreover, we demonstrate that YDR314C is not merely acting as a substitute when Rad4 is absent, but that preferential repair of the RNA pol I transcribed strand specifically requires YDR314C. The effect is especially evident in the GGR deficient *rad16* background, in which there is a clear difference in repair of the transcribed and non-transcribed strand. This strand bias is completely absent in *rad16ydr314c* double mutants, demonstrating that YDR314C, despite the presence of Rad4, is essential for preferential repair of the transcribed strand. The specific decrease in repair of the transcribed strand suggests that YDR314C is involved in TCR, however, we have not shown that in RNA pol I transcribed rDNA the preferential repair of the transcribed strand is dependent on active transcription. We therefore can not exclude the possibility that the YDR314C dependent repair in *rad16* cells is independent of transcription, but only occurring in the template strand.

Deletion of *YDR314C* has no effect on dimer removal from both strands of the RNA pol II transcribed *RPB2* gene. This suggests that YDR314C solely acts on RNA pol I transcribed regions and is unable to substitute for Rad4 in TCR of RNA pol II transcribed genes. The absence of UV sensitivity of *ydr314c* cells shows that removal of lesions from rDNA does not significantly contribute to survival. Considering that YDR314C was reported to co-immunoprecipitate with Rad23 and the fact that repair of rDNA is defective in *rad23* but not in *rad4* mutants, we assume that YDR314C functions, like Rad4, in complex with Rad23.

The two homologues Rad4 and YDR314C appear to have non-overlapping roles. Rad4 is essential for repair of both strands of RNA pol II transcribed genes and is unable to act in strand specific repair of genes transcribed by RNA pol I. YDR314C on the other hand is essential for preferential repair in RNA pol I transcribed rDNA and can not replace Rad4 in repair of RNA pol II transcribed regions. A simple explanation for the non-overlapping functions could be that Rad4 and YDR314C are prevented from travelling in and out the nucleolus respectively. However, the requirement of Rad4 for GGR of rDNA demonstrates that the inability of Rad4 to act in preferential repair of the transcribed strand of rDNA is not due to exclusion of Rad4 from the rDNA locus. Moreover, YDR314C appears not to be restricted to the nucleolus, since proteome-wide GFP localization experiments show that YDR314C is present throughout the nucleus (Huh *et al.*, 2003). Given that Rad4 and YDR314C are not spatially confined, we conclude that although Rad4 and YDR314C have homologous functions in analogous processes, they are unable to substitute for each other.

In *Schizosaccharomyces pombe* two Rad4 homologues are present as well. Involve-

ment of these proteins in repair of RNA pol I transcribed rDNA has not yet been studied. In contrast to Rad4 and YDR314C, Rhp41 and Rhp42 both seem to function, to different degrees, in GGR and TCR of RNA pol II transcribed genes (Fukumoto *et al.*, 2002; Marti *et al.*, 2003). Moreover, *rhp41rhp42* double mutants exhibit enhanced UV sensitivity compared to either single mutant, showing that the *Schizosaccharomyces pombe* Rad4 homologues have redundant functions. In addition to their role in NER, Rhp41 and Rhp42 are involved in NER dependent short-patch mismatch repair during meiosis (Marti *et al.*, 2003). A possible involvement of YDR314C and Rad4 in this type of DNA repair in *Saccharomyces cerevisiae* has not yet been investigated.

In human cells, XPC appears to be the only homologue of Rad4 since a second gene encoding a Rad4pfam-A domain containing protein is not present in the human genome (Bateman *et al.*, 2004). There are marked differences between the roles of XPC and Rad4 in NER. In *rad4* cells, repair of RNA pol II transcribed genes is completely defective whereas lesions in the RNA pol I transcribed strand of rDNA can still be removed. In human cells on the other hand, XPC is essential for repair of both strands of RNA pol I transcribed rDNA (Christians and Hanawalt, 1994) but not required for TCR in RNA pol II transcribed regions (Venema *et al.*, 1991). Here we show that in *Saccharomyces cerevisiae*, the Rad4-independent repair is explained by the involvement of YDR314C. It remains unclear how NER in humans can process lesions in the transcribed strand without XPC.

The reason why Rad4 and YDR314C are unable to replace each other at different loci in the genome is yet unknown. Possibly, differences in chromatin structure at different chromosomal positions determine the requirement for either Rad4 or YDR314C. The poorly conserved N-terminal region might harbor the properties that are necessary to perform NER at different loci in the genome. The difference in the N-termini among the Rad4 family members could also reflect additional functions of the Rad4 homologues, apart from their role in the NER reaction. Further studies are necessary to identify the factors that influence the requirement of either YDR314C or Rad4 to facilitate NER.

4 Experimental procedures

Strains and media

All experiments were conducted in the *Saccharomyces cerevisiae* W1588-4a background. The strains used in this study are listed in table 1. W1588-4a (Mortensen *et al.*, 2002) was kindly provided by R. Rothstein. Strain MGSC 471 (*rad16::hisG)* and MGSC 479 (*rad4::HisGURA3HisG)* were constructed analogous to the previously described MGSC 268 and MGSC 283 respectively (Jansen *et al.*, 2000), using a W1588-4a instead of a W303-1B background. *YDR314C* deletions were constructed by transforming target strains with a loxLEU2lox disruption cassette, created by ligating a loxLEU2lox fragment to PCR generated *YDR314C* flanking regions, using the following primers:
5'-TGGAACAGTGCTGAAAATGCGT, 5'-TTCGGTGACCGGTTTCAAGGTTTGACCCTTCG, 5'-CATGGTTACCGATTCGACGCTGTTTCGCAGAG and 5'-GGAGGCGATTCCACGTCGCTAT. Underlined sequences contain a BstEII restriction site by which the flanking regions were ligated to the loxLEU2lox sequence. Correct integration of the constructs was confirmed by Southern blot analysis. Strains MGSC 471, 537, W1588-4a and MGSC 517 were transformed with an URA3 fragment to obtain the $URA3^+$ strains MGSC 578-581 respectively.

UV survival

Cells were grown for 3 days in YPD and diluted in water to appropriate OD_{600} values. The diluted cells were plated on YPD. NER^+ cells were irradiated with 0, 20, 40 and 80 J/m^2, *rad16* cells with 0, 5, 20 and 35 J/m^2 and *rad4* cells with 0, 1, 2.5 and 4 J/m^2 respectively. 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.

Table 1. Yeast strains

Strain	Genotype	Source
W1588-4a	MATa leu2-3,112 ade2-1 can1-100 his3-11,15	R. Rothstein
	ura3-1 trp1-1	This study
MGSC 471	*rad16*::hisG*	This study
MGSC 479	*rad4::*hisGURA3hisG*	This study
MGSC 517	*ydr314c::*loxLEU2lox*	This study
MGSC 518	*rad4*::hisGURA3HisG *ydr314c::*loxLEU2lox*	This study
MGSC 537	*rad16::*HisG *ydr314c*::loxLEU2lox URA3*	This study
MGSC 578	*rad16*::hisG URA3*	This study
MGSC 579	*rad16::*HisG *ydr314c*::loxLEU2lox URA3*	This study
MGSC 580	URA3*	This study
MGSC 581	*ydr314c::*loxLEU2lox URA3*	This study

*The remainder of the genotype is identical to that of W1588-4a

Sensitivity towards various chemical agents
Serial dilutions of stationary cells were made in water. Of each dilution 2ml was spotted on YPD or YNB plates with a concentration varying from 0 to 0.03% methyl methanesulfonate (MMS), 0 to 15 mg/ml cisplatin, 0 to 3 % dimethylsulfoxide (DMSO), 0 to 6 mM H_2O_2, 0 to 6 mM caffeine and 0 to 100 mg/ml 6-aza-uracil respectively. For the 6-aza-uracil test *URA3*$^+$ cells were used. Cells were grown for 2 days at 30°C.

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 Li and Smerdon (2002), with the following modifications. After the RNAse A+T treatment, ammonium acetate was added to a final concentration of 2.5M. The solution was kept on ice for 30 minutes. Following the removal of insoluble components by centrifugation the DNA was precipitated with ethanol. Repair of rDNA was measured as described previously (Verhage *et al.*, 1996a). Analysis of *RPB2* repair was performed as described previously (Jansen *et al.*, 2000). 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 3 independent experiments and the error-bars indicate standard deviations.

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Chapter

4.1

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

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.

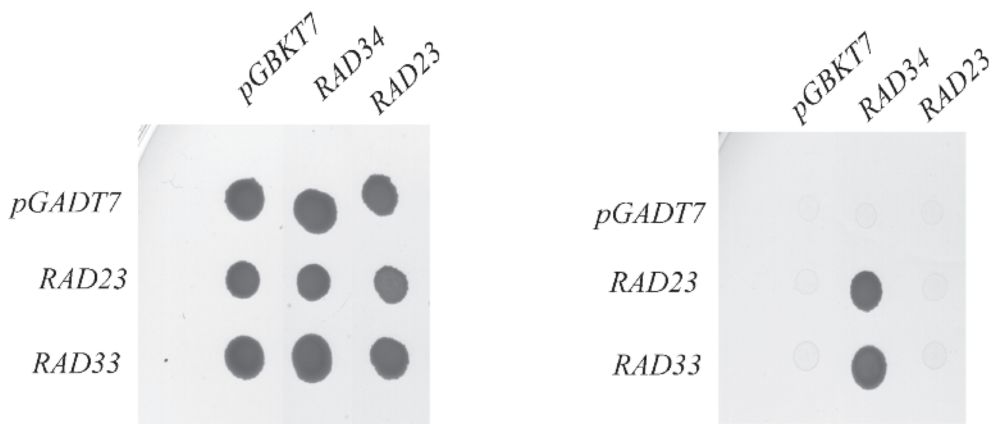


Figure 1:
Two Hybrid test. The coding regions of *RAD23, RAD33* and *RAD34* were cloned in the pGBKT7 or pGADT7 vectors (Clonetech Matchmaker 3) as indicated in the figure. The empty vectors and constructs were transformed to Y187 and AH109 cells as described in materials and methods. The cells were 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.

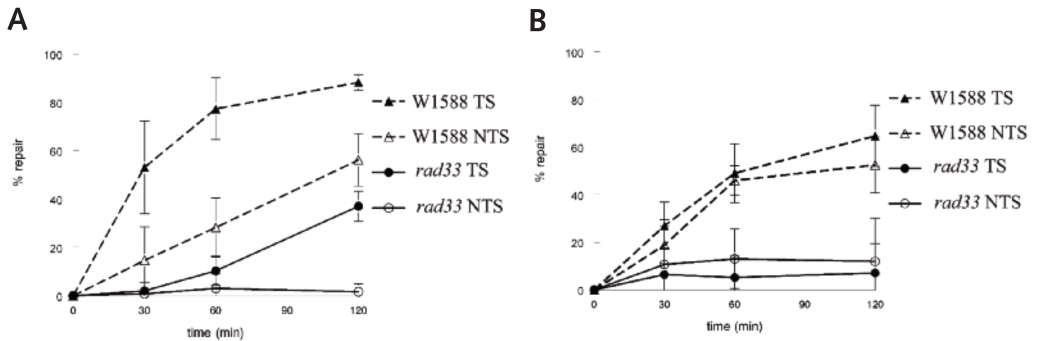


Figure 2:
Repair analysis.
(**A**) Strand specific repair analysis of the *RPB2* gene in wildtype (W1588) and *rad33* cells.
(**B**) Strand specific repair analysis of the RNA pol I transcribed rDNA locus. See also chapter 5.

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-

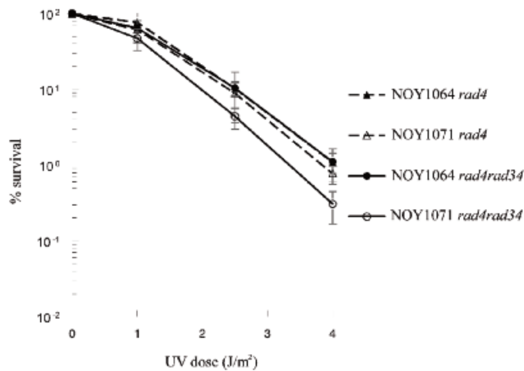


Figure 3
UV dose response curve. Suitable dilutions of exponentially growing cells were irradiated with the doses indicated. Plates were incubated 72 hours in the dark at 30°C. Colonies were counted and the percentage of surviving cells was calculated. Average of 4 independent experiments are shown.

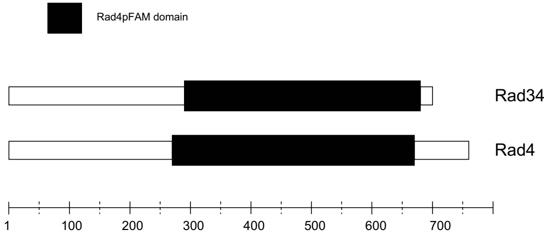
gion is also essential for the interaction with Rad23, as well as for binding to TFIIH and DNA (Bunick *et al.*, 2006; Uchida *et al.*, 2002). The amino terminal half varies considerably between Rad4 homologues and the contribution of this region to NER is unclear. The factor that determines that Rad4 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).**

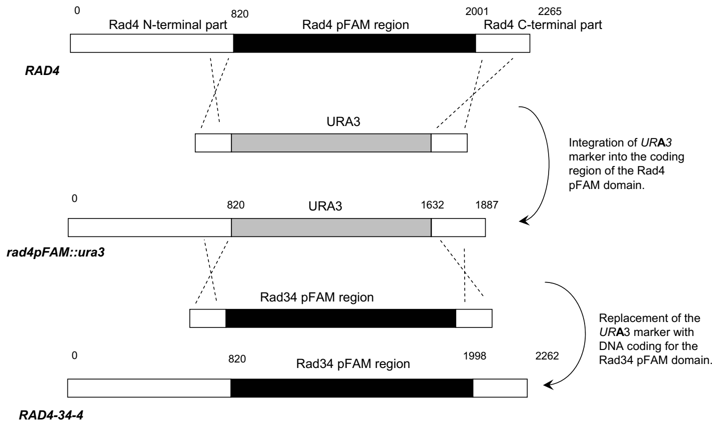
If the Rad4-34-4 protein is functional, our hypothesis predicts that it will (partially) substitute for the Rad4 protein, but not for the Rad34 protein. *RAD4-34-4 rad34* cells will thus be (partially) UV resistant but defective in rDNA repair. For *rad4* cells expressing the Rad34-4-34 protein we expect 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.

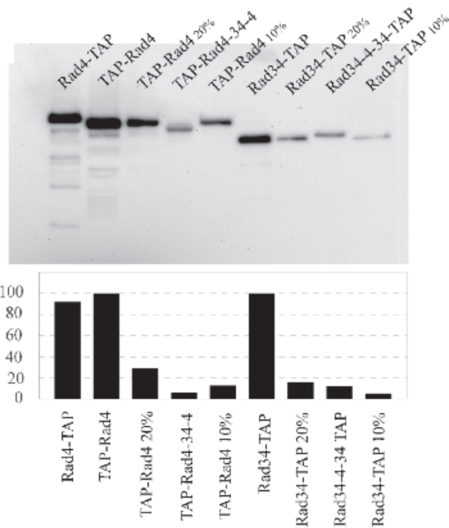
A



B



C



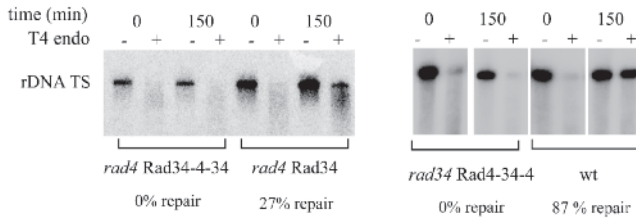


Figure 4
(**A**) Schematic representation of the location of the pFAM domain in the Rad4 and Rad34 proteins.
(**B**) Schematic representation of the construction of the *RAD4-34-4* gene. The *URA3* gene was amplified using primers with 5' flanks homologous to the flanking region of the pFAM region of *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.

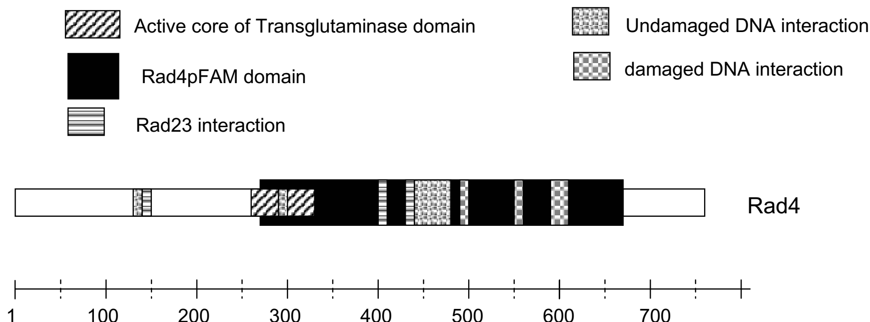


Figure 5
Schematic representation of the approximate location of the residues in Rad4 required for interaction with Rad23, undamaged dsDNA segment and the damaged DNA segment (Min and Pavletich, 2007).

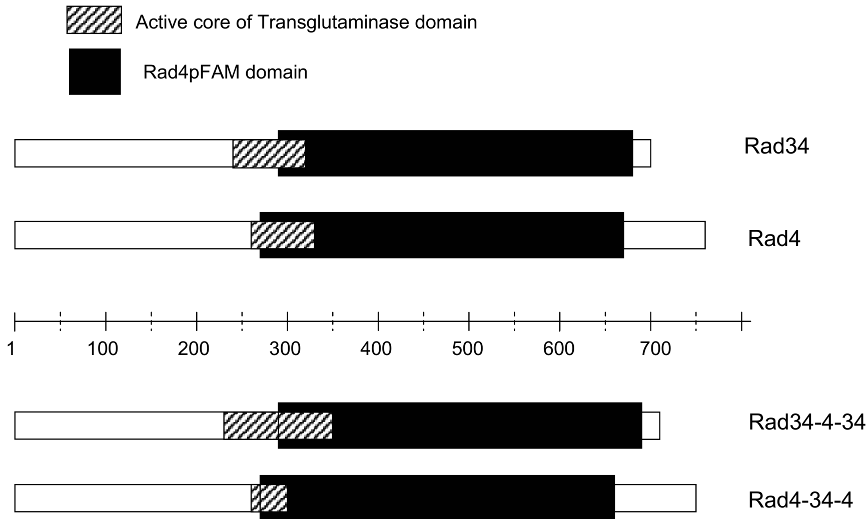


Figure 6:
Schematic representation of location of the pFAM domain and the TGD in Rad4, Rad34, Rad4-34-4 and Rad34-4-34 proteins.

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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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

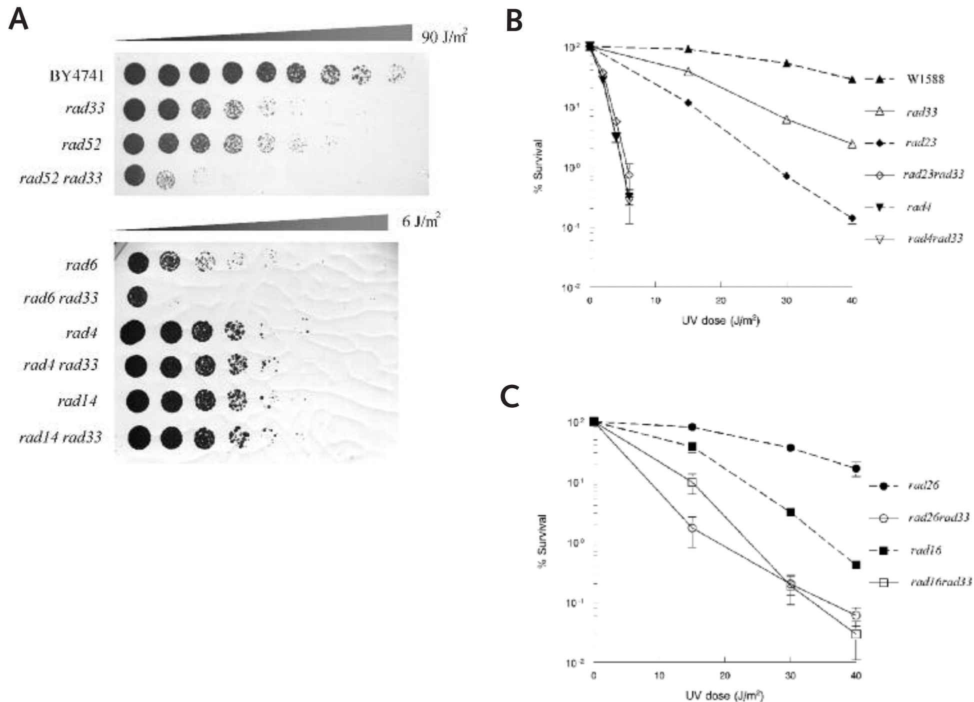

Figure 1

(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

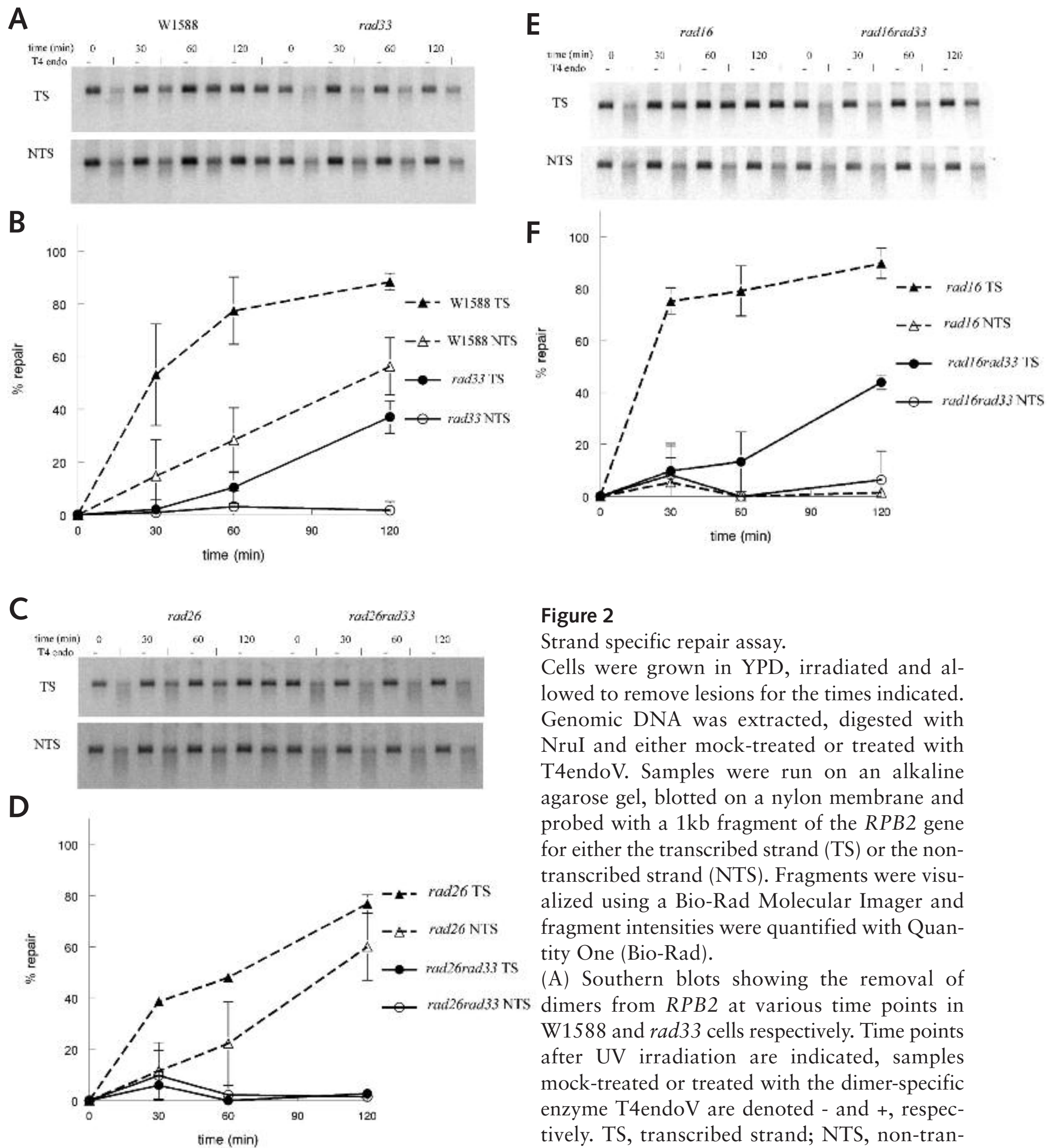

Figure 2
Strand specific repair assay.
Cells were grown in YPD, irradiated and allowed to remove lesions for the times indicated. Genomic DNA was extracted, digested with NruI and either mock-treated or treated with T4endoV. Samples were run on an alkaline agarose gel, blotted on a nylon membrane and 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.

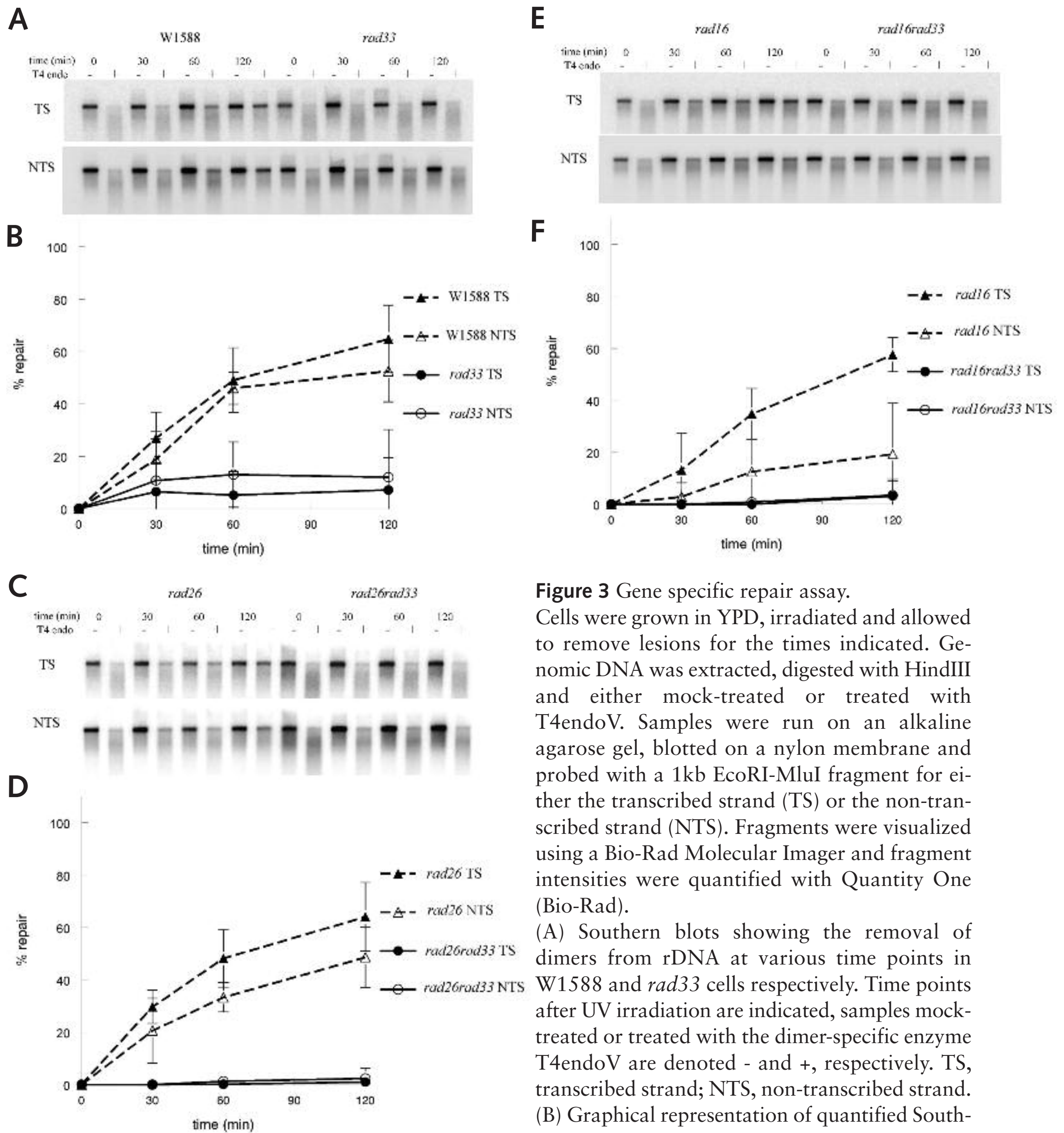

Figure 3 Gene specific repair assay.
Cells were grown in YPD, irradiated and allowed to remove lesions for the times indicated. Genomic DNA was extracted, digested with HindIII and either mock-treated or treated with T4endoV. Samples were run on an alkaline agarose gel, blotted on a nylon membrane and 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.

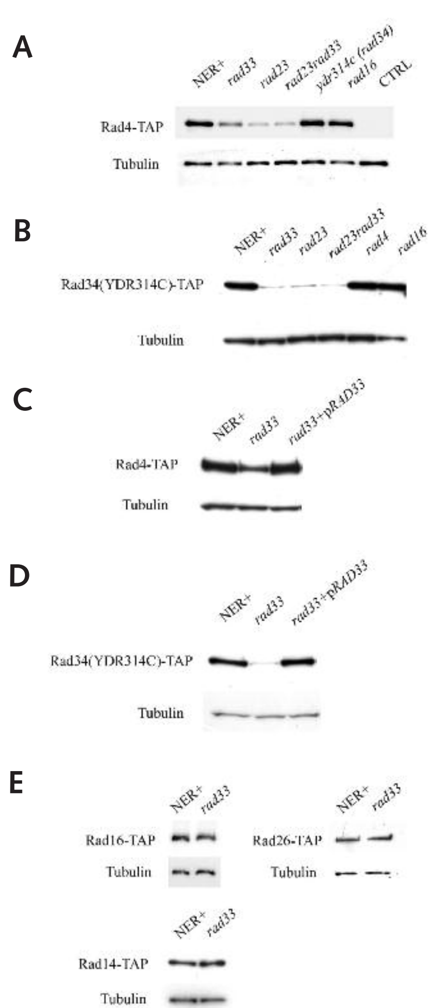

Figure 4
Western blot analysis.
Cells containing Rad4-TAP, YDR314C-TAP, Rad16-TAP, Rad26-TAP or Rad14-TAP in various NER deficient backgrounds were grown in YPD till stationary phase, proteins were extracted, run on SDS gel and transferred to western blot. Blots were hybridized with PAP or alpha tubulin 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.

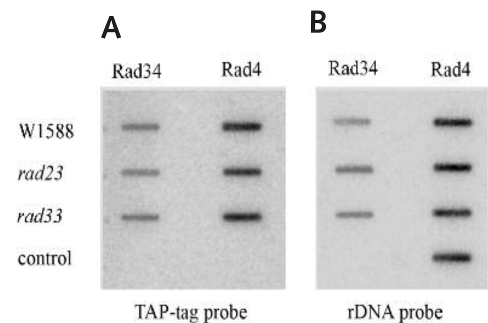

Figure 5
mRNA level analysis
Cells containing Rad4-TAP or Rad34-TAP (YDR314C-TAP) in *rad23*, *rad33* or W1588 background were grown to OD600 ~0.5; RNA was isolated and transferred to a nylon membrane. (A) Left column, total RNA isolated from cells containing Rad34-TAP in a W1588, rad23 or rad33 background. 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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Chapter 6

The NER protein Rad33 shows functional homology to human Centrin2 and is involved in modification of Rad4

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The NER protein Rad33 shows functional homology to human Centrin2 and is involved in modification of Rad4.

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Abstract

In the yeast *Saccharomyces cerevisiae* the Rad4-Rad23 complex is implicated in the initial damage recognition of the Nucleotide Excision Repair (NER) pathway. NER removes a variety of lesions via two subpathways: Transcription Coupled Repair (TCR) and Global Genome Repair (GGR). We previously showed that the new NER protein Rad33 is involved in both NER subpathways TCR and GGR. In the present study we show UV induced modification of Rad4 that is strongly increased in cells deleted for *RAD33.* Modification of Rad4 in *rad33* cells does not require the incision reaction but is dependent on the TCR factor Rad26. The predicted structure of Rad33 shows resemblance to the Centrin homologue Cdc31. In human cells, Centrin2 binds to XPC and is involved in NER. We demonstrate that Rad4 binds Rad33 directly and via the same conserved amino acids required for the interaction of XPC with Centrin2. Disruption of the Rad4-Rad33 interaction is sufficient to enhance the modification of Rad4 and results in a repair defect similar to that of a *rad33* mutant. The current study suggests that the role of Rad33 in the Rad4-Rad23 complex might have parallels with the role of Centrin2 in the XPC-HHR23B complex.

6.1 Introduction

Nucleotide Excision Repair (NER) is a DNA repair system characterized by its ability to remove various structurally unrelated lesions from DNA, including the UV induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine pyrimidone (6-4) photoproducts. Completion of the core-NER reaction requires a set of 15 highly conserved proteins. In the yeast *Saccharomyces cerevisiae* the reaction is initiated by binding of the Rad4-Rad23 complex to the lesion (Guzder *et al.*, 1998; Jansen *et al.*, 1998; Min and Pavletich, 2007; Sugasawa *et al.*, 1998). This event triggers the recruitment of the other NER factors to the site of the damage. Once the DNA adjacent to the lesion is locally unwound, single strand incisions are made on both sides of the damage, followed by the removal of the oligonucleotide containing the lesion. The DNA is restored to its pre-damaged state by the actions of DNA polymerase and ligase (de Laat *et al.*, 1999; Guzder *et al.*, 1995; Park and Choi, 2006; Volker *et al.*, 2001). Defects in the human NER system lead to severe disorders, mostly associated with a highly elevated risk of skin cancer (de Boer and Hoeijmakers, 2000; Kraemer *et al.*, 2007; Leibeling *et al.*, 2006).

Lesion removal by NER is not homogenous as relative fast repair by the subpathway Transcription Coupled Repair (TCR) occurs only in DNA which is transcriptionally active. Global Genome Repair (GGR) on the other hand is slower and removes lesions throughout the entire genome. The TCR pathway, in yeast mainly dependent on the CSB homologue Rad26 (van Gool *et al.*, 1994), is initiated when the RNA polymerase is obstructed at the site of the damage. The presence of this highly lethal structure triggers the recruitment of NER proteins to the site of the lesion (Sarasin and Stary, 2007; Svejstrup, 2002). The second NER subpathway, GGR, requires the Rad7-Rad16 complex in yeast (Verhage *et al.*, 1996) and is suggested to function as a damage sensor in the context of chromatin (Guzder *et al.*, 1997). In humans, no homologues of Rad7 and Rad16 are identified, but DDB1 and DDB2 appear to be the functional equivalents of the yeast GGR proteins (Gillette *et al.*, 2006). Yeast cells lacking the Rad7-Rad16 complex or the Rad26 protein can still rely on TCR or GGR respectively and are therefore only partially UV sensitive. Mutations in genes encoding for core NER factors on the other hand lead to a complete NER defect. In recent years various studies challenged the traditional view of the GGR and TCR systems. For instance, the Rad7 and Rad16 proteins have been shown to function in a post-incision event (Yu *et al.*, 2004) and the TCR factor Rad26 can contribute to repair of the non-transcribed strand in transcriptionally inactive genes (Li *et al.*, 2007).

Although NER is similar in yeast and humans, the role of Rad4 differs from that of its homologue XPC. In yeast TCR is dependent on the Rad4 protein whereas in human cells repair of the transcribed strand can be completed without XPC. Rad4 is therefore considered a core NER protein and XPC a GGR factor. The basis for the different requirement of Rad4 or XPC in transcription coupled repair remains to be elucidated. In yeast, the binding of the Rad4-Rad23 complex to damaged DNA occurs through Rad4 (Guzder *et al.*, 1998; Jansen *et al.*, 1998), a process recently visualized by elegant work showing the crystal-structure of Rad4 bound to DNA containing a CPD lesion (Min and Pavletich, 2007). Rad23 provides stability to Rad4 and enhances its affinity for DNA (Xie *et al.*, 2004). An additional role of Rad23 in NER is mediated via the ubiq-

uitin-like domain (UbL domain) and two ubiquitin associated domains (UBA domains), which link NER to the ubiquitin/proteasome pathway (Reed and Gillette, 2007; Schauber *et al.*, 1998).

Although a defined role for the ubiquitin proteasome system in NER is not yet clear (Bergink *et al.*, 2007), it was shown that ubiquitylation is involved in damage recognition in human NER. The GGR factor UV-DDB consists of the DDB1 and DDB2 proteins which can bind Cul4A and Roc1 to form a Cullin-RING ubiquitin ligase (Petroski and Deshaies, 2005; Shiyanov *et al.*, 1999). After binding of UV-DDB to a lesion XPC is recruited and ubiquitylated by the DDB1-DDB2-Cul4A-Roc1 complex. At the same time the UV-DDB-ubiquitin ligase complex is auto-ubiquitylated, leading to the degradation of DDB2. The modified XPC is stable and possesses increased affinity for the damage, subsequently replacing UV-DDB at the site of the lesion (Sugasawa, 2006; Wang *et al.*, 2005).

There are indications that ubiquitylation is also involved in GGR of *S. cerevisiae*. The Rad7-Rad16 complex is thought to be part of a Cullin-RING ubiquitin ligase (Ho *et al.*, 2002; Ramsey *et al.*, 2004) similar to the UV-DDB complex. The Rad7-Rad16 ubiquitin ligase was purified and shown to be able to mono-ubiquitylate Rad4 *in vitro* (Gillette *et al.*, 2006). Whether ubiquitylation plays a role in Rad4 damage recognition is not clear however, since mutation of the SOCS site in Rad7, essential for its function in the Cullin-RING ligase, does not lead to increased UV sensitivity (Gillette *et al.*, 2006).

We previously reported that Rad33, a 20kD protein with no clear homology to any known repair factor, is involved in NER in *S. cerevisiae* (den Dulk *et al.*, 2006). Deletion of *RAD33* leads to moderate UV sensitivity, however, a synergistic effect is observed in the *rad23rad33* double mutant which displays UV sensitivity indicative of a complete NER defect. In *rad33* cells only partial repair of the transcribed strand (TS) and no repair of the non-transcribed strand (NTS) is detected implicating that Rad33 is involved in both GGR and TCR (den Dulk *et al.*, 2006).

Large scale affinity capture studies show co-purification of Rad33 with both Rad4 and Rad23 (Krogan *et al.*, 2006) indicating that Rad33 might be part of the Rad4-Rad23 complex. Our previous results show that deletion of *RAD33* causes a reduction of Rad4 protein levels (den Dulk *et al.*, 2006). Here we study the role of Rad33 in relation to the Rad4 protein in more detail. We show that the Rad4 levels in wildtype cells as well as the reduced levels of Rad4 in a *rad33* background are reasonably stable. Furthermore, we find that deletion of *RAD33* strongly enhances UV induced modification of Rad4 *in vivo*. Interestingly, the data in this paper suggests that Rad33 and the human XPC binding protein Centrin2 might have a comparable role in the NER damage recognition complex.

6.2 Materials and methods

6.2.1 Strains and plasmids

The strains used in this study are listed in table 1. The *RAD4TAP rad33* mutants with an additional deletion of either *RAD6, UBC4, UBC5, UBC7, UBC8, UBC11, UBC12,* or *UBC13* were made by crossing MGSC825 cells with BY4741 cells that were deleted for one of the above mentioned genes (Euroscarf). Spores were dissected to obtain haploid cells. YEp112K was constructed by insertion of the EcoRV-PvuII KANMX fragment from pUG6 (Guldener *et al.*, 1996) into the BstXI site of YEp112 (Hochstrasser *et al.*, 1991). TAP tag constructs were created as described previously (Puig *et al.*, 2001). Cells expressing the amino-terminally HA tagged *RAD4* gene were created by targeting *RAD4* with a modified version of the pBS1761 plasmid (Puig *et al.*, 2001) in which the TAP tag sequence was replaced by a triple HA-tag. The *rad4AAA* mutations were introduced via the two-step gene replacement method (Sherman, 2002) using the YIpLAC211 vector containing the *RAD4* sequence encoding the carboxy-terminal part of Rad4 in which the residues W649, L652 and L656 were replaced by alanines.

6.2.2 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 *RAD4* region coding for amino acids 1-277, the conserved part of *RAD4* coding for residues 274-667 and the full length *RAD23* gene were fused to the *GAL4* binding domain (BD) in pGBKT7. pGBKT7-*RAD4AAA* was created as described for the conserved *RAD4* region (coding for residues 274-667).

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.

6.2.3 Western blot analysis

All cell cultures were grown for three days in YPD before extracts were prepared. Optical densities were typically around an OD_{600} of 10 and were found comparable for all strains used. 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 7,5% 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 detected using rabbit Peroxidase-anti-Peroxidase antibodies (American Qualex, P2250). The blots were stripped and re-probed with an antibody against alpha tubulin as loading control (Genetex, GTX76511). Native Rad4 was shown using anti-Rad4 antibody (Gillette *et al.*, 2006), a kind gift from Dr. Simon Reed. For these blots an unknown protein which reacted aspecifically with anti-Rad4 was used as loading control.

Mouse anti-Ubiquitin antibodies used were purchased from Zymed laboratories (Catalog No. 13-1600), Goat anti-ubiquitin was purchased from Abcam (ab14372).

For the western blot detection of ubiquitin the blots were chemically denatured prior to detection to enhance the sensitivity as described previously (Pagano, 1997). All western blots were developed using Pierce supersignal west Pico and exposed to hyperfilm (GE healthcare) for 30 minutes or overnight for detection of Rad4 modification in wild-type background. For quantitative analysis used in the results sections 3.1 and 3.2 western blots were analyzed with a BioRad Chemidoc XRS and quantified using BioRad Quantity one.

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
BY4741	*MATa his3Δ leu2Δ ura3Δ met15Δ*	Euroscarf
BY4742	*MATα his3Δ leu2Δ lys2Δ ura3Δ*	EuroScarf
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80,Δ LYS2 : : GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3 : : MEL1UAS-MEL1 TATA-lacZ*	Clontech
Y186	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4D, met-, gal80Δ, URA3 : : GAL1UAS-GAL1TATA-lacZ*	Clonetech
MGSC 623	*RAD4TAP-URA3*[a]	This laboratory[b]
MGSC 624	*RAD4TAP-URA3 rad33::KAN*[a]	This laboratory[b]
MGSC 625	*RAD4TAP-URA3*[a] *rad23Δ::loxLEU2lox*[a]	This study
MGSC 771	*3*HA-RAD4TAP*[a]	This study
MGSC 772	*3*HA-RAD4TAP rad33Δ::KAN*[a]	This study
MGSC 825	*RADTAP-URA3 rad33::HIS3*[c]	This study
MGSC 640	*RAD14TAP-URA3*[a]	This laboratory[b]
MGSC 641	*RAD14TAP-URA3 rad33 ::KAN*[a]	This laboratory[b]
MGSC 690	*TAP-RAD23*[a]	This study
MGSC 695	*TAP-RAD23 rad33Δ::KAN*[a]	This study
MGSC 699	*RAD4TAP-URA3 rad33Δ::KAN*[a] *rad2::TRP1*	This study
MGSC 685	*RAD4TAP-URA3 rad33Δ::KAN*[a] *ra16:TRP1*	This study
MGSC 698	*RAD4TAP-URA3 rad33Δ::KAN*[a] *rad14::LEU2*	This study
MGSC 788	*RAD4TAP-URA3 rad33Δ::KAN*[a] *elc1::HIS3*	This study
MGSC 792	*RAD4TAP-URA3 rad33Δ::KAN*[a] *rad26::HIS3*	This study
MGSC 479	*rad4::hisG*[a]	This laboratory [b]
MGSC 662	*rad33::KANMX*[a]	This laboratory [b]
MGSC 779	*rad4AAA (W649A, L652A, L656A ::EcoRI)*[a]	This study
MGSC 780	*rad33::KANMX rad4AAA (W649A, L652A, L656A ::EcoRI)*[a]	This study
MGSC 810	*rad4AAATAP::URA3*[a]	This study
MGSC 811	*rad33::KANMX rad4AAA::URA3*[a]	This study

[a]Remainder of the genotype identical to that of W1588-4a
[b]Constructed as described previously (den Dulk *et al.*, 2006)
[c]Remainder of the genotype identical to that of BY4742

Figure 1
Western blot analysis. TCA extracts of cells expressing Rad4TAP were prepared as described in the section materials and methods. Extracts were analysed on western blot using PAP antibody, anti-Rad4 antibody or alpha tubulin antibody (loading control).
(A) Steady state Rad4TAP levels in wildtype or *rad33* cells.
(B) Left papel: Westernblot showing Rad4TAP levels in wildtype cells incubated in the absence (lane 1-4) or presence (lane 5-8) of cycloheximide (CHX). Stationary cells were resuspended in YPD medium with or without cycloheximide and incubated at 30°C, samples were taken 0, 30, 60 and 120 minutes after resuspension in YPD medium. Right panel: Intensity of the Rad4 bands at 30,60 and 120 minutes related to time point zero. Open triangles represent Rad4 from CHX treated cells, solid triangles represent Rad4 from untreated cells.
(C) As (B) but for *rad33* cells.
(D) Left panel: Western blot showing Rad4TAP levels in wildtype (lane 1-4) and *rad33* cells (lane 5-8) harvested 0, 30, 60 and 120 minutes after transfer to YPD with cycloheximide. Right panel: Intensity of the Rad4 bands at 30,60 and 120 minutes relative to time point zero. Solid triangles represent Rad4 from wildtype cells, open triangles represent Rad4 from *rad33* cells.
(E) As (D), except that cells expressing untagged Rad4 were used. Blots were analysed using anti-Rad4 antibody. The a-specific band which is visible using the Rad4 antibody functions as loading control.

3 Results

3.1. Levels and stability of Rad4 in rad33 background.

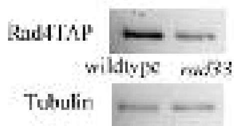
The steady state protein levels of Rad4 are lower in the absence of Rad33 (den Dulk *et al.*, 2006) (Fig. 1A). The most obvious explanation for this effect is that Rad4 is unstable in *rad33* cells. We here examined the levels of Rad4 in wildtype and *rad33* cells incubated in the presence of cycloheximide (CHX), blocking *de novo* protein synthesis.

Figures 1B and 1C show the Rad4 protein levels in wildtype and *rad33* cells respectively. Quantifications of the blots are shown in the right panels (Fig 1BC). Figure 1D shows data similar to those in figures 1B and 1C but here extracts from wildtype and *rad33* cells are loaded on the same gel (only with CHX) in order to be able to compare the levels in the different backgrounds. Consistent with our previous results (den Dulk *et al.*, 2006) and the data in figure 1A, figure 1D shows that the amount of Rad4 is about 3 fold lower in Rad33 deficient cells. Furthermore, the quantification data (Fig. 1B-D, right panels) indicate that CHX treatment does not markedly influence the levels of Rad4 protein in wildtype or *rad33* cells. We find similar results when the native Rad4 levels in wildtype and *rad33* cells are compared using anti-Rad4 (Fig 1E).

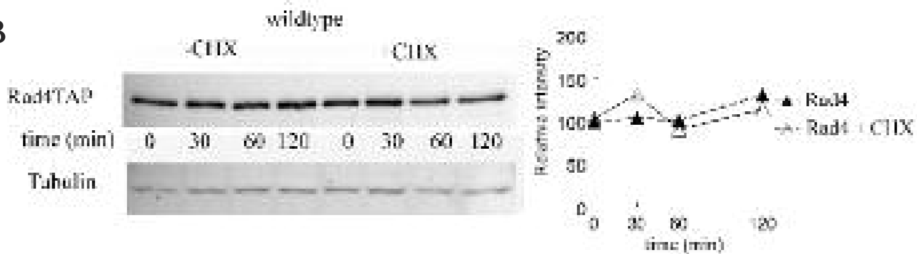
3.2 Rad4 is modified after UV irradiation in rad33 cells.

The stability of Rad4 in *rad33* cells discussed above (Fig. 1B-E) is not altered after UV irradiation (Fig. 2A). Interestingly, longer exposures of the blot shown in figure 2A reveal a clear UV induced modification of Rad4 in *rad33* cells (Fig. 2B). The modification of Rad4 is visible at low doses of UV, starting at 2 J/m^2 and does not further increase with doses higher than 8 J/m^2 (Fig. 2D). Under the same conditions, no modification of Rad4 is observed in wildtype cells (Fig. 2C) or in *rad23* cells (data not shown). In *rad33* cells exposed to a UV dose of 8 J/m^2 the modification appears ~5 minutes after UV irradiation and reaches a maximum around 25 minutes after UV treatment (Fig. 2F). During identical treatment Rad4 modification is not detected in Rad33 proficient cells. However, prolonged exposure of the film also reveals some UV

A



B



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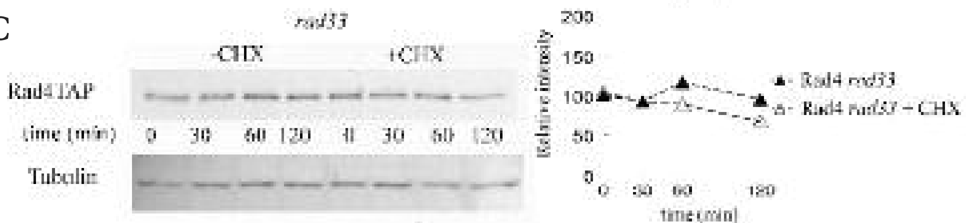

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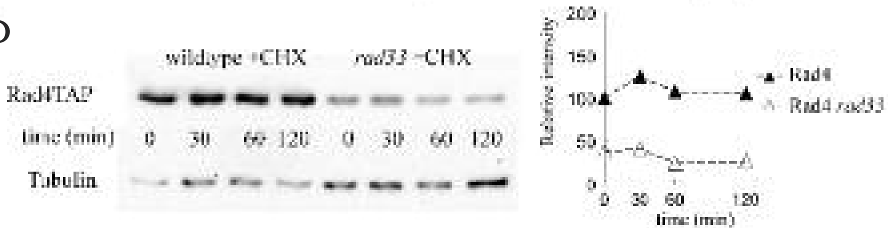

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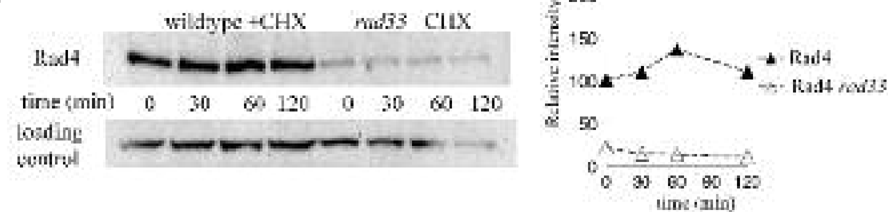

irradiation induced modification of Rad4 in wildtype background (Fig. 2G).

Based on quantification of several western blots we estimate that in *rad33* cells the ratio of modified Rad4 in relation to non-modified Rad4 is increased ~50 fold compared to the ratio in wildtype cells. In the absence of Rad33 roughly 10% of the Rad4 protein is modified 30 minutes after UV irradiation. After 120 minutes, the Rad4 modification in *rad33* cells was reduced to ~4%. In wildtype cells ~3 fold more Rad4 protein is present, here we find a modified fraction of only ~0,2%. Thus, the absolute amount of modified Rad4 in wildtype cells is ~15 fold lower compared to the amount

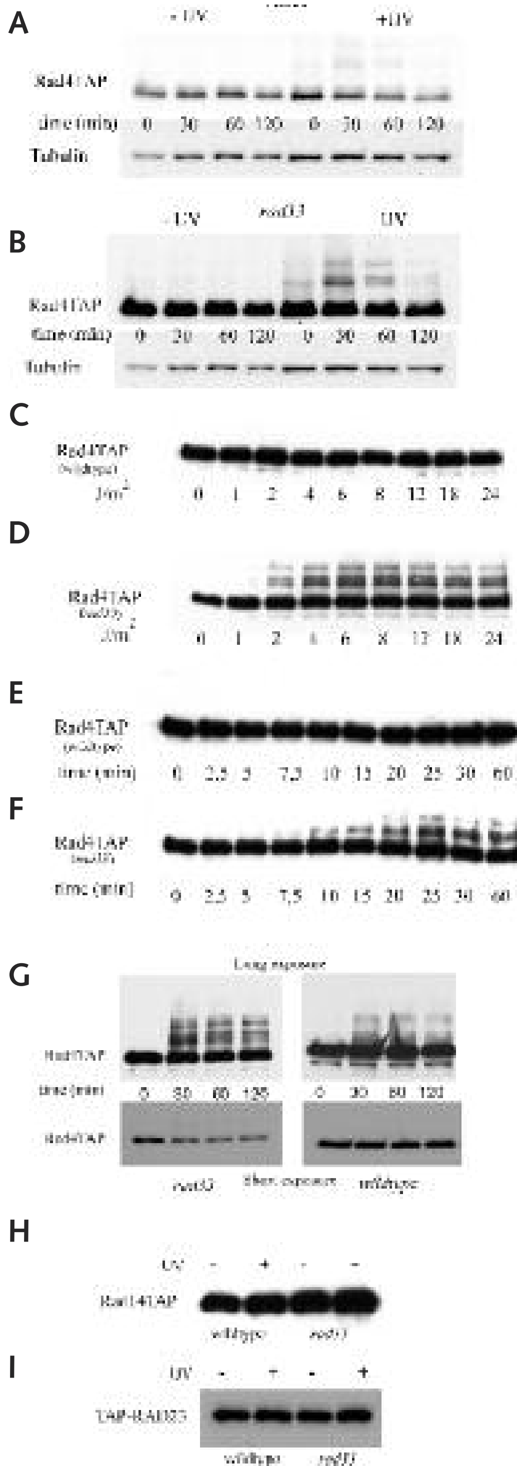

Figure 2
Western blot analysis. TCA extracts of cells with TAP-tagged Rad4 were analysed on western blot using PAP antibodies. Cells were suspended in Phosphate Buffered Saline (PBS), irradiated and resuspended in YPD. At time-points indicated, cells were harvested and TCA extracts were prepared.
(A) Rad4TAP levels in *rad33* cells analysed 0, 30, 60 and 120 minutes after UV irradiation or mock treatment. Cells were recovered in the presence of cycloheximide.
(B), as (A), but showing a longer exposure of the film.
(C) Rad4TAP in wildtype cells after UV irradiation with the doses indicated. Cells were irradiated in PBS and were recovered in YPD for 30 minutes.
(D) As (C), but for *rad33* cells.
(E) Rad4TAP in wildtype cells irradiated with 8 J/m^2 harvested at the indicated time points after irradiation.
(F) as (E), but for *rad33* cells.
(G) Left panels: Rad4TAP from *rad33* cells 0, 30, 60 and 120 minutes after UV irradiation. The lower left panel is identical to the upper panel with the exception of the exposure time. The right panels are similar to the left panels but here Rad4TAP from wildtype cells is analysed. Quantification indicates that, relative to the band intensity of the unmodified Rad4, the modification is ~50 times more intense in *rad33* cells compared to that in wildtype cells.
(H) Rad14TAP in UV irradiated (+) or mock treated (-) wildtype or *rad33* cells. Cells were recovered in YPD for 30 minutes before preparation of TCA extracts.
(I) As (H), but for TAP-Rad23 .

in *rad33* cells.

Two other TAP tagged NER proteins involved in the early steps of NER, Rad14 and Rad23, are not modified in irradiated *rad33* cells, indicating that the UV induced modification, which is strongly increased in *rad33* cells, is not a general effect (Fig. 2H and I).

3.3 The nature of the Rad4 modification

It was reported that XPC, the human homologue of Rad4, is ubiquitylated upon UV irradiation (Sugasawa *et al.*, 2005; Sugasawa, 2006; Wang *et al.*, 2005) and ubiquitylation of Rad4 was demonstrated *in vitro* (Gillette *et al.*, 2006). It is therefore possible that ubiquitin is also involved in the modification of Rad4 we observe here. To examine this, modified Hemagglutinin (HA) tagged Rad4 was purified from wildtype and *rad33* cells (Fig. 3A) and analyzed on western blot for interaction with anti-ubiquitin antibodies. However, no signal could be detected when the blot was probed with anti-ubiquitin antibodies from two different suppliers (data not shown). In an alternative approach beads coated with UBA (Ubiquitin Associating) domains were used, which were shown to bind to ubiquitin or ubiquitylated proteins (Wilkinson *et al.*, 2001). Cell extracts of UV irradiated *rad33* cells were incubated with the UBA coated beads. The bound fraction of the beads incubated with extracts from UV irradiated cells shows a marked increase of the modified species of Rad4 whereas these bands are hardly detectable in the bead-bound fraction of cell extracts from unirradiated *rad33* cells. From quantification of the blots it is estimated that for the UV irradiated cells the ratio of modified versus non-modified Rad4 is increased ~8 fold in the bead bound fraction compared to the whole cell extract. The binding to the UBA beads was most clear using extracts from cells in which ubiquitin is overexpressed (Fig. 3B). To check whether the UBA beads actually enriched the ubiquitylated protein pool we stripped the

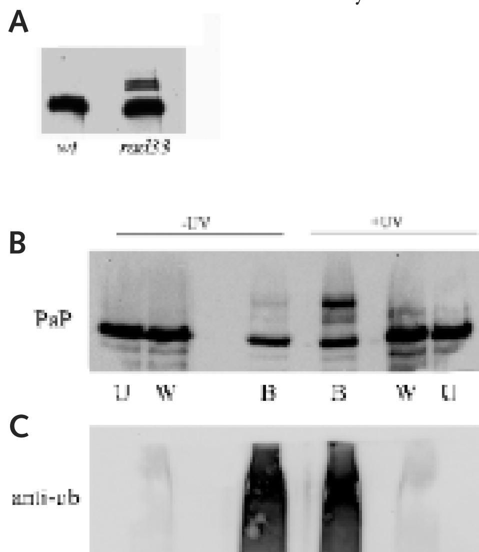

Figure 3
(A) HA-Rad4 immunoprecipitation. Wildtype and *rad33* cells expressing a genomic amino-terminally HA tagged *RAD4* gene were grown for three days. Cell extracts were made in RIPA buffer using glass-beads. Cell extracts were bound for three hours to ProtA beads (Amersham) coated with 12CA5 anti-HA antibodies. The beads were washed and the remaining proteins were boiled in loading buffer, run on 7,5% SDS/PAGE and analysed on western blot. Western blots were probed with 12CA5 anti-HA antibodies.
(B) UBA-beads binding experiment. Cell extracts from UV irradiated or mock treated Rad4TAP*rad33* cells in which additional ubiquitin is expressed from a YEp112K plasmid (Hochstrasser *et al.*, 1991) were incubated for 4 hours with BIOMOL ubiqapture beads. The beads were washed thoroughly with RIPA buffer and the bead-bound fraction (denoted 'B' in the figure) was boiled in loading buffer, analyzed on western blot and compared with the whole cell extract 'W' and the unbound fraction 'F'. Rad4TAP is visualized using PAP antibody.
(C), as (B), but blot probed with mouse anti-ubiquitin antibody (Zymed laboratories).

blot and probed it with anti-ubiquitin. The results clearly show enrichment of ubiquitylated proteins (Fig. 3C), confirming the functionality of the beads. Whereas the results from the UBA bead experiment imply that Rad4 is ubiquitylated the modification was still present in *rad33* cells deleted for any of the genes encoding non-essential E2 ubiquitin conjugases (*RAD6, UBC4, UBC5, UBC7, UBC8, UBC11 UBC12, UBC13)* (data not shown), indicating that these E2 enzymes are probably not involved in the observed modification.

3.4 Modification of Rad4 is dependent on Rad26.

The notion that Rad4 modification is triggered by UV irradiation suggests a relationship with the NER process. To examine this we checked whether the core NER proteins Rad2 and Rad14 are required for the modification of Rad4. Figure 4A shows that the deletion of neither *RAD2* nor *RAD14* affects the modification, demonstrating that the incision reaction is not required for the modification event.

In human cells ubiquitylation of XPC is dependent on the UV-DDB complex. UV-DDB functions in complex with Cul4A and Roc1 and acts as a Cullin-RING ubiquitin ligase (Sugasawa *et al.*, 2005). There are no clear sequence homologues of DDB1 or DDB2 in *S. cerevisiae* but several indications suggest that the Rad7-Rad16 complex may have a similar function. The Rad7-Rad16 complex binds to Cul3 and the elongin C homologue Elc1. This four-protein complex bears the hallmarks of a typical Cullin-

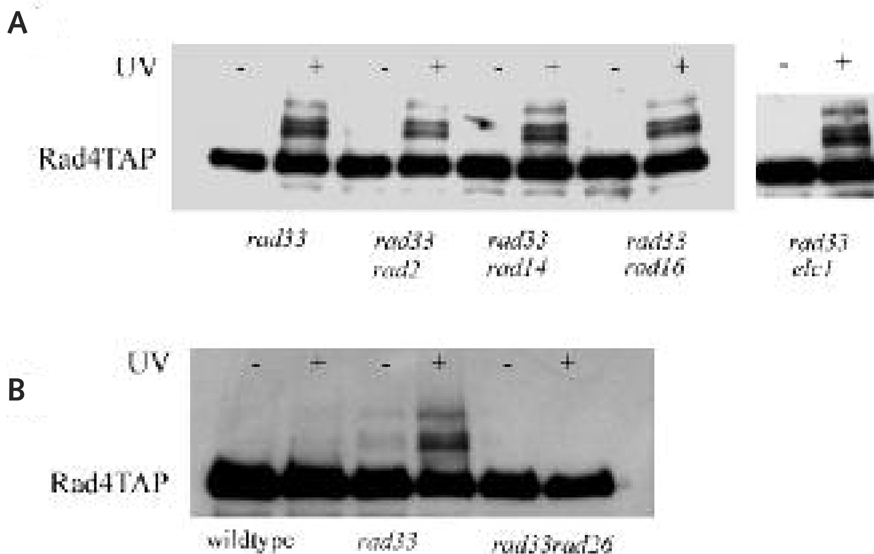

Figure 4

Western blot analysis. TCA extracts of cells containing TAP-tagged Rad4 were analysed on western blot using PAP antibody. Cells were harvested and suspended in PBS, irradiated and resuspended in YPD medium. After 30 minutes TCA extracts of the cells were prepared.

(A) Rad4TAP in *rad33* cells, *rad33rad2* cells, *rad33rad14* cells, *rad33rad16* cells and *rad33elc1* cells 30 minutes after UV irradiation (+) or mock treatment (-).

(B) Rad4TAP in wildtype cells, *rad33* cells or *rad33rad26* cells 30 minutes after UV irradiation (+) or mock (-) treatment.

RING ubiquitin ligase and is suggested to be functionally homologous to UV-DDB (Ramsey *et al.*, 2004). Moreover, it was shown that this complex is able to mono-ubiquitylate Rad4 *in vitro.* (Gillette *et al.*, 2006). We studied the possible role of the Rad7-Rad16-Cul3-Elc1 complex in the UV induced modification of Rad4 in *rad33* cells. Figure 4A shows that the modification of Rad4 is not dependent on Rad16 or Elc1 which implies that the modification we observe is regulated in a different way. Surprisingly, the TCR factor Rad26 appeared essential for the increased modification as it was abolished in *rad33* cells deleted for *RAD26* (Fig. 4B). The requirement of Rad26 suggests that Rad4 modification in *rad33* cells depends on the coupling of transcription to NER.

3.5 Rad33 binds to Rad4 via direct interaction

In large scale interaction screens the Rad33 protein is found to co-precipitate with both the Rad4 and Rad23 proteins (Gavin *et al.*, 2002; Krogan *et al.*, 2006). However, the affinity capture experiments do not reveal whether Rad33 binds Rad4 via a direct interaction or via Rad23 or other proteins. To gain more insight in the binding of Rad33 to the Rad4-Rad23 complex, we assessed interactions between Rad4, Rad23 and Rad33 using a yeast two-hybrid system. Since the full-length Rad4 protein is lethal in *E.coli* (Fleer *et al.*, 1987; Siede and Eckardt-Schupp, 1986), the amino-terminal domain of Rad4 (residues 1-277) and the evolutionary conserved domain between residues 274 and 667 (Bateman *et al.*, 2004; Finn *et al.*, 2006) were tested separately.

Figure 5 shows that there is no interaction of the amino terminal Rad4 fragment (residues 1-277) with either Rad23 or Rad33. Rad4(274-667) binds to both Rad23 and Rad33 (Fig. 5A). On the other hand, Rad33 shows no interaction with Rad23, indicating that Rad33, like Rad23, binds Rad4 directly.

3.6 Rad33 binds Rad4 via conserved residues which are essential for the XPC-Centrin2 interaction.

No sequence homologue of Rad33 was found in the human genome database. In order to gain insight in the structure of Rad33 we used mGenthreader to predict the structural features of Rad33 and compared the results with a database of solved structures

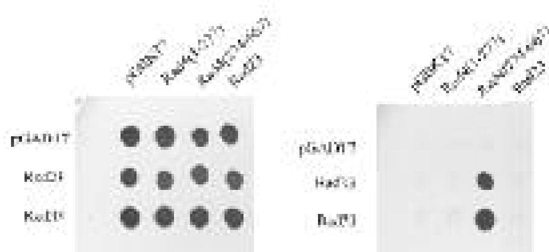

Figure 5
Two hybrid test. The *RAD4* region coding for residues 1-277, the *RAD4* region coding for residues 274-667 and the *RAD23* and *RAD33* genes were cloned in the pGBKT7 or pGADT7 vectors (Clontech Matchmaker 3) as indicated in the figure. The vectors and constructs were introduced into Y187 and AH109 as described in materials and methods. The cells were assayed for growth on YNB medium selective for the presence of both plasmids (left panel) and on YNB medium selective for transcription activation of the reporter genes (*HIS* and *ADE)* (right panel), which is indicative of interaction of the proteins tested.

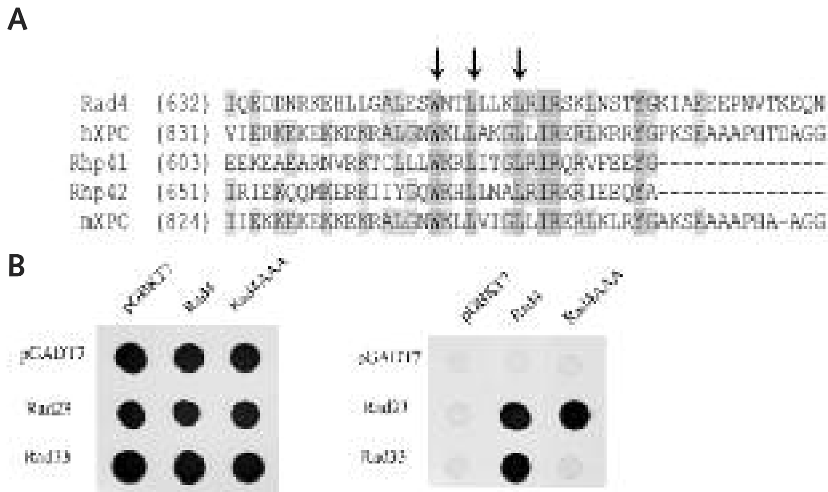

Figure 6
(A) Alignment of Rad4 homologues. The Rad4 protein was aligned with the human and mouse XPC proteins and the two *S. pombe* Rad4 homologues Rhp41 and Rhp42 using Invitrogen AlignX. The conserved WLL residues required for the XPC-Centrin2 interaction are marked with arrows.
(B) Two hybrid test. The Rad4 and Rad4AAA clones contain the GAL4 binding domain (BD) fused to the coding sequences for residues 274-667 (bp 822-2262) of the wildtype and the mutant gene respectively. Rad23 and Rad33 are GAL4 activator domain (AD) fusion clones as described in figure 5. pGADT7 and pGBKT7 are the empty vectors. Interactions are assayed as described in the materials and methods section.

(Jones, 1999). The blast search returned several candidates which, albeit with medium or low confidence, showed resemblance with the predicted structure of Rad33. It is noticeable that all proteins retrieved from the database are calmodulin-like proteins. The most similar structure is that of the yeast Cdc31 protein (table 2).

Interestingly, XPC also binds a calmodulin like protein, Centrin2 (Araki *et al.*, 2001), which is one of the human homologues of Cdc31. The interaction between XPC and Centrin2 has been intensively studied and three amino acids in XPC required for this interaction were identified (Nishi *et al.*, 2005; Popescu *et al.*, 2003; Thompson *et al.*, 2006; Yang *et al.*, 2006). The residues involved in the XPC-Centrin2 interaction are conserved and present in all known Rad4 homologues (Nishi *et al.*, 2005 and Fig. 6A). The two-hybrid experiments (Fig. 5) show that Rad4 interacts directly with Rad33 and Rad23 via the highly conserved domain between residues 274 and 667 (Bateman *et al.*, 2004; Sonnhammer *et al.*, 1997). In XPC this conserved domain also contains the binding site for both HHR23B and Centrin2. To investigate a possible parallel between the

Table 2. mGenThreader results of Rad33 structural resembling proteins.

Protein	Confidence	PBD-ID	Species
Cdc31	Medium	2doq-C0	*Saccharomyces cerevisiae*
Myocin light chain	Low	2bl0-C0	*Physarum polycephalum*
E-LC	Low	1wdc-C0	*Aequipecten irradians*
Calcium-binding protein	Low	2scp-A0	*Nereis diversicolor*
Calcium-binding protein	Low	1jfj-A0	*Entamoeba histolytica*

roles of Rad33 and Centrin2 in the yeast and human damage recognition complexes respectively we examined whether the conserved residues in XPC required for binding of Centrin2 to XPC are also implicated in the binding of Rad33 to Rad4.

The three residues W649, L652 and L656 (WLL residues, figure 6A) in the conserved region of Rad4 were replaced by alanines (referred to as the *rad4AAA* mutation). The *RAD4* fragment containing the *rad4AAA* mutation was cloned in the pGBKT7 vector and used for two-hybrid experiments. The Rad4AAA protein does bind Rad23 which implies that the overall conformation has not changed dramatically (Fig. 6B). Interestingly, Rad33 does not interact with the Rad4AAA protein (Fig. 6B) showing that Rad33 binds Rad4 via the WLL motif.

3.7 Cells lacking the Rad4-Rad33 interaction.

To examine the UV sensitivity of cells in which the Rad4-Rad33 interaction is disrupted we introduced the *rad4AAA* mutation in wildtype cells and in *rad33* cells and checked the UV sensitivity of the resulting mutants. Figure 7A shows that the introduction of the *rad4AAA* mutation leads to moderate UV sensitivity, similar to that of *rad33* mutants. Interestingly, the additional deletion of *RAD33* in a *rad4AAA* mutant does not lead to an increase in UV sensitivity (Fig. 7A). This demonstrates that the NER defect of the *rad4AAA* cells is the specific result of the disrupted Rad4-Rad33 interaction.

The *rad4AAA* mutation allows us to study the effect of the loss of Rad33 from the Rad4-Rad23 complex on the modification of Rad4, without interfering with any other possible roles of Rad33. The modification of the Rad4AAA protein from UV irradiated wildtype or *rad33* cells was compared to the modification of wildtype Rad4. In agreement with earlier experiments (Fig. 2C and D) the high molecular species of Rad4 were observed in UV irradiated *rad33* cells but not in wildtype background (Fig. 7B). In lane 6 of figure 7B the modification of the Rad4AAA protein is shown. It is evident that the modification of Rad4AAA is very similar to that of the wildtype Rad4 protein analyzed in *rad33* background as seen in lane 4. Importantly, additional deletion of *RAD33* in *rad4AAA* mutants does not alter the modification. This proves that the increase in UV dependent modification of Rad4 in *rad33* cells is not caused by an indirect effect linked to the *rad33* deletion, but due to the disruption of the interaction between Rad4 and Rad33.

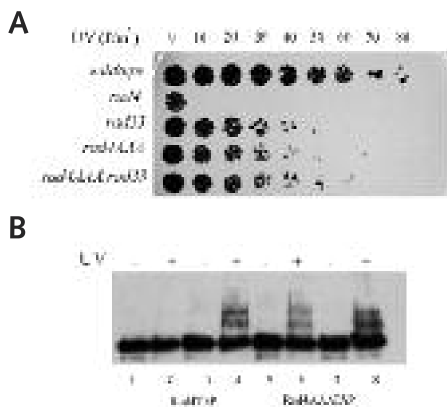

Figure 7.
(A) UV survival droptest. Wildtype, *rad4*, *rad33*, *rad4AAA* and *rad4AAArad33* cells were grown for 3 days in YPD. Appropriate dilutions of the cells were spotted on YPD plates and irradiated with UV as indicated. Cells were grown for 3 days in the dark at 30°C. **(B)** Western blot analysis. TCA extracts of UV irradiated (+) or mock treated (-) wildtype and *rad33* cells expressing Rad4TAP or Rad4AAATAP were prepared as described in the materials and methods and were analysed on western blot using PAP antibody. Lanes 1-4 show Rad4TAP in wildtype (lane 1-2) or *rad33* (lane 3-4) extracted from UV irradiated (+) or mock (-) treated cells. Lanes 5-8 are similar to lane 1-4, but here cells expressing Rad4AAATAP are analysed.

4 Discussion

The Rad4-Rad23 complex is responsible for initial damage recognition in NER in *Saccharomyces cerevisiae* (Guzder *et al.*, 1998; Jansen *et al.*, 1998; Min and Pavletich, 2007). We recently identified Rad33, a NER factor that might also be involved in this process (den Dulk *et al.*, 2006). Rad33 is found in complex with both Rad4 and Rad23 (Krogan *et al.*, 2006). Our data demonstrate that Rad33 binds directly to Rad4 and not to Rad23. Moreover, distinct sites on the Rad4 protein are involved in the binding of Rad33 and Rad23 respectively, indicating that the three proteins might exist in one complex.

We have previously shown that Rad4 steady state levels are lower in cells lacking Rad33 (den Dulk *et al.*, 2006). By analyzing protein levels in the absence of *de novo* protein synthesis we here demonstrate that the decreased amount of Rad4 protein in *rad33* cells are not the result of instability of Rad4. The reduction might be the consequence of lower Rad4 synthesis when Rad33 is absent. In UV irradiated *rad33* cells Rad4 is also stable, thus, in contrast to what is reported on Rad4 in *rad23* cells (Lommel *et al.*, 2002; Ortolan *et al.*, 2004; Xie *et al.*, 2004) our observations show the reduced levels of Rad4 protein in *rad33* cells are relatively stable.

Our studies here show that UV induced modification of Rad4 is strongly increased in *rad33* cells. Since XPC is ubiquitylated upon UV irradiation (Sugasawa *et al.*, 2005; Wang *et al.*, 2005) we examined whether the modification of Rad4 is the result of ubiquitylation as well. We obtained evidence that ubiquitin is involved but we could not unambiguously determine the nature of the modification. None of the non-essential E2 ubiquitin conjugases appear to be involved in the modification and also anti-ubiquitin antibodies from two different suppliers failed to detect the modified Rad4. Also when we precipitated modified Rad4 from cells over-expressing MYC-tagged ubiquitin we yet could not detect any MYC-ubiquitin in the Rad4 precipitates (data not shown). In an alternative approach, beads coated with Ubiquitin Associating (UBA) domains were used. The relative amount of modified Rad4 was increased in the bead-bound fraction, indicating that Rad4 is ubiquitylated. It is noticeable that the higher band of the modification is especially enriched. This band is the faintest of the modified Rad4 in whole cell extracts and hardly visible in Rad4-precipitates. This might explain the difficulty to obtain a signal using anti-ubiquitin (or anti-MYC).

Our observations here do not provide elucidation of the nature of the modification and at this point we cannot exclude that Rad4 is modified by post translational modifications different from ubiquitylation, like sumoylation.

The modification of Rad4 in *rad33* cells is not dependent on the incision reaction since it is not inhibited in the absence of the core NER proteins Rad2 and Rad14. The recently identified Rad7-Rad16-Cul3-Elc1 E3 ligase complex, that is shown to monoubiquitylate Rad4 *in vitro* (Gillette *et al.*, 2006), is also not involved since deletion of *ELC1* or *RAD16* does not affect the modification. Surprisingly, we find that Rad26 is essential for the increased UV induced modification of Rad4, suggesting that the coupling of NER to the transcription machinery is essential for the modification to take place. The faintly visible modification of Rad4 in wildtype cells appears however not dependent on Rad26. Long exposures of the blots showed Rad4 modification in *rad26* cells reminiscent of that in wildtype cells. This type of modification is apparently not

related to Rad26 dependent TCR.

The augmented modification of Rad4 is likely to be related to the NER defect of *rad33* cells. However, it is unclear whether this effect is the cause or the consequence of the impaired NER reaction. If the increased modification of Rad4 is assumed to be the (partial) cause of the repair defect in *rad33* cells, it is expected that inhibition of the modification event would (partially) suppress the repair defect of *rad33* cells. However, this assumption is opposed by the fact that *rad33rad26* cells lack Rad4 modification but are severely NER deficient compared to *rad33* single mutants. It is therefore more likely that the Rad4 modification is the result rather than the cause of the defective NER in *rad33* cells. The modification of Rad4, like ubiquitylation of XPC, might be required for efficient NER of certain lesions. The observation that modified Rad4 is hardly detectable in wildtype cells might indicate that the modified Rad4 is quickly processed in the presence of Rad33. Deletion of *RAD33* possibly causes a delay or blockage in the processing of the reaction intermediate involving the modified Rad4, thereby causing a net increase in modified protein.

No clear sequence homologue of Rad33 was identified in higher eukaryotes, however, fold recognition analysis (Jones, 1999) in our study shows structural resemblance of Rad33 to the yeast Cdc31 protein. Cdc31 is the yeast homologue of the human Centrin2 protein. Noticeably, Centrin2 binds to the human Rad4 homologue XPC (Araki *et al.*, 2001). Centrin2 is one of the three identified centrin isoforms in humans which are essential for duplication and segregation of the microtubule organization centers (MTOC), known as the spindle pole bodies (SPB) in yeast (Baum *et al.*, 1986; Middendorp *et al.*, 2000; Salisbury *et al.*, 2002). However, more than 90% of the Centrin proteins in the cell are not associated with the centrosome (Paoletti *et al.*, 1996) indicating involvement of these proteins in other processes.

In recent years a role of Centrin2 in human NER has been described. Addition of Centrin2 to *in vitro* NER reactions stimulates NER activity, possibly by stabilization of XPC (Araki *et al.*, 2001). The Centrin2-XPC complex is extensively studied and three amino acids in XPC that are essential for the interaction with Centrin2 are identified (Nishi *et al.*, 2005; Popescu *et al.*, 2003). Cells in which the XPC-Centrin2 interaction is disrupted show significantly reduced GGR. Biochemical analysis of XPC, HHR23B and Centrin2 shows that the binding of Centrin2 stimulates the DNA-binding activity of XPC (Bunick *et al.*, 2006). HHR23B has a similar effect but in contrast to Centrin2 is found to dissociate upon the binding of XPC to DNA (Bunick *et al.*, 2006; Nishi *et al.*, 2005; You *et al.*, 2003).

There is yet no indication that Cdc31, the only Centrin homologue in *S. cerevisiae*, is involved in NER since all the large scale interaction studies performed failed to show an interaction of Cdc31 with Rad4, Rad23 or any other NER protein. In this study we show that Rad33 binds Rad4 via the three amino acids that correspond to the residues required for the XPC-Centrin2 interaction. Importantly, genetic disruption of this interaction results in mutants with a phenotype similar to *rad33* deletion cells, showing that the role of Rad33 in NER is completely dependent on its interaction with Rad4. The data presented here and in our previous paper show similarities between the defects of yeast and human cells in which the Rad4-Rad33 and XPC-Centrin2 interaction is disrupted respectively; in both mutants GGR appears to be defective and the protein levels of both Rad4 and XPC are lower compared to wildtype cells. This observation,

combined with the predicted structural resemblance and the fact that Rad33 and Centrin2 bind to Rad4 or XPC via the same conserved motif, might suggest that the role of Rad33 in the Rad4-Rad23 complex is similar to that of Centrin2 in human XPC-HHR23B complex.

A striking resemblance is observed when XPC is modeled on the recently published crystal structure of Rad4 (Min and Pavletich, 2007), suggesting that Rad4 and XPC recognize lesions via a comparable mechanism. The Rad4 protein of which the crystal structure was determined lacks the carboxy-terminal part that is essential for the interaction with Rad33 (Min and Pavletich, 2007). This region is presumed to be unstructured and flexible in both Rad4 and XPC (Charbonnier *et al.*, 2007; Min and Pavletich, 2007). Data derived from the crystal structure of Centrin2 bound to the carboxy-terminal fragment of XPC implies that the binding of Centrin2 stabilizes the unstructured XPC region. Binding of Rad33 to Rad4 might have a comparable effect. The conformational change in the carboxy-terminal part of Rad4 upon binding of Rad33 may stimulate the affinity for damaged DNA, as is shown for XPC by Nishi *et al.* (Nishi *et al.*, 2005). Since this region is also involved in the XPC-TFIIH interaction (Uchida *et al.*, 2002; Yokoi *et al.*, 2000) it is also suggested that the ability to recruit TFIIH might be altered by binding of Centrin2 (Charbonnier *et al.*, 2007). Given the analogy with Centrin2, Rad33 might be involved in the same mechanism.

The role of Rad23 in the Rad4-Rad23 complex as well as the function of the post-translational modification of Rad4 and XPC is still under debate (Bergink *et al.*, 2007; Reed and Gillette, 2007; Sugasawa, 2006). Yet, several studies imply that Rad23, sumoylation and ubiquitylation cooperate in the tight regulation of the protein levels and the DNA binding properties of Rad4 and XPC, thereby providing flexibility to the NER damage recognition process. Our present data suggest that Rad33 is an additional factor affecting damage recognition by the Rad4-Rad23 complex. The adaptive nature of damage recognition could be essential for quick initiation of NER only when it is required, avoiding interference with other metabolic processes acting on undamaged DNA (Bergink *et al.*, 2007; Bunick *et al.*, 2006; Gillette *et al.*, 2006; Wang *et al.*, 2007). The parallels between Centrin2 and Rad33 might imply that these proteins modulate the function of XPC and Rad4 respectively. The elucidation of their roles will contribute to the understanding of the mechanism of damage recognition and the involvement of post translational modifications in this process.

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Chapter 7

Summary and concluding remarks

Summary and concluding remarks

DNA constitutes the basis of heredity, but does not possess the molecular characteristics to provide the stability required to maintain genetic information. DNA is subjected to many modifications that are caused by various endogenous and exogenous agents of both chemical and physical nature. These DNA adducts interfere with essential cellular processes like transcription and replication, the failure of which can lead to cell death. Moreover, incorrect replication of damaged bases can give rise to mutations in the genome which can have several deleterious effects, including premature ageing and cancer. To safeguard the integrity of the DNA, lesions are continuously removed by a variety of DNA repair mechanisms. In **chapter 1** a general introduction to repair mechanisms is given.

The topic of this thesis is the repair system Nucleotide Excision Repair (NER), introduced in **chapter 2.** NER recognizes and removes a broad spectrum of structurally unrelated DNA lesions, including CPDs (cyclobutane pyrimidine dimers) and (6-4)PPs (pyrimidine (6-4) pyrimidone photoproducts), caused by UV irradiation emitted by the sun. The biological significance of the NER system is exemplified by the severe clinical disorder Xeroderma Pigmentosum (XP) that is the result of a defect in NER and characterized by a dramatic increase in the occurrence of skin cancer.

NER operates in multiple steps and requires the action of several proteins. First, the lesion is detected by NER damage sensors. The two strands are then separated after which single stranded nicks are made both 5' and 3' to the lesion. A 24-32 residue oligonucleotide containing the lesion is then removed and the DNA is restored by the replication machinery. NER is divided in two sub-pathways: Global Genome Repair (GGR), which removes lesions throughout the entire genome, and Transcription Coupled Repair (TCR), specifically involved in repair of transcriptionally active genes. These sub-pathways differ in the way by which the lesion is detected: GGR relies on specific NER factors that probe the genome for lesions whereas TCR makes use of damage-arrested transcription machinery as a signal to initiate the NER reaction. The basic NER system is conserved throughout evolution and in the eukaryotic kingdom the proteins that carry out the NER reaction display considerable homology. In this thesis the yeast *Saccharomyces cerevisiae* is used as a model organism to study the NER mechanism.

Chapter 3 discusses a particular challenging phase in GGR, the recognition of different, seemingly unrelated, lesions within a vast excess of undamaged nucleotides. A possible model how recognition is achieved is described. The NER factor Rad4-Rad23/XPC-hHR23B binds DNA that exhibits a general deviation from the standard Watson-Crick basepairing. These irregular DNA regions are then further inspected by the subsequently recruited NER factor TFIIH, which functions in strand separation. While using its helicase activity to displace the strands of the DNA, TFIIH will arrest when a chemically modified nucleotide is encountered. This blockage might be essential for the assembly of the NER complex at the site of the damage.

It is generally established that Rad4, the yeast homologue of XPC, is a central protein in the damage recognition process. The following chapters describe studies on the Rad4 protein, its homologue Rad34 in yeast (**chapter 4**) and a new binding factor Rad33 (**Chapter 5-6**). In contrast to the other NER proteins that are essential for the

core-NER reaction (i.e., the NER reaction reconstituted *in vitro*), the requirement of Rad4 in NER differs from that of its human homologue XPC. In human cells XPC is essential for GGR but is not required in the TCR sub-pathway whereas yeast Rad4 is necessary for both TCR and GGR of RNA polymerase II transcribed DNA. NER does not only operate in regions transcribed by RNA polymerase II (which comprises the great majority of the genome), but is also active in the RNA polymerase I transcribed ribosomal DNA (rDNA) region. The rDNA locus is comprised of ~150 tandemly repeated units of 9.1 kb. Around 50% of these repeats is transcriptionally active, the remainder is silenced. We earlier showed that Rad4 is required for GGR of the *rRNA* genes whereas preferential repair of the RNA polymerase I transcribed strand occurs independent from the Rad4 protein.

In **chapter 4** we report that the Rad4-independent NER in the rDNA locus is carried out by a newly identified Rad4 homologue in *S. cerevisiae*, Rad34 (YDR314C). This protein is exclusively required for preferential repair (most likely TCR) of the transcribed strand of the rDNA region. Rad34 can not substitute for Rad4 in NER of RNA polymerase II transcribed DNA, and Rad4 is unable to replace Rad34.

In a supplement of **chapter 4** (**chapter 4.1**) we investigated what properties of the Rad4 homologues determine that they are specifically active in their respective target loci. Although it seemed obvious that the poorly conserved amino-terminal domain is responsible for the divergent roles of Rad4 and Rad34, hybrid proteins in which these non-conserved regions were swapped between the Rad4 homologues are not functional. This indicates that certain elements of the amino terminal domains of Rad4 and Rad34 specifically cooperate with their respective carboxy terminal domains, and can not do so with the carboxy terminal domain of the other Rad4 homologue.

The results presented in **chapter 4** raise the question why a specific Rad4 homologue exists that functions only in NER of the RNA pol I transcribed strand of the relatively small rDNA region. The data suggest that the different RNA polymerase functioning in the rDNA region is the reason for the requirement of Rad34. To conclusively show that Rad34 is a TCR factor additional experiments are required. For example, the transcriptionally active and inactive rDNA fractions can be separated and subjected to DNA repair assays. In addition, elongating and damage-arrested RNA polymerase I complexes can be isolated and analysed for associated NER factors. This will show whether Rad34 is physically associated with RNA polymerase I and/or the NER complex, and if so, whether Rad34 is recruited in response to genotoxic treatment or already present on the elongating transcription complex. As *rad34* cells are not UV sensitive, it is yet uncertain whether Rad34 is primarily a NER factor. In this light it will be interesting to investigate whether Rad34 binds (damaged) DNA and if and how Rad34 contributes to an *in vitro* NER reaction (lacking Rad4) on naked DNA.

In **Chapter 5** another newly identified NER factor, Rad33, is introduced. Cells lacking Rad33 are moderately UV sensitive. DNA repair analysis showed that GGR is completely abolished in *rad33* cells while TCR is severely impaired. Two-hybrid tests showed that Rad33 binds to both Rad4 and Rad34, but not to Rad23, suggesting that Rad33 is part of the Rad4-Rad23 and Rad34-Rad23 complexes. The amount of Rad4 and Rad34 protein is reduced in the absence of Rad33. However, the data discussed in

chapter 5 indicate that the lower level of Rad4 protein is not the sole cause of the NER defect in *rad33* cells.

Chapter 6 reports the involvement of Rad33 in UV-induced post-translational modification of Rad4. We showed that this modification is at least in part consisting of ubiquitin. In wildtype yeast cells the UV induced modification of Rad4 is hardly visible, but in *rad33* mutants, or in cells in which the interaction between Rad4 and Rad33 is genetically disrupted, the relative amount of modified Rad4 protein increases dramatically. Rad4 seems to be modified when engaged in the TCR sub-pathway, as deletion of *RAD26* in *rad33* cells abolishes the augmented modification. This may indicate that the modification of Rad4 is an integrated part of the TCR reaction. In the absence of Rad33 TCR is clearly less efficient (~50%) and for this reason the relative amount of modified Rad4 may be enhanced in *rad33* cells. Alternatively, Rad33 may regulate the activity of Rad4 by physically shielding Rad4 from modification. The modification of Rad4 is reminiscent of the ubiquitylation of the human Rad4 homologue XPC. For XPC, it is known that the ubiquitylated species possess an enhanced DNA binding activity. However, the modification of Rad4 seems to be related to TCR whereas the UV induced ubiquitylation of XPC in human cells is a GGR specific event. Yet, we cannot exclude that ubiquitylation also plays a role in the GGR reaction. The absence of Rad33 may render the Rad4-Rad23 complex incapable of reaching lesions in non-transcribed DNA. To learn more about the role of the modification of Rad4 the complete nature of the modified Rad4 species has to be determined. It will also be useful to investigate whether the modified Rad4 protein possesses an altered affinity for (damaged) DNA.

Rad33 does not have a clear human sequence homologue, however, the data presented in **chapter 6** show that the predicted structure of Rad33 bears resemblance to the solved structure of the Cdc31 protein. Cdc31 is the homologue of human Centrin2. In human cells Centrin2 is part of the XPC-hHR23B complex and known to enhance the efficiency of the NER reaction *in vivo* and *in vitro*. We showed that Rad4 binds to Rad33 via the same conserved residues that connect XPC to Centrin2, indicating that the function of Rad33 may be similar to that of Centrin2 in NER in human cells. It was recently reported that Cdc31 also binds to Rad4, but the relevance of the interaction between Cdc31 and Rad4 for the NER reaction remains to be explored. Possibly, Cdc31 and Rad33 are both involved in the regulation of the Rad4-Rad23 complex.

Concluding remarks

The experiments presented in this thesis show that Rad33 is an important factor for NER in *S. cerevisiae*, but more studies will be required to fully elucidate the role of this protein. *In vitro* studies of Rad33 in DNA binding experiments and in reconstituted NER assays will provide a hint on the contribution of Rad33 to the NER reaction. It also will be interesting to monitor the interaction of Rad4 and Rad33 in different phases of cell growth, with and without DNA damage. Moreover, to investigate a possible role of Rad33 in the recruitment of other NER factors, the assembly of the NER complex at the site of the lesion can be studied in the presence and absence of Rad33.

As pointed out in chapter 3, damage recognition is an extremely complex mechanism. This is probably the reason that Rad4 functions in complex with two proteins,

Rad23 and Rad33, which both modulate and/or regulate the action of Rad4 and of its homologue Rad34. Unravelling the function of these proteins will be imperative in the understanding how damage recognition is organized. The fact that the Rad4 homologues in *Saccharomyces cerevisiae* have distinct roles emphasizes the delicacy of the interplay between NER and transcription. The experiments in this thesis demonstrate that more proteins than previously assumed are involved in the damage recognition step of the NER reaction in *S. cerevisiae*.

Samenvatting

DNA is de drager van onze genetische informatie, maar bezit niet de moleculaire eigenschappen om de stabiliteit te verklaren waarmee genetische eigenschappen worden overgeërfd. De structuur van het DNA wordt continu beschadigd door factoren buiten de cel (straling, sigarettenrook) en intern door producten van cellulair metabolisme. Beschadigingen aan het DNA hinderen verschillende essentiële processen binnen de cel, zoals transcriptie en replicatie. Defecten in deze processen kunnen dodelijke gevolgen hebben voor de cel. Daarnaast kunnen DNA schades worden omgezet in mutaties wanneer gemodificeerde basen foutief worden gerepliceerd. Deze mutaties kunnen leiden tot velerlei defecten, waaronder versnelde veroudering en, voor meercellige organismen, kanker. De reden dat het DNA molecuul in staat is om genetische eigenschappen stabiel te herbergen is dat de vele beschadigingen die het DNA dagelijks oploopt worden hersteld door meerdere zogenaamde DNA-herstel mechanismen. In **hoofdstuk 1** wordt een algemene introductie van deze herstelmechanismen gegeven.

Het onderwerp van dit proefschrift is nucleotide excisie herstel (afgekort NER, van Nucleotide Excision Repair), geïntroduceerd in **hoofdstuk 2**. Het NER systeem is in staat een grote verscheidenheid aan DNA beschadigingen, die qua structuur geen duidelijke overeenkomsten vertonen, te repareren. NER is van bijzonder belang voor de verwijdering van DNA beschadigingen veroorzaakt door UV licht afkomstig van de zon, zoals cyclobutaan pyrimidine dimeren (CPDs) en (6-4) fotoproducten ((6-4)PPs). Personen die lijden aan de erfelijke ziekte Xeroderma Pigmentosum hebben een defect NER systeem en deze aandoening is daarom geassocieerd met een sterk verhoogde kans op huidkanker.

De NER reactie wordt uitgevoerd door meerdere eiwitten die elk een specifieke taak vervullen in de verwijdering van het beschadigde DNA. De eerste stap in NER is de herkenning van beschadigd DNA, uitgevoerd door zogenaamde 'schade sensor eiwitten', die het genoom afzoeken naar aanwezige beschadigingen. Als er beschadigd DNA is gevonden worden andere NER factoren gerekruteerd die er voor zorgen dat de twee DNA strengen rondom de schade van elkaar worden gescheiden. Uit de beschadigde streng wordt een fragment van ongeveer 30 nucleotiden verwijderd. Nieuw DNA wordt aangemaakt door het DNA polymerase dat de onbeschadigde streng repliceert. Het DNA is weer volledig intact wanneer DNA ligase het nieuw gesynthetiseerde DNA vastplakt aan het bestaande DNA.

NER kan worden onderverdeeld in twee subsystemen: globaal genoom herstel ("*Glo-*

bal Genome Repair", GGR), een systeem dat DNA schades in het gehele genoom kan verwijderen, en transcriptie-gekoppeld herstel ('*Transcription Coupled Repair*', TCR), specifiek betrokken bij het herstel van schades in actief getranscribeerd DNA. Deze twee subsystemen verschillen in de methode waarop de schade wordt gedetecteerd. In GGR wordt het genoom doorzocht door specifieke schadeherkennings-eiwitten terwijl in TCR het RNA polymerase, dat strandt wanneer het DNA schades zoals CPDs of (6-4)PPs probeert te transcriberen, als signaal dient om de NER reactie te beginnen. Het NER systeem is evolutionair geconserveerd en onder eukaryote organismen vertonen de betrokken eiwitten een aanzienlijke homologie. In dit proefschrift wordt de gist *Saccharomyces cerevisiae* gebruikt als modelorganisme om NER te bestuderen.

Hoofdstuk 3 is gewijd aan een bijzonder ingewikkelde fase in GGR, de herkenning van verschillende, schijnbaar structureel ongerelateerde, beschadigde nucleotiden binnen een grote overmaat van onbeschadigd DNA. De NER factor Rad4-Rad23 bindt aan DNA dat afwijkt van de standaard Watson-Crick conformatie. Deze afwijkende gebieden in het DNA worden vervolgens verder geïnspecteerd door de daarna gerekruteerde factor TFIIH, benodigd voor het scheiden van de twee DNA strengen. Wanneer TFIIH zijn helicase activiteit gebruikt om de verbindingen tussen de twee strengen te verbreken, strandt het enzym op het moment dat het een beschadigde nucleotide probeert te verwerken. Deze blokkering is wellicht essentieel voor de verdere opbouw van het NER complex op de plaats van de schade.

Rad4, de gist homoloog van humaan XPC, is een centraal eiwit in het schadeherkenningsproces. De volgende hoofdstukken behandelen het Rad4 eiwit, de homoloog Rad34 in gist (**hoofdstuk 4**) en een nieuwe Rad4-bindingsfactor genaamd Rad33 (**hoofdstuk 5-6**). In tegenstelling tot andere NER eiwitten die essentieel zijn voor de NER reactie *in vitro,* is de betrokkenheid van Rad4 bij GGR en TCR anders dan die van zijn humane homoloog XPC. In humane cellen is XPC nodig voor GGR maar niet betrokken bij TCR. In gist is Rad4 echter essentieel voor zowel GGR als TCR van RNA polymerase II getranscribeerd DNA. NER is niet alleen maar actief in gebieden getranscribeerd door RNA polymerase II (het overgrote deel van het genoom), maar ook in ribosomaal DNA (rDNA) getranscribeerd door RNA polymerase I. Het rDNA locus bestaat uit een set van ~150 kopieën van de *rRNA* genen. Ongeveer 50% van de kopieën worden actief getranscribeerd, de overigen kopieën zijn niet actief. In eerdere studies hebben wij aangetoond dat Rad4 nodig is voor GGR in rDNA terwijl preferentieel herstel van de RNA polymerase I getranscribeerde streng plaatsvindt onafhankelijk van Rad4.

In **hoofdstuk 4** tonen we aan dat voor het Rad4-onafhankelijk herstel in het rDNA locus een homoloog van Rad4 nodig is, Rad34 (YDR314C). Dit eiwit is exclusief betrokken bij preferentieel herstel van de RNA polymerase I getranscribeerde streng (waarschijnlijk TCR). Rad34 en Rad4 kunnen elkaar niet vervangen en hebben dus strikt gescheiden rollen in NER.

In een supplement van **hoofdstuk 4** (**hoofdstuk 4.1**) wordt onderzocht welke eigenschappen van Rad4 en Rad34 bepalen dat deze eiwitten specifiek actief zijn in verschillende regionen in het DNA. In het algemeen kunnen Rad4-homologen worden onderverdeeld in twee domeinen: een geconserveerd deel aan het carboxyl-uiteinde van het eiwit, en een niet-geconserveerd deel aan het amino-uiteinde van het eiwit. Hoewel het te verwachten viel dat de niet geconserveerde delen van Rad4 en Rad34 verant-

woordelijk zijn voor de specificiteit van de eiwitten zijn hybride eiwitten, waarin de niet-geconserveerde delen van Rad4 en Rad34 verwisseld zijn tussen de twee eiwitten, niet functioneel. Dit geeft aan dat elementen in de niet-geconserveerde delen van Rad4 en Rad34 specifiek samenwerken met de bijbehorende geconserveerde delen en dit niet kunnen met het carboxyl-uiteinden van de andere Rad4 homoloog.

De resultaten in **hoofdstuk 4** roepen de vraag op waarom er een Rad4 homoloog bestaat die exclusief nodig is voor NER van de RNA polymerase I getranscribeerde streng van het relatief kleine rDNA locus. De meest aannemelijk verklaring is dat het feit dat er in rDNA een ander RNA polymerase actief is de reden is dat Rad34 nodig is voor NER in rDNA. Om deze aanname te bewijzen zullen er additionele experimenten uitgevoerd moeten worden. DNA herstel zou bijvoorbeeld gemeten kunnen worden in de gescheiden fracties van transcriptioneel actieve en inactieve rDNA regionen. Daarnaast kunnen RNA polymerase I complexen worden geïsoleerd die *of* actief aan het transcriberen zijn *of* zijn vastgelopen op een schade. Deze geïsoleerde complexen kunnen geanalyseerd worden voor associatie met Rad34 en andere NER factoren. Dit zal aangeven of Rad34 al deel uitmaakt van het RNA polymerase I complex alvorens dit op een schade is vastgelopen of wordt gerekruteerd na inductie van DNA schade. Gezien *rad34* mutanten niet UV gevoelig zijn is het zeer de vraag of de primaire rol van Rad34 in NER ligt. Wellicht is Rad34 betrokken bij andere processen binnen de cel. Het is daarom interessant om te testen of Rad34, net als Rad4, aan (beschadigd) DNA bindt, en om te onderzoeken of Rad34 de rol van Rad4 kan overnemen in herstel van naakt DNA in een *in vitro* NER reactie.

In **hoofdstuk 5** wordt een tweede nieuw geïdentificeerde factor, Rad33, geïntroduceerd. Cellen waaruit het *RAD33* gen is verwijderd zijn duidelijk gevoelig voor UV straling. DNA herstel experimenten laten zien dat GGR volledig defect is in *rad33* cellen. Daarnaast is de efficiëntie van TCR ernstig verminderd. Uit '*two-hybrid*' interactie proeven blijkt dat Rad33 direct bindt aan Rad4 en Rad34, wat impliceert dat Rad33 deel uitmaakt van het Rad4-Rad23 complex. De hoeveelheid Rad4 en Rad34 eiwit in de cel is aanzienlijk minder in afwezigheid van Rad33. De resultaten die worden besproken in **hoofdstuk 5** duiden er echter op dat het lagere niveau van Rad4 eiwit niet de enige oorzaak is van het NER defect in *rad33* cellen.

Hoofdstuk 6 rapporteert over de betrokkenheid van Rad33 in een UV geïnduceerde, post translationele, modificatie van het Rad4 eiwit. We laten hier zien dat deze modificatie voor een deel bestaat uit ubiquitine. In cellen met functioneel NER is de UV geïnduceerde Rad4 modificatie nauwelijks waarneembaar, maar in *rad33* cellen, of in cellen waarin de interactie tussen Rad4 en Rad33 genetisch is verbroken, is de aanwezigheid van het gemodificeerde Rad4 eiwit duidelijk zichtbaar. Rad4 lijkt te worden gemodificeerd terwijl het actief is in de TCR reactie, gezien deletie van het *RAD26* gen de toegenomen Rad4 modificatie teniet doet. Dit kan betekenen dat de modificatie van Rad4 een intrinsiek onderdeel is van de TCR reactie. Gezien in *rad33* cellen TCR veel minder efficiënt verloopt, is het denkbaar dat in deze mutant de gemodificeerde Rad4 eiwitten relatief langer aanwezig zijn en daardoor duidelijker waarneembaar. Een andere mogelijke verklaring is dat Rad33 normaliter de activiteit van Rad4 reguleert door het eiwit in bepaalde situaties af te schermen van de modificatie.

De Rad4 modificatie doet denken aan de UV geïnduceerde ubiquitinering van het humane Rad4 homoloog XPC, waarvan is aangetoond dat de modificatie leidt tot een

verhoogde affiniteit van XPC voor DNA. Een verschil tussen de modificatie van Rad4 en XPC is dat Rad4 modificatie gerelateerd is aan TCR terwijl ubiquitinering van XPC een GGR specifieke gebeurtenis is. Het kan echter niet worden uitgesloten dat modificatie van Rad4 ook een rol speelt in GGR, maar dat in afwezigheid van Rad33 het Rad4-Rad33 complex niet in staat is om DNA schades te bereiken in niet-getranscribeerd DNA. Om meer te leren over de rol van het gemodificeerde Rad4 in gist zal de volledige aard van de modificatie bepaald moeten worden. Het is ook interessant om te bepalen of de gemodificeerde Rad4 eiwitten een veranderde affiniteit voor (beschadigd) DNA bezitten.

Er is geen duidelijk humaan sequentie homoloog van Rad33. Echter, de data in **hoofdstuk 6** laat zien dat de voorspelde structuur van Rad33 overeenkomsten vertoond met de structuur van het Cdc31 eiwit. Cdc31 is de gist homoloog van het humane Centrin2, een eiwit dat deel uitmaakt van het XPC-hHR23B complex en bijdraagt aan een efficiënt NER proces. We laten zien dat Rad4 bindt aan Rad33 via dezelfde geconserveerde aminozuren die XPC verbinden aan Centrin2, een vinding die mogelijk aanduidt dat de rol van Rad33 functioneel vergelijkbaar is aan die van Centrin2 in NER in humane cellen.

Curriculum vitae

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