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

Dulk, B. den

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.**

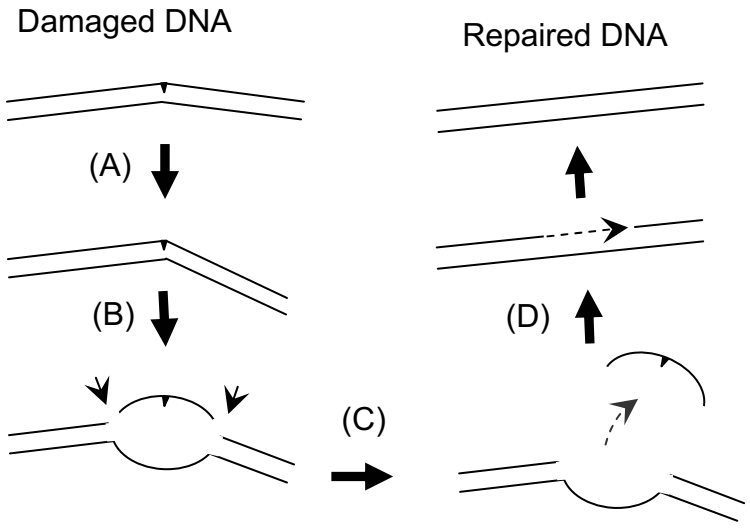

Figure 1
Schematic representation of the NER reaction. (A) Damaged DNA is recognized, possibly leading to a conformational change in the DNA (discussed in chapter 3). (B) Incisions are made on both sides of the lesion. (C) A ~30nt oligonucleotide containing the lesion is removed. (D) DNA is re-synthesized 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.

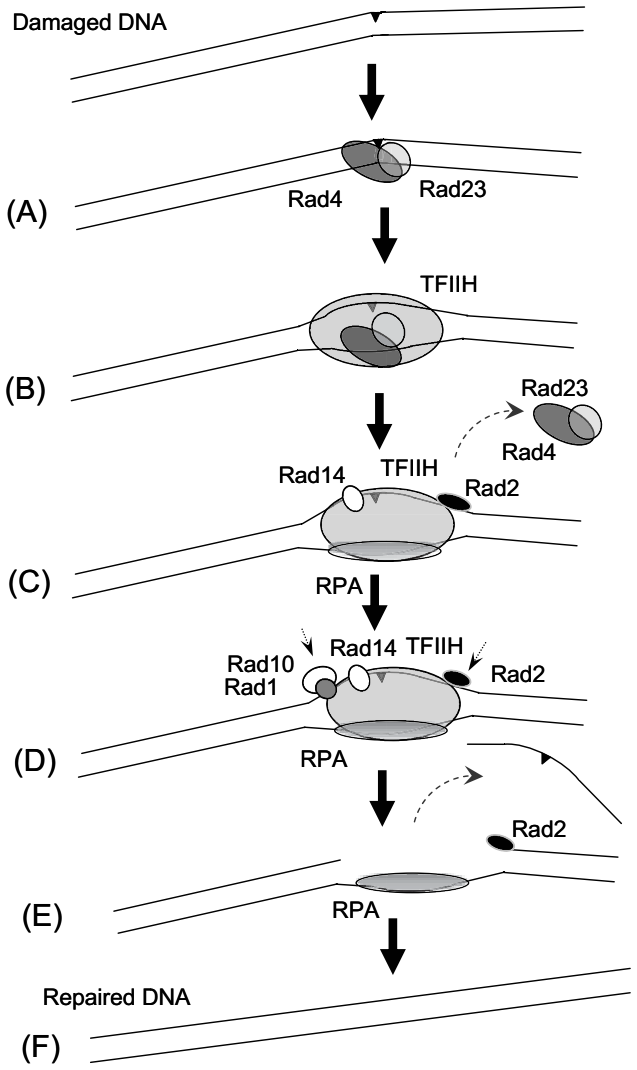

Figure 2
Schematic outline of the steps in the eukaryotic core-NER reaction. The names of the proteins refer to the Saccharomyces cerevisiae proteins. The names of the human homologues can be found in table 1. (A) Damaged DNA is bound by the Rad4-Rad23 complex (the Rad33 protein, not shown here, is expected 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.