Nucleotide excision repair in yeast

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Nucleotide Excision Repair in Yeast

Patrick van Eijk

Nucleotide Excision Repair in Yeast

PROEFSCHRIFT

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de graad van Doctor aan de Universiteit Leiden,
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1. General Introduction

DeoxyiboNucleic acid (DNA) is the carrier of genetic information for most forms of life [1, 2]. DNA exists as a double helix of complementary strands that resides in the nucleus of eukaryotic cells. Prokaryotes lack a cell nucleus and hence maintain their genome in the cytoplasm in the form of a nucleoid structure [3]. The primary structure in DNA is the linear arrangement of nucleotides into genes that code for proteins and a variety of RiboNucleic Acid (RNA) molecules. Both pro- and eukaryotes maintain their genome in a higher order structure required for compaction of the DNA to fit inside the cell or nucleus. More importantly, this structure functions in regulation of gene expression [4-6]. The higher order structure seen in eukaryotes contains many levels of organization of which the basic unit is the nucleosome [7].

Historically, the stable propagation of genetic traits has been recognized for many years, but it was hard to reconcile that a simple biological molecule could harbor such stability. The chemical nature of DNA makes it inherently unstable and subject to many chemical alterations under physiological conditions [8]. Moreover, our genome faces many natural challenges altering our genetic information that would collectively compromise faithful inheritance via any type of biomolecule. Endogenous sources of DNA damage are deamination, depurination and importantly, Reactive Oxygen Species (ROS) produced by cellular metabolism. Exogenous agents, on the other hand, are both chemical and physical in nature including but not limited to UV radiation from sunlight, ionizing or gamma radiation, smoke-related carcinogens, anti-cancer drugs and environmental pollutants [8]. UV radiation causes formation of thymine-thymine dimers in the form of cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) [9]. Ionizing and gamma radiation damage the DNA by inducing nicks, breaks and base modifications [10, 11]. This constant pressure of DNA insults requires an ample maintenance and repair capacity. Indeed nature has devised a large spectrum of DNA repair and damage response pathways with different degrees of conservation amongst the three kingdoms of life [12]. It is thus the concerted action of a plethora of DNA repair and response pathways that is responsible for the very stable transmission of genetic traits and not the stability of the carrier per se.

If DNA damages are left unrepaired they can lead to mutations. The cellular response to DNA damage regardless of the source is in essence twofold: that of the DNA Damage Response (DDR) and of DNA repair. Defects in either of these cellular processes can lead to genomic aberrations ranging from point mutations to gross chromosomal rearrangements, referred to as genomic instability [13]. Failure to remove DNA damages can be due to impaired DNA repair, a defective DDR, an extreme high damage dose or a combination of the three. The presence of genomic instability in a majority of human cancers indicates that genomic instability drives carcinogenesis [13-16]. Indeed, most genetic defects of DNA repair pathways lead to predisposition to cancer in humans [17],

putting into perspective the importance of DNA repair in maintaining genome stability and preventing carcinogenesis [18-20].
Firstly, in the next sections of the introduction the importance of genome stability and the DNA Damage Response (DDR) will be discussed (2). Secondly, the DNA repair pathways will be described in more detail in section 3 including: Post-Replication Repair (PRR) and Translesion DNA Synthesis (TLS) (3.1), Mismatch Repair (MMR) (3.2), Direct DNA damage reversal (3.3), Base Excision Repair (BER) (3.4), and repair of Double Strand Breaks (DSB) via Homologous Recombination (HR) and Non-Homologous End-Joining (NHEJ) (3.5). In light of the research described in this thesis special emphasis is placed on Nucleotide Excision Repair (NER) described in sections 4 to 6..

2. The DNA Damage Response

During the initial response to DNA damage cells recruit the DNA Damage Response (DDR), a pathway of damage detectors and kinase protein that signal the cell to halt cell cycle progression and alter the expression of many gene targets [21]. Before introducing damage detection that fuels the DDR it is critical to cover some of the basics around replication. Elements of the replication complex are vital for DDR signaling as will become evident in the following paragraph.
DNA replication is achieved at an appreciably low error-rate but is sensitive to aberrant bases or other DNA damages that can block the replication machinery or lead to misincorporation of bases [14, 22]. The replication fork is built up around origins of replication as a multi-protein complex starting as the pre-replication complex. The double stranded nature of DNA requires a denaturing step by the MCM (Mini Chromosome Maintenance) helicase that individually runs ahead of the replication complex. At each origin two replication complexes, called replisomes, are formed that synthesize DNA in opposite direction. The Replication Factor Complex C (RFC) loads the Proliferating Cell Nuclear Antigen (PCNA) clamp onto the DNA that stimulates the processivity of the replisome. The replicative DNA polymerases are recruited to the replication fork by PCNA. The presence of partial duplex DNA (stretches of ss- an dsDNA) puts the replication complex at risk of DNA strand breaks or recombination. Moreover, the MCM helicase activity is uncoupled from the main replication machinery and will generate large stretches of ssDNA ahead of a stalled replication fork, creating another substrate for DNA breaks or recombination. To illustrate, genetic mutation or deletion of genes encoding for components of the replication complex results in the accumulation of replication intermediates that are prone to recombination leading to genomic instability [19]. Other endogenous sources that can impede on replication are DNA secondary structures in repetitive DNA, protein-DNA and transcription complexes [19].

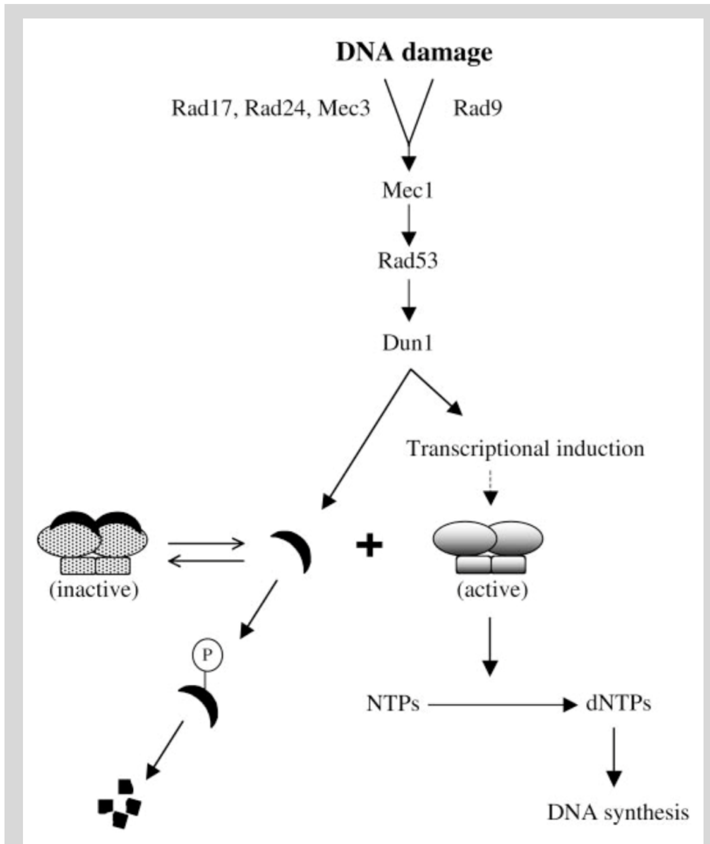

Figure 1 – A model for the DDR directed control of dNTP synthesis, DNA damage detection via the Rad17, Rad24, Mec3 and Rad9 pathways funnel into the core Mec1-Rad53-Dun1 DDR signaling cascade. The dual function of Dun1 is highlighted. Dun1 phosphorylates the RNR inhibitor Sml1 and concurrently induces transcription of the *RNR2* to 4 genes. In its free form Sml1 is degraded. Taken from [50].

More relevant to this thesis is the role of DNA damage in generating genome instability. Any DNA damage that will prevent the replication machinery from progressing is a potential risk for genome stability. This includes but is not limited to UV-induced pyrimidine dimers and chemically induced bulky DNA adducts [19]. The natural response to these types of bulky lesions, initially, is the DDR discussed here followed by DNA repair. If however, damages persist into S phase when replication commences, an important 'last resort' exists, referred to as DNA damage tolerance pathways. This is Post-Replication Repair (PRR) which includes translesion DNA synthesis (TLS). Sub-pathways of this response can be both error-free or error-prone and will be discussed in more detail in their respective sections below. At this stage I introduce these pathways as part of the holy trinity of DDR, DNA repair and damage tolerance all working in concert to promote cellular survival after DNA damage. It is important to appreciate that even though these three major processes are all highly redundant within and amongst each other, failure at any stage is a potential hazard for genomic stability.

In order to provide cells time and to produce the resources and conditions to repair DNA damages, the so-called DNA Damage Response is initiated. A variety of DNA lesions can trigger the DDR in order to halt cell cycle progression and prepare the cell for DNA

damage repair. The DDR signaling pathway is comprised of DNA damage sensors, transducers and effectors. However, many factors in the DDR fulfill hybrid functions that overlap these three archetypes in cell signaling.
In budding yeast the central spine of the DDR is a signaling cascade involving the Mec1-Tel1-Rad53-Dun1 (ATM-ATR-RAD53 in humans) protein kinases that signal the detection of DNA damage into a cellular response to halt cell cycle progression and alter gene expression [21]. Mec1 interacts with the ssDNA associated Ddc2-Rpa (Replication Protein A) complex [23]. This Mec1-Ddc2-Rpa-DNA complex can originate whenever RPA is bound to ssDNA or at partial duplex DNA such as sites formed at a stalled replication fork. Subsequently, the budding yeast PCNA-like Ddc1-Mec3-Rad17 complex, which is analogous to the 9-1-1 complex in higher eukaryotes and fission yeast, is loaded onto partial duplex DNA by the Rad24-Rfc2-5 clamp loader [24, 25]. The interplay between the clamp-loaders and PCNA is the start of PRR/TLS discussed in more detail later. In this context however, this PRR intermediate also serves as a substrate for the DDR since Mec1-Ddc2-Rpa associates with these damage sites leading to activation of Mec1 [26, 27]. This is as an important interplay between DNA repair (or in this instance more accurately described as DNA damage tolerance) pathways and the DDR that will be a recurring theme in chapter 2. Similar interfaces between DNA repair and the DDR exist as reviewed in [28].
Tel1 binds to the Mre11-Rad50-Xrs2 complex (MRX - MRN in higher eukaryotes) that resides at a DSB [29]. The Xrs2 subunit of the complex can be viewed as the regulatory component that is phosphorylated after DSB induction in the context of damage signaling [30]. As with the yeast PCNA-like 9-1-1-complex serving both as an initiator of TLS and the DDR, the MR(X)N complex also directs DSB repair in yeast, discussed in more detail in section 3.5 [30, 31]. Mec1 and Tel1 are not entirely redundant, however, some overlap in function exists, as double mutants missing both factors are more sensitive to DNA damaging agents [32]. It is generally accepted that Mec1 is more important for the DDR response during the S and G_2 cell cycle phases, while Tel1, as the name implies, is vital for maintaining telomere length and signaling of DSBs [33, 34].
The central function of the DDR is to halt cell cycle progression in response to DNA damage. Chk1 and Rad53 (Chk2 in higher eukaryotes) are the prime Mec1 and Tel1 downstream substrates and are important for the checkpoint function of the DDR [21]. This part of the pathway makes sure that the signal is both amplified and transmitted away from the site of damage. Both Rad53 and Chk1 are diffusible protein kinases and activated Rad53 can autophosphorylate thus multiplying the signal [21]. In yeast Chk1 and Rad53 in concert help to stabilize the anaphase suppressor Pds1, but later stages of mitotic exit are also repressed in the presence of DNA damage [35-37]. In higher eukaryotes Chk1 inhibits Cyclin Dependent Kinases (CDK) that drive cell cycle progression, which is not required for cell cycle arrest in yeast [38, 39]. Furthermore, the intra S phase checkpoint inhibits replication by slowing down the progression of ongoing

replication forks, but also by inhibiting the firing of new origins of replication. Importantly, Rad53 phosphorylates and activates the Dun1 kinase that is responsible for the upregulation of RiboNucleotide Reductase (RNR) genes that control cellular dNTP synthesis [40, 41], described in more detail in the next section.

In addition to regulation of the cell cycle checkpoints and dNTP synthesis, the DDR regulates a DNA repair, chromatin, cytoplasmic and transcriptional response in concert to promote a coordinated response to DNA damage. For more details on these targets of the DDR the reader is referred to [21] and references therein.

Linking back to the topic of genomic instability, DDR checkpoint mutants, like *mec1*, *dun1* and *ddc2*, show an increase in genome instability [42]. Therefore, it is thought that the S phase replication checkpoints described here help to maintain the protein complexes formed at stalled replication forks to allow replication to restart [18, 43]. Breakdown or reversal of replication forks is believed to be detrimental to genome stability as a result of the partial duplex DNA being processed into a DSB and/or long stretches of ssDNA. Thus, inability to maintain stalled forks in DDR mutants drives genomic instability [19, 43].

The importance of an intact DDR to maintain genome stability is now widely recognized and is underscored by the human disorder ataxia telangiectasia. This disorder is caused by homozygous mutations in the human homologs of Tel1 and Mec1, Ataxia Telangiectasia mutated (ATM) and Ataxia Telangiectasia and Rad3 related (ATR) [32, 44, 45]. Patients suffering from ATM display cerebral degeneration, sensitivity to radiation and predisposition to cancer [46]. Thus, maintenance of genomic stability at the level of regulation of the DNA repair pathways via the DDR is vital to counteract carcinogenesis. Importantly, the coordinated events that make up the DDR facilitate and drive DNA repair, but the DDR itself does not result in DNA damage removal or reversal.

2.1 RNR pathway and dNTP synthesis

The RiboNucleotide Reductase (RNR) family of genes and associated repressors and activators form the hallmark example of a DDR target. The concerted action of these factors allows for the damage induced activation of dNTP synthesis that has been shown to facilitate survival in response to DNA damage and to protect against DNA damage [47]. The RNR enzyme consists of 4 subunits, 2 major catalytic subunits coded by *RNR1* and *RNR3* and 2 regulatory proteins Rnr2 and 4 [48]. The Rnr1 subunit holds the active site for final step in dNTP synthesis and alosteric product inhibition. This first level of inhibition is part of a negative feedback loop, whereby increased dNTP concentrations inhibit enzyme activity. In yeast, this mode of regulation is active but relatively insensitive compared to that in higher eukaryotes [49]. Another mechanism of regulation exists at the protein level by direct inhibition of the RNR complex by Sml1. Sml1 interacts directly with the RNR complex, an interaction that is disrupted when Sml1 is phosphorylated in a Mec1 and Rad53 dependent fashion. Specifically, Sml1 is phosphorylated by Dun1, after

which it can no longer interact with the RNR enzyme complex and is subject to ubiquitin dependent degradation [50, 51].
RNR activity is also regulated at the level of gene expression by Crt1 and Dun1. Crt1 represses the expression *RNR2*, *RNR3* and *RNR4*, and this is relieved after phosphorylation of Crt1 by Dun1 [52]. The expression of all RNR genes is DNA damage inducible and *RNR3* is most significantly upregulated after DNA damage (up to 100-fold) [48]. Rnr3 is therefore referred to as the damage specific subunit that can form a heterodimeric subcomplex with Rnr1. Rnr1 is present at levels in excess of Rnr3 in the absence of DNA damage [49]. However, Rnr3 by itself does not exhibit significant catalytic activity, it requires Rnr1 to form an enzyme that has any appreciable catalytic activity [49]. Rnr3 is therefore thought to contribute to the regulation of dNTP synthesis in response to DNA damage by promoting enhanced interactions with Rnr1 resulting in increased levels of the heterodimeric catalytic subunits for the RNR enzyme complex,
Summarizing, in response to initiation of the DDR, both Sml1 and Crt1 are phosphorylated. Sml1 releases the RNR enzyme creating a catalytically active complex permitting dNTP synthesis to occur. This activity is reinforced since phosphorylated Crt1 no longer represses RNR gene expression, while DNA damage induced expression of the RNR genes is mediated via the concomitant activation of Dun1. These events culminate into an upregulation of dNTP levels in the order of 10-20-fold in response to DNA damage. In a similar fashion, but in a different context, dNTP production can be upregulated during replication in S phase by cycling of the Sml1 inhibitor [48, 53].

3. DNA Repair

DNA repair mechanisms can be classified based on their mode of action. We can distinguish between several strategies among the different repair pathways which are damage bypass (MMR and PRR/TLS), DNA damage reversal (photolyase repair), DNA damage removal (BER, NER) and DNA break repair (HR/NHEJ). The two error avoidance pathways Post-Replication Repair (PRR) and Mismatch Repair (MMR) are historically included in the assortment of DNA repair mechanisms, while they do not repair DNA damage per se. Instead, they are the last line of defense for survival if DNA damages persist to interfere with replication and to remove the resulting mismatches. The conceptually straightforward direct damage reversal pathways include photolyase and DNA alkyltransferases. By directly removing the chemical alteration or reversing the structural change to the DNA, these mechanisms revert the DNA to its original state without otherwise complex processing of the damaged DNA. DNA damage removal on the other hand, removes the modified base or an entire oligonucleotide to repair the DNA. In these repair pathways the complementarity of the DNA is utilized to fill the empty ssDNA gap making both pathways inherently non-mutagenic. Finally, DNA double strand breaks (DSB) are uniquely different from other types of DNA damage. The state of the cell cycle and the nature of the break determine which of the repair pathways is

utilized to bring the two severed DNA ends together. Collectively, these processes are referred to as DNA repair pathways and throughout this section I will provide background information on these main repair mechanisms covering their mode of action, the damages they repair and when relevant, their link to genome instability and cancer.

3.1 Post-Replication Repair

Regardless of the efficiency of the intricate DNA repair mechanisms described in the second half of this section, DNA damage might persist to interfere with replication during S phase if cells are unable to repair the lesion in a timely fashion. This could be the result of an excessive damage load that overwhelms the cell's repair ability or due to reduced capacity of the repair system. More importantly, DNA damage will be induced when replication is already underway. Thus cells have a range of damage tolerance pathways at their disposal, collectively referred to as Post Replication Repair (PRR) [54, 55]. Even though these processes do not result in repair per se, they are mentioned here because the contribute to survival and for historic context. PRR is a rather archaic term to describe repair of large ssDNA gaps into higher molecular weight dsDNA first identified in bacteria [56].

PRR encompasses translesion DNA synthesis (TLS) and recombinational or template switching repair [55, 57, 58]. The founding members of the PRR pathway in yeast are Rad6 and Rad18 as part of the *RAD6* epistasis group [54]. The PRR proteins mainly consist of ubiquitination enzymes and TLS polymerases. Rad6 is an E2 ubiquitin conjugating enzyme [59-61] that exists in complex with the RING finger E3 ligase Rad18 [62]. Rad18 has affinity for ssDNA and recruits Rad6 to ssDNA that occurs at a stalled replication fork [63, 64]. At the replication fork Rad6-Rad18 monoubiquitinates PCNA at a defined lysine residue [65, 66]. Monoubiquitinated PCNA serves as a docking site for low-fidelity TLS DNA polymerases [66]. These enzymes lack exonuclease proofreading activity and can synthesize DNA opposite a variety of lesions by virtue of their excessively large active site that can accommodate different large aberrant bases [67]. These characteristics of TLS polymerases make them inherently error-prone. Hence DNA synthesis by these polymerases is tightly kept in check and is exclusively used during TLS. After synthesizing DNA opposite the lesion high-fidelity polymerases are recruited to continue replication. It is thought that through ubiquitination of the resident low-fidelity polymerase the second high-fidelity polymerase can be recruited [68]. This dual polymerase mechanism is supported by an ubiquitin interaction domain shown to reside in some polymerases. Low and high-fidelity TLS polymerases in this way can work in concert to synthesize DNA opposite the lesion via interacting with each other and monoubiquitinated PCNA.

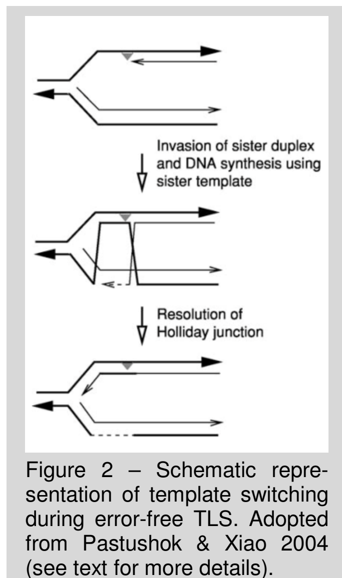

Figure 2 – Schematic representation of template switching during error-free TLS. Adopted from Pastushok & Xiao 2004 (see text for more details).

Interestingly, PCNA can also be polyubiquitinated by the Rad5-Ubc13-Mms2 complex of proteins [65]. Ubc13-Mms2 forms a heterodimeric E2 conjugating enzyme that associates with the RING finger E3 ligase Rad5 [65]. Rad5 also holds ssDNA-dependent ATPase and helicase activity [69]. The factors in this Rad5 protein complex act downstream of Rad6-Rad18 and have been identified to drive error-free PRR [65, 70]. Indeed a *rad5* mutant is sensitive to UV but still displays normal UV-induced mutagenesis that is otherwise reduced in *rad6* and *rad18* mutants [70]. Moreover *rad5* and *rad18* mutants are known to be hyper-recombinant, recombination that is dependent on HR factors Rad51 and Rad52 [55]. This mode of error-free damage avoidance is most likely directed by template switching using homologous recombination proteins. The stalled replisome can be diverted to a homologous sequence on the sister chromatid to continue DNA replication and thereby bypassing the lesion (see figure 2). However, the exact mechanism behind template switching is not fully understood and requires further investigation [71]. Polyubiquitination of PCNA is mutually exclusive to monoubiquitination thus the decision to either mono- or polyubiquitinate PCNA inadvertently directs the system towards error-prone or error-free template switching [71]. However, it remains to be discerned how cells regulate the progression through these PRR pathways.

Human Rad5 homologs have been identified that are inactivated or mutated in colorectal, gastric and ovarian cancers [72-75]. The functional characterization of the human Rad5 homologs HLTF and SHPRH fully implicates these human proteins in polyubiquitination of PCNA and error-free PRR synonymous to Rad5 in yeast [76, 77]. The downregulated expression or inactivation of HLTF or SHPRH in a subset of human cancers and the established role for these proteins in maintaining genome stability via error-free PRR makes them important tumor suppressors [78, 79].

3.2 Mismatch Repair

The inherently high fidelity of DNA replication is a testament to the concerted action of the DNA polymerase fidelity, proof-reading and Mismatch Repair (MMR) activity, resulting in a mispaired base only once in every 10^9 to 10^{10} bases per cell division [80]. However, mutation rates in eukaryotic cells have been shown to be lower than 10^{-10} [81]. The error-rates of the DNA polymerases in replication are in the order of 10^{-3} to 10^{-6}. Proofreading and mismatch correction reduce the error-rate even further to the observed fidelity of canonical DNA replication [81]. Secondary substrates for MMR are single

nucleotide mismatches that originate from DNA damage that persists through S phase and is tolerated by the error-prone PRR pathway. These mismatches arise due to misincorporation of nucleotides by the low fidelity TLS DNA polymerases. Other important MMR targets are mismatches generated by insertion and deletion events in microsatellites. These mismatches result from misalignment of the polymerase during synthesis of highly repetitive DNA of these microsatellites that is prone to polymerase slippage [82, 83]. This makes microsatellites inherently unstable and even more unstable when MMR is deficient. Therefore, MMR is widespread in nature because it actively contributes to replication fidelity which when solely dependent on polymerase fidelity and proof-reading is not sufficient to replicate large genomes at an appreciably low error-rate. MMR genes were initially described in both prokaryotes and simple eukaryotes as 'Mut' genes due to their spontaneous and UV-induced mutator phenotype and accumulation of replication errors when deleted [82]. Bacterial MMR has been completely reconstituted *in vitro* which lead to the deciphering of the mechanism behind MMR [84]. Repair of these lesions, as with any DNA anomaly, starts with the recognition of the mismatch [85]. In bacteria the MutS homodimer recognizes a host of mismatches and interacts with the β-clamp (homologous to PCNA in eukaryotes) that is believed to help deliver MutS to newly replicated DNA [86, 87]. Next, the MutL ATPase protein is recruited to attract and activate the MutH endonuclease [88, 89]. The concerted action of MutL and MutS is believed to serve as a mode of licensing damage recognition for downstream processing [90]. MutL is referred to as a 'matchmaker' as it lacks specific enzymatic activity but instead collaborates with MutS in damage verification, recruitment and activation of downstream MMR factors. MutL has aspecific affinity for DNA underscoring its matchmaker function [91, 92]. Since mismatches are not damaged bases or nucleotides, the discrimination between the parental and newly synthesized strand, containing the mismatch, is crucial for MMR to prevent mutagenesis. It is thus not the recognition of the mismatch per se but the selection of the newly synthesized strand that allows MMR to actively contribute to replication fidelity. In *E.coli* the temporal hemimethylated state of newly synthesized duplex DNA makes this possible due to *dam* DNA methylation [84]. The MutH endonuclease specifically cleaves the newly synthesized strand at the hemimethylated GATC site [93]. Following the strand-selective nicking of the DNA, MutL is responsible for loading a DNA helicase onto the nick [94, 95], that together with ssDNA binding protein (SSB) generates a ssDNA stretch that can be refilled by a DNA polymerase [84]. The basic steps of recognition, strand-selection, nicking and repair synthesis are conserved mechanistically [82]. Similarly, genes and proteins involved in MMR are conserved from bacteria to man [82, 83, 96].

Interestingly, higher eukaryotes maintain multiple MutS (MSH) and MutL homologs (MLH) that consistently act as heterodimers [82, 96]. It has been shown that the eukaryotic MMR proteins have affinity for different mismatches [82, 96] ranging from single basepair mismatches to insertion and deletion loops [97, 98]. Similarly, specific

MSH and MLH proteins are involved in meiotic recombination [99] and mitochondrial MMR [100]. Intriguingly, MutH homologs have not been identified in higher eukaryotes and endo- or exonucleases associated with MMR have only recently been described [90]. In mammals, EXO1 and the PMS2 subunit of MutLα are responsible for the digestion and nicking steps of MMR, respectively [90]. Importantly, the underlying mechanism of repair is conserved despite these minor differences.

Strand selection in higher eukaryotes cannot make use of hemimethylated DNA because *dam* DNA methylation is unique to a subset of bacteria [83]. MSH and MLH proteins in eukaryotes have been shown to interact with PCNA [87], drawing on the parallel with the bacterial system. However, for eukaryotes it is suggested that this interaction is important for the strand specificity of MMR [82, 96, 101]. The latent endonuclease activity of PMS2 in MutLα is activated by its interaction with PCNA and MutS [102]. The PMS2 subunit is spatially restricted within the complex to only nick one strand by the orientation of PCNA which in turn makes the nick specific for the mismatched strand [90]. In this way PCNA is thought to give MMR its directionality as PCNA is always loaded onto heteroduplex DNA with the same orientation that cannot switch [58, 90]. Another possible mechanism for strand-specificity in higher eukaryotes is thought to arise from strand discontinuities associated with replication like Okazaki fragments, confining this mode of strand-selection to the lagging strand [86, 103, 104].

Long stretches of simple DNA repeats like mono-, di- and trinucleotide repeats pose difficult substrates to faithfully replicate. These microsatellites have been known to be highly unstable in MMR deficient model organisms [82, 83, 96]. An important link between cancer and MMR deficiency arose when it was identified that 60 to 70% of hereditary non-polyposis colon cancer (HNPCC) patients have mutations in MLH1 and MSH2 [82, 105]. Similarly, a minority of HNPCC cases is associated with mutations in PMS1, PMS2 or MSH6 (reviewed in [106]). Thus, loss of MMR activity increases mutagenesis and tumor development [82]. Increase in cancer susceptibility has also been shown for numerous mouse MMR knockouts that suffer from increased tumorigenesis of internal organs confirming the importance of MMR in supporting DNA replication to maintain genome stability [82, 96].

3.3 Direct DNA damage reversal

The majority of organisms studied thus far feature one or both of the following direct reversal pathways: photolyase and AlkylGuanine Transferase (AGT). Photolyases are widely spread through all kingdoms of life, but most placental mammals including humans do not have a photolyase [107]. A photolyase is a monomeric enzyme that catalyzes the reversion of UV-induced lesions by using energy quanta of visible light [108, 109]. Originally, the first *E.coli* photolyase that was described is a CPD photolyase, only able to revert CPDs [110] and it was not until much later that the first 6-4PP photolyase was described in *Drosophila melanogaster* [111, 112]. Both CPD and 6-4PP

photolyases have different degrees of sequence similarity amongst species but are all expected to have fairly similar structures and reaction mechanisms based on available sequence and structure data [108, 112, 113]. The photolyase protein binds UV damaged DNA due to the detection of a slight kink that is induced by the helix distortion of the UV lesion not present in undamaged DNA [114]. Upon binding the photolesion flips out of the DNA double helix into the photolyase active site where the reaction takes place [115].

All photolyase enzymes carry the FAD flavin chromofore as cofactor that is pivotal in the cyclic redox reaction mechanism described here [109, 113]. The flavin cofactor is accompanied by a second chromophore that acts as a photoreceptor antenna commonly in the form of MTHF (5,10-methenyltetrahydrofolate) or more rarely, 8-HDF (8-hydroxy-5-deazariboflavin) [109, 113]. After DNA damage binding the energy of a single photon is absorbed by the folate cofactor and transferred to the flavin chromophore, generating an excited $FADH^{-}$* molecule. The energy quantum is not sufficient to break the cyclobutane ring by itself but it increases the redox potential of the $FADH^{-}$ molecule. The excited $FADH^{-}$* molecule transfers an electron to the pyrimdine dimer splitting the cyclobutane ring. The cyclic nature of the reaction makes that the FADH• radical is also reverted back to its groundstate $FADH^{-}$ by electron back-transfer. As a result the pyrimidine dimer is completely reverted to the original DNA conformation as is the flavin cofactor [108, 109, 113].

AlkylGguanine Transferases (AGTs) are widespread in nature, but are not found in plants and *Schizosaccharomyces pombe* [116]. AGTs protect cells from the effects of both endogenous and exogenous alkylating agents. This family of damage reversal proteins was originally characterized based on the properties of the human and *E.coli* MGMT enzymes (O^6-methylguanine-DNA methyltransferase) that were shown to remove the methyl group from a O^6-methylguanine [110, 117-119]. However, more recent literature describes a more generic alkyltransferase function to this family of proteins by showing that more bulky adducts and tobacco-induced carcinogens are also a substrate for these enzymes [120, 121]. Therefore, these enzymes are now collectively referred to as alkyltransferases [116]. AGTs are thought to be able to actively slide acrross the DNA double helix in search for a damage, however, the exact nature of the force driving this process is currently unknown [116]. Upon DNA damage binding AGTs bend the DNA slightly, allowing it access to the minor groove. In this conformation the enzyme can flip out the substrate into its active center where a reactive cysteine attacks the alkyl group and covalently transfers it onto the enzyme itself [122, 123]. This restores the guanine and leaves the enzyme catalytically dead. In this state the occupied reactive site brings about a conformational change in the enzyme that is thought to expose a ubiquitination site leading to ubiquitin mediated degradation of the protein in eukaryotes [124, 125].

A confounding factor of AGTs is that increased AGT activity in certain types of cancer protects the malignant cells from alkylating agents used in cancer treatment, while at the

same time reduced AGT activity might result in accumulation of alkylating damages that could result in cancer in people that are exposed to exogenous alkylating agents [126, 127]. This makes AGT inhibitors prime agents for cancer therapy, specifically sensitizing cancer cells for treatment with alkylating agents [126, 128]. Importantly, AGTs help protect the cell from smoke derived carcinogens that bind covalently to DNA. The smoking related nitrosamine carcinogens are a substrate for AGTs and interindividual differences in MGMT expression can predispose those smokers with reduced MGMT expression to lung cancer even more [126].

3.4 Base Excision Repair

BER is responsible for the repair of altered bases and including oxidative damages [129, 130]. Oxidative damage is a rather frequent event in aerobic organisms resulting in the order of ~100-500 8-hydroxy-guanine (8-oxoG) lesions for instance in a human cell, per day [8]. Other chemically altered bases in DNA are also substrate for BER including uracil induced by cytosine deanimation and alkylation generated 3-methyladenine [127, 131]. Most of these damages are not bulky and will therefore not block replication. Instead they will mispair during replication resulting in mutagenesis. The high potential for mutation and the abundance of oxidative damages due to respiration are a likely explanation for the strong evolutionary conservation of most genes involved in BER [130].

Mechanistically, BER can be described by 5 separate enzymatic steps starting with the recognition and excision of the damaged base [132]. DNA glycosylases bend DNA upon binding [133] and flip-out the damaged base into an extra helical position to fit inside the catalytic pocket [134]. The DNA-enzyme interaction leads to a severe kink in the DNA of up to 70° probing the DNA for damage. Only when a damaged base is bound will it be flipped into the active site [135, 136]. DNA damage recognition is followed by enzymatic cleavage of the N-glycosyl bond between the base and the sugar connected to the DNA backbone. This initial step is performed by an abundance of DNA glycosylases with different substrate affinity. Individual enzymes can recognize a subset of lesions making the damage recognition system in BER highly redundant. After cleavage the damaged base is released and an apurinic-/apyrimidinic (AP) site remains [137, 138]. The resulting AP-site is a substrate for the AP-endonuclease APE1, that generates a 5' nick at the AP-site in the DNA backbone. Alternatively, some DNA glycosylases are bifunctional and contain a protein domain with AP-lyase function [139]. In this way the glycosylase itself can generate a nick after excising the damaged base. The small single nucleotide gap and DNA nick are now substrate for DNA Polymerase β (POLB) to perform the third and forth enzymatic steps. POLB removes the 5’-terminal deoxyribophosphate (dRP) left over by the AP-endonuclease making it a suitable substrate to insert the correct nucleotide into the AP-site [140, 141]. Finally, DNA Ligase I or III will ligate the nick returning the DNA to its undamaged state [142].

Depending on how the nick is generated and processed, BER can funnel into short-patch or long-patch BER after DNA damage recognition and base excision [143]. A suitable 5' and 3' substrate for POLB allows short-patch BER to take place according to the mechanism described above. During long-patch BER, however, displacement DNA synthesis by POLB or POLD occur as a result of the residual 5' terminal dRP not being amenable for POLB induced removal. After removal of the 4 to 6 basepair flap by the endonuclease FEN1 [144] the synthesized patch is suitable for DNA Ligase I or DNA Ligase III mediated ligation.

Interestingly, BER is very important in sustaining life as gross defects in BER are not found in nature. Knocking out genes essential for BER like POLB, APE1 or FEN1 in mice results in embryonic lethality [141, 145, 146]. It is interesting to note that 30% of all tumors described contain a mutant variant of POLB highlighting that defective BER can drive mutagenesis leading to cancer [129]. A few examples of human disease as a result of specific BER deficiencies have been described in literature but are beyond the scope of this introduction [129, 130, 147].

3.5 Double Strand Break Repair via Homologous Recombination & Non-Homologous End-Joining

DNA Double strand breaks (DSBs) are uniquely different to other DNA damages as both backbones of the DNA double helix are severed, in essence breaking a chromosome in two. The gross chromosomal aberrations that result from their faulty repair has a large impact on the genome opposed single nucleotide mutations described previously. Instead severed genes can lose or gain function and the fusion of genes can drive many different malignant processes that underlie tumorigenesis. DSBs are, therefore, highly lethal and their repair is pivotal to maintain genome stability and prevent cancer.

DSBs can be induced by ionizing radiation [148]. Moreover, certain chemicals and ROS induced by cellular metabolism also have the potential to block replication and result in DSBs [17, 149]. Under high damage load, repair mechanisms can induce nicks in opposite backbones of the DNA in close enough proximity to produce DSBs. Similarly, ssDNA nicks can result in DSBs when the replisome attempts to synthesize DNA opposite these lesions. Importantly, some processes require the cell to generate DSBs, for instance to create genetic diversity during meiotic recombination [150]. Induction of these types of DSBs is of course tightly controlled.

If a homologous template is available it can be applied for its complementarity by Homologous Recombination (HR) which is therefore inherently limited to S phase [151]. A second mode of repair exists that results in the annealing of the two severed ends of a DSB that is referred to as Non-Homologous End-Joining (NHEJ) [148]. A mode of repair that is not restricted to cell cycle but is believed to be downregulated during S phase since it can have deleterious effects during this stage of the cell cycle [151]. In the absence of a homologous template NHEJ efficiently anneals a two-ended DSB [149].

Depending on how a DSB is generated, the DNA ends caused by an exogenous damage source are chemically altered and will not consist of a 5' and 3' terminus that is amenable for ligation. Thus the processing of both ends of the DSB is a prerequisite for both HR and NHEJ [30, 152]. Endogenous breaks, on the other hand, typically result in less aberrant DNA end structures that are a suitable substrate for direct ligation [30, 148, 152]. Interestingly, end processing is suggested to play an important role in regulation of HR and NHEJ. NHEJ can already ligate two ends with 4 or less basepair overhangs whereas HR requires long sections of ssDNA for strand invasion [153]. The selection to progress through the HR pathway is fixed when the DNA end is irreversibly processed into long ssDNA stretches to facilitate strand invasion [154]. Vice versa, stabilizing short resected NHEJ substrate ends will drive NHEJ in favor of HR [154]. Moreover, it has been shown that end resection is tightly regulated during the cell cycle adding a level of control to inhibit NHEJ and stimulate HR during S phase [34, 155].

NHEJ is conceptually the most straightforward way of fixing a DSB. Native damages such as blunt-ends or short 5' or 3' complementary overhangs at a break are amenable for *in vitro* repair by only the core eukaryotic NHEJ factors Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and DNA ligase IV [156]. The Ku70-Ku80 heterodimer has affinity for DNA ends and forms a ring structure around the DNA end protecting it from degradation [157] and recruiting DNA-PKcs and other downstream targets [148, 152]. DNA-PKcs in higher eukaryotes hold the two DNA ends together during the repair process [158]. The association of Ku70-Ku80 and DNA-PKcs changes the conformation of the DNA-protein complex exposing the end for processing by the endonuclease Artemis [159-161]. This protein complex can process a large variety of altered DNA structures at the DNA end, making the end available for repair [161, 162]. After a DNA polymerase is recruited that fills any ssDNA gaps, ligation can take place [163, 164]. Finally, DNA Ligase IV is recruited to close the remaining nicks [165, 166].

As mentioned earlier HR is restricted to S phase because of the need for a homologous DNA strand. HR is based on the exchange of homologous or semi-homologous sequences between a broken and intact DNA strand [149, 152]. The basic steps of HR involve end-resection, strand invasion, DNA synthesis and resolving the repair intermediate DNA structure [30]. Depending on the order of events that result in strand invasion HR culminates into different sub-pathways of which only the classic double-strand break repair (DSBR) pathway is discussed here [30].

The first step in DSBR is 5' end-resection producing a 3' ssDNA stretch. The MR(X)N complex (see section 2) is required for end-resection but the nature of the specific exonuclease is unclear [167, 168]. Currently, redundant nuclease activity has been described for several factors that contribute to end-resection [30]. Rad50 of the MRX complex is thought to hold the DNA ends together during HR, as it does for NHEJ in yeast [169, 170]. The processed ssDNA stretch is bound by RPA quickly after resection, which prevents formation of secondary DNA structure [171]. RPA in turn interacts with

Rad52, a core recombination protein that is required for most DSB repair pathways [30]. Rad52 and possibly MR(X)N actively load the Rad51 recombinase onto the ssDNA replacing the RPA molecules resulting in a Rad51 nucleofilament [152, 172]. The Rad51-DNA complex is ready for homology search and strand invasion resulting in D-loop formation interlinking the broken DNA strand and the intact template. However, the exact mechanism behind homology search is not fully understood. Recent progress has identified chromatin remodeling activity of Rad54 to be a driving force for the search for homology [30]. After strand invasion, replication can synthesize DNA across the homologous template. The resulting DNA structure of interwoven dsDNA is attached via Holliday junctions that need to be actively resolved [152]. After untangling of the intricate DNA structures both DNA strands are restored to intact dsDNA molecules.

4. Nucleotide Excision Repair in eukaryotes

4.1 A short history of Nucleotide Excision Repair

An abundant natural exogenous source of DNA damage is UV radiation from sunlight. UV radiation results in dimer formation between neighboring pyrimidines. These dimers are Cyclebutane Pyrimidine Dimers (CPD) and 6-4 PhotoProducst (6-4PP) that are the major components of UV induced DNA damage (see figure 3). The common denominator amongst lesions repaired by NER is that they distort the DNA double helix, unwinding it slightly. Mechanistically, NER is highly conserved from bacteria to higher eukaryotes. Amongst eukaryotes homology is high but not perfect. Because of its nature NER is not mutagenic and deficiency of this mode of repair due to genetic defects is associated with the cancer prone disorder Xeroderma pigmentosum in humans. If UV damages persist through S phase they can become lethal or mutagenic by blocking replication and transcription. The focus of this thesis and the research described in the following chapters is on NER. Therefore, more emphasis is put on this repair pathway.

The first mode of DNA repair that was discovered was photoreactivation: repair of UV induced damage that is dependent on visible light, later shown to be the result of photolyase DNA damage reversal (3.3). However, cells after UV treatment showed

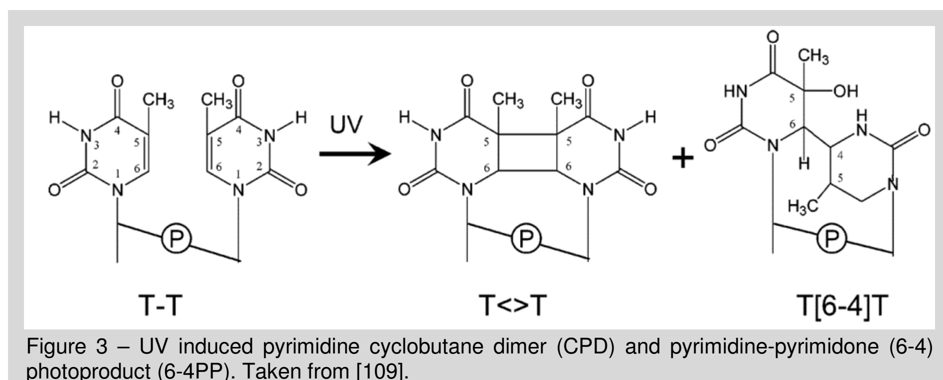

Figure 3 – UV induced pyrimidine cyclobutane dimer (CPD) and pyrimidine-pyrimidone (6-4) photoproduct (6-4PP). Taken from [109].

increased survival and reduced mutagenesis also when kept in the dark in suspension. This mode of 'dark repair' was shown to also occur if cells were not kept in medium and thus does not require cell division [173]. This procedure of liquid holding recovery was shown to be independent of light and thus a repair pathway separate to photoreactivation must exist [174]. From experiments with UV irradiated bacteria it became apparent that short oligonucleotides were 'excised' containing pyrimidine dimers and mutants deficient in this process, were identified. At the same time synthesis of short stretches of DNA was shown to occur independent of replication when microorganisms were grown on media containing radioactive nucleotides referred to as Unscheduled DNA Synthesis (UDS). These independent discoveries paved the way for the description of NER. Seminal work following the discovery of NER uncovered the link between defective DNA repair and cancer proneness. The fact that cells from Xeroderma pigmentosum patients are sensitive to UV *and* the patients display an increase in sunlight induced skin cancer, made it possible for researchers to link a DNA repair defect with cancer proneness for the first time [175, 176].

4.2 The Mechanism of Nucleotide Excision Repair

The mechanism that describes NER can be divided into damage recognition, bubble formation, incision, excision, repair DNA synthesis and ligation (see figure 4). The concerted action of 25-30 proteins in eukaryotes achieves this myriad of functions [177], that in *E.coli* requires the action of at least 6 proteins [178]. Therefore, I introduce the basic mechanism of NER by treating the bacterial system first, after which we carry on to describe the eukaryotic counterparts based on the *in vitro* reconstitution of the eukaryotic NER reaction.

In bacterial NER the UV induced lesion is recognized by the UvrA-UvrB heterotetramer containing two subunits of each protein. Upon DNA damage binding the UvrB protein specifically flips the damaged base into an extra-helical position after which the UvrA moiety dissociates from the complex. The UvrC endonuclease is recruited that incises the DNA 5' and 3' of the lesion. The incision is followed by the recruitment of the UvrD helicase that drives excision i.e. the removal of the incised oligonucleotide. Next, DNA polymerase I is recruited to close the ssDNA gap and remove UvrB from the DNA. Finally, the action of DNA ligase I anneals the DNA backbone to its original state [178].

Based on the *in vitro* reconstitution of the entire eukaryotic repair system the same basic steps were identified [179-181]. Over 20 proteins are necessary to perform NER in a test tube complementing the reaction with a DNA Polymerase and ligase [179, 180]. In yeast the core NER reaction starts with DNA damage binding by Rad4-Rad23 [182-184] (Figure 4A). The next step consists of the recruitment of Transcription Factor II H (TFIIH) [185, 186] that locally unwinds the DNA and is instrumental in recruiting the structure specific endonucleases Rad2 and Rad1-Rad10 that incise the DNA [187] (Figure 4B & C). The ssDNA created by TFIIH is bound by Rad14 and RPA in concert stabilizing the

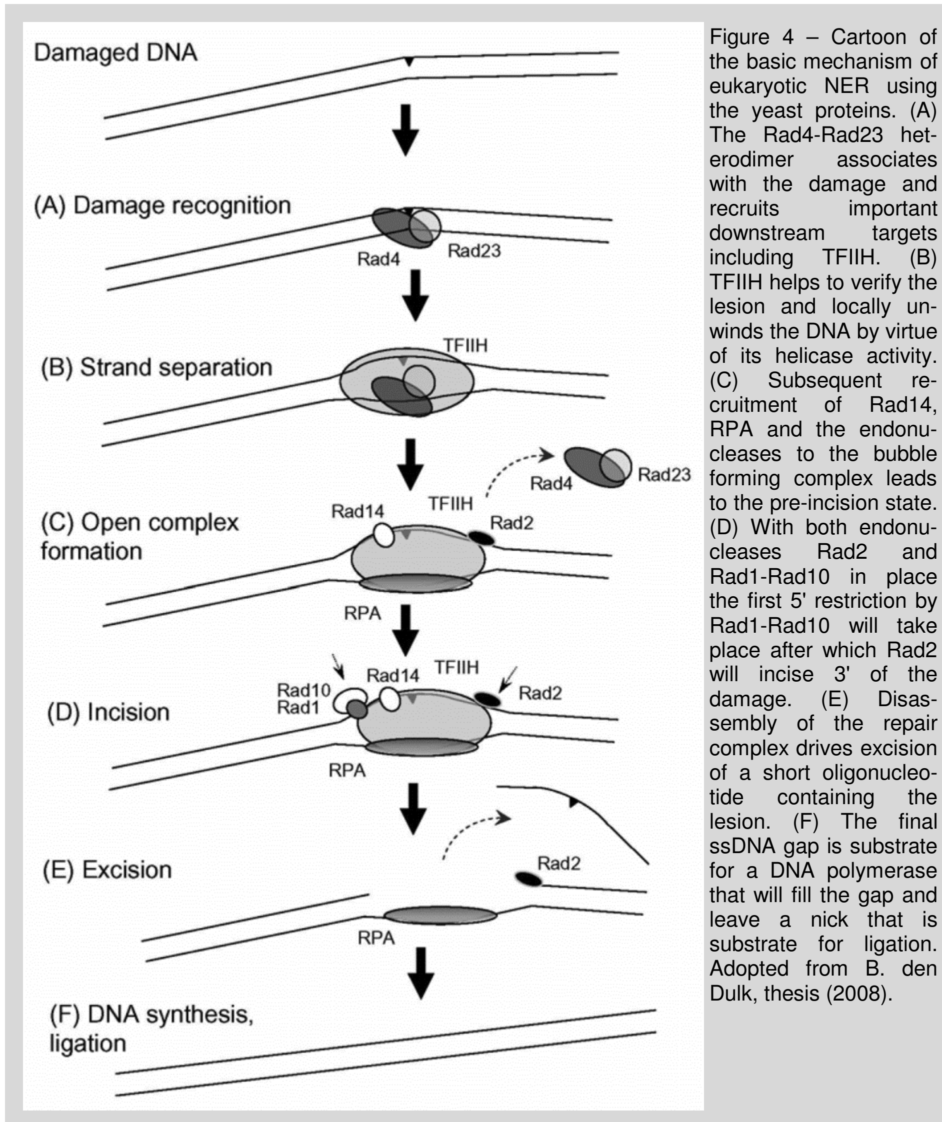

Figure 4 – Cartoon of the basic mechanism of eukaryotic NER using the yeast proteins. (A) The Rad4-Rad23 heterodimer associates with the damage and recruits important downstream targets including TFIIH. (B) TFIIH helps to verify the lesion and locally unwinds the DNA by virtue of its helicase activity. (C) Subsequent recruitment of Rad14, RPA and the endonucleases to the bubble forming complex leads to the pre-incision state. (D) With both endonucleases Rad2 and Rad1-Rad10 in place the first 5' restriction by Rad1-Rad10 will take place after which Rad2 will incise 3' of the damage. (E) Disassembly of the repair complex drives excision of a short oligonucleotide containing the lesion. (F) The final ssDNA gap is substrate for a DNA polymerase that will fill the gap and leave a nick that is substrate for ligation. Adopted from B. den Dulk, thesis (2008).

pre-incision complex [188, 189]. Finally, the recruitment of a DNA polymerase that fills the ssDNA gap [190] and a DNA ligase that rejoins the DNA backbone at the remaining nick, completes the repair reaction (Figure 4F). Mechanistically, NER utilizes the DNA double stranded nature for damage recognition and incision to completely remove an oligonucleotide containing the damaged base or nucleotides. The complementarity of the resulting ssDNA is then a perfect substrate for faithful replication of the excised DNA changing the DNA back to its undamaged state.

4.3 DNA damage recognition

NER recognizes helix distortion by probing the DNA double helix for loss of integrity, rather than direct recognition of an aberrant base or nucleotide. This has been described for both bacterial and eukaryotic NER and makes this repair pathway exquisite due to its diverse substrate specificity. The core NER complex Rad4-Rad23 is responsible for discriminating damaged from non-damaged DNA *in vitro*. The Rad4 protein has a propensity to interact with bubble structured DNA or ssDNA inherent to helix distorted lesions. With Rad4-Rad23 present at a site of damage all downstream factors described in the following sections can be recruited as in the absence of Rad4 no appreciable repair can be detected. The Rad4-Rad23 complex from yeast was purified and subjected to *in vitro* characterization that lead to the discovery of its higher affinity for 6-4PPs over CPDs and its capacity to complement *in vitro* repair [182, 183, 191]. Collectively, data on the main DNA damage binding factor in NER indeed shows that the affinity for bubble structure DNA opposite a lesion is what drives damage recognition in NER [182-184, 192-200]. Follow-up research identified in more detail the affinity of Rad4 and XPC for UV damaged DNA and more specifically the importance of the C-terminal aromatic residues that coordinate interaction with ssDNA inherent to helix distorting lesions [193, 196, 201]. With the advent of the Rad4-Rad23 structure bound to damaged and non-damaged DNA the underlying mechanism that drives damage recognition is now largely unraveled (Figure 5 on page 35) [184]. Indeed, Rad4 binds damaged DNA opposite to the pyrimidine dimer interacting with the unpaired bases that result from such lesions. The insertion of a beta-hairpin into the DNA double helix results in flipping out of the CPD into an extra-helical confirmation. These data were further confirmed by *in vitro* studies that already suggested that positioning a bubble structure in the vicinity of a lesion stimulates its recognition [200, 202]. Thus, the high substrate versatility of NER is made possible by the molecular detection of damage-associated helix distortion.

4.4 Bubble formation and Incision

After damage recognition the next major step towards repair of the damaged DNA is recruitment of TFIIH that is responsible for the local unwinding of the dsDNA helix around the DNA lesion. In yeast TFIIH is recruited by Rad4 analogous to the XPC-TFIIH interaction in higher eukaryotes [185, 203, 204]. Pioneering work on TFIIH established that it possessed a dual function in transcription and repair [185, 205]. At the same time, different forms of TFIIH were described that functioned differently in transcription and/or repair *in vitro* [206]. TFIIH drives transcription by melting the region around the promoter via its 5'-3' directional helicase subunit, Rad3 in yeast or XPD in humans [207]. The unwinding activity of Rad3/XPD further supports the recruitment of the two structure specific endonucleases Rad1-Rad10/XPF-ERCC1 and Rad2/XPG [207, 208]. The second helicase subunit Rad25/XPB is responsible for transferring large conformational changes of the TFIIH complex driven by ATP hydrolysis to anchor the complex at the site

of damage [207, 209]. Due to the local unwinding of the DNA by Rad3/XPD more ssDNA becomes available that is bound by Rad14/XPA and RPA traditionally referred to as damage verifiers [188]. The process of verification is not understood on a mechanistic level, but the interaction of Rad14/XPA with the pre-incision complex is essential because in its absence NER cannot take place making Rad14/XPA a core NER factor similar to Rad4. ssDNA coated with RPA is thought to initiate DDR signaling when NER does not repair the damage [23, 210]. Thus TFIIH interacts with Rad4/XPC [203, 204, 211] and supports damage verification by Rad14 [186] and the recruitment of the two endonucleases [206].

When TFIIH is anchored to the site of damage and the local bubble is formed the structure specific endonucleases can be recruited that incise the DNA at ss-dsDNA junctions 5' and 3' of the damage. For the human proteins a detailed description of the coordination of incision has been described recently [188, 212]. This tight coordination is not trivial as nicks in the DNA double helix are damages themselves as described in detail in the previous sections. Rad2/XPG is the first endonuclease to be recruited by direct interaction with TFIIH. Rad2/XPG binding to the pre-incision complex initially stabilizes the complex [188, 212]. In NER the presence of both the structure specific endonucleases forms the first mutually exclusive condition that drives incision 5' of the damage by XPF-ERCC1 [188]. The first incision requires the presence of XPG in the pre-incision complex but not its catalytic activity. The structural conformation changes in XPG that drive the 3' incision reaction do not take place before 5' incision by XPF-ERCC1 has occurred [188, 212]. Interestingly, DNA repair synthesis can start in the absence of the second 3' XPG-induced incision but not the protein, shown by the recruitment of PCNA, RFC and DNA Pol δ and detection of UDS in a catalytic dead XPG background [212]. This implication prompted the authors to suggest a cut-patch-cut-patch mechanism [212]. This mechanism describes the crucial first 5' incision by XPF-ERCC1 (cut) and the presence of both endonucleases as a precedent for starting DNA repair synthesis (patch). These initial steps are followed by displacement synthesis inducing incision of XPG 3' of the lesion (cut) to allow repair synthesis to finish filling the gap (patch) [212]. This tight coordination prevents the creation of ssDNA and is reminiscent of long-patch BER [130, 213]. A model for regulation of incision in yeast has not been described to date. However, based on the extensive homology between the yeast and human proteins and protein functions involved in incision, incision in yeast no doubt occurs via a similar mechanism.

4.5 Oligonucleotide excision and repair synthesis

Obviously, the final stages of NER are as much crucial as damage recognition and all the steps described before. However, excision (i.e. removal of the incised oligonucleotide) and DNA repair synthesis and ligation did not receive ample attention as compared to the damage recognition and early repair steps, because DNA synthesis had

already been described in much detail. Knowledge of DNA replication can be extended to show that short patch UDS occurs via a similar mechanism because all of the *in vitro* reconstituted systems use replicative DNA polymerases and DNA ligase I to achieve NER of an artificial substrate [179, 180, 214, 215]. By extension it was assumed that the replicative B-family polymerases do the job *in vivo* as well since NER is non-mutagenic. However, more recent data describes the involvement of error-prone Y-family polymerases associated with NER complexes [216]. Therefore, the regulation and active recruitment of DNA polymerases to NER foci is less well described.

PCNA is recruited to NER foci by the concerted action of the endonucleases XPF and XPG, and XPA [217-220]. With the PCNA platform present different DNA polymerases are actively recruited to the NER post-excision complex supporting DNA synthesis. It appears that, depending on the ubiquitination state of PCNA and the relevant clamp loader at the repair site, three different polymerases can be recruited post-incision [221]. These authors propose that the nature of the incision product and the chromatin context drives recruitment of either of the polymerases. If a repair site is swiftly incised 5' and 3' of the lesion leading to the relatively unhampered disassembly of the repair complex and release of the oligonucleotide. This substrate is amenable for repair synthesis by a replicative DNA polymerase [221]. However, if the 5' and 3' incisions do not take place in quick succession the NER intermediate structure is subject to displacement synthesis; repair synthesis that displaces the overhanging flap structure of the ssDNA protruding from the non-excised 3' end of the lesion. This mode of repair is what was described as the cut-patch-cut-patch model in the previous section [189]. The different substrates not only recruit the specific polymerase suited for the job, but the association of the different polymerases are mutually exclusive by actively inhibiting recruitment of the other polymerases which are less suited for the job [221]. Collectively, the final stages of NER drive excision and repair synthesis, resulting in perfect repair of the DNA making full use of the inherent complementarity of DNA.

4.6 From in vitro to in vivo NER

Interestingly, some NER factors in yeast and human are dispensable for the *in vitro* reconstituted NER reaction but are required for efficient *in vivo* repair [179, 180]. These factors in yeast include Rad7, Rad16 and Rad26. Deletion of *RAD7* or *RAD16* results in moderately UV sensitivity [222]. It turns out that these NER factors are responsible for DNA damage recognition in a chromatin context. These repair proteins detect lesions that are located anywhere in the genome and are part of the Global Genome NER (GG-NER) pathway.

Moreover, a bias in repair of active versus inactive genes exists elucidated by gene-specific repair assays [223, 224]. More specifically, by using strand-specific repair assays a strand-bias in active genes can be detected. This means that the transcribed strand (TS) of an active gene is repaired more quickly than the non-transcribed strand

(NTS) [225]. This pioneering work fully implicated transcription into NER and led to the description of Transcription Coupled NER (TC-NER) [223-225]. Mutants were identified that where specifically disrupted in this mode of NER, leading to the characterization of the TC-NER factor Rad26 in yeast [226]. DNA damage that is specifically located in the transcribed strand (TS) of active genes is detected and repaired by TC-NER. Thus extending the *in vitro* data it is now evident that the *in vivo* chromatin context of DNA adds a level of complexity to NER that resulted in the GG- and TC-NER pathways in NER that contribute to DNA repair and UV survival by specifically tackling *in vivo* hurdles to the core NER reactions.

4.7 Global Genome NER

In yeast, GG-NER specifically requires the Rad7-Rad16-Abf1 protein complex [227-229]. Rad16 shows homology to the family of SWI/SNF chromatin remodelers. Moreover, the yeast GG-NER proteins have been shown to be part of an E3 ligase complex that contributes to repair and survival by ubiquitinating Rad4 in yeast [230, 231]. Rad7 and Rad16 were shown to be important for repair of the silent mating type locus in yeast and the NTS of active genes, all bona fide substrates for GG-NER [232].

In higher eukaryotes, the functional homolog of Rad7-Rad16 is most probably UV-DDB made up of DNA Damage Binding proteins 1 and 2 (DDB1 & DDB2) [233, 234]. Moreover, the XPC-hHR23B-CEN2 complex is also exclusively required for GG-NER. In higher eukaryotes the presence of UV-DDB and XPC-hHR23B-CEN2 is most likely warranted by the different substrate specificity of these two complexes. Whereas UV-DDB has affinity for both 6-4PP and CPDs, XPC-hHR23-CEN2 has more affinity for 6-4PPs. It has actually tremendous difficulty in recognizing CPDs and requires UV-DDB for recruitment to CPD photolesions *in vivo* [195, 235, 236]. In yeast, this type of substrate affinity and diversification of GG-NER factors recognizing them does not exist. Rad7-Rad16-Abf1 is solely required for GG-NER of all NER substrates *in vivo*.

Detailed biochemical characterization of GG-NER in human cells provided important insight into the mechanism of DNA damage binding and the so called 'damage handover' between UV-DDB and XPC [237]. The interesting notion that UV-DDB [237] is involved in an E3 ubiquitin ligase complex appeared to be crucial in this process. UV-DDB resides in an E3 ligase complex made up of DDB1-DDB2, CUL4A and ROC1 [238]. Interestingly, the UV-DDB E3 ligase ubiquitinates XPC and DDB2 after DNA damage induction [201, 237]. The fate of these targets is remarkably different: ubiquitinated XPC has increased damage affinity while autoubiquitination of DDB2 leads to loss of UV-DDB at the site of damage facilitating the so-called damage handover from UV-DDB to XPC-hHR23B-CEN2 [237]. This dual action damage recognition could possibly underlie the GG-NER mechanism in yeast.

No structure data for the yeast Rad7-Rad16-Abf1 complex bound to damaged DNA is available, however, the structure of UV-DDB bound to a lesion has been solved [239].

The interaction of the DDB2 subunit with a lesion is entirely opposite to what has been described for Rad4. UV-DDB recognizes and flips out the 6-4PP into its binding pocket. Spatial 3D modeling of the known UV-DDB structure and Rad4-based XPC model showed that UV-DDB can expose the ssDNA opposite a lesion and accommodate XPC binding *in trans*. The concerted action of two GG-NER complexes in higher eukaryotes is thus required to cover a wider spectra of substrates that apparently in yeast is covered by Rad7-Rad16-Abf1 alone.

4.8 Transcription Coupled NER

UV-induced lesions are potent transcription blockers and the presence of a stalled RNA polymerase at a site of damage makes it both a damage signal as well as an obvious block to repair. By blocking a UV lesion the RNA polymerase makes the lesion less accessible for GG-NER to repair the lesion, explaining why a dedicated set of proteins is required to process the RNA polymerase at lesions in the transcribed strand (TS) of active genes. How a cell discriminates between a stalling RNA Pol II complex and a transcription complex blocked at a DNA damage is an important question regarding TC-NER. How a cell exactly targets blocked polymerases is still under debate but several mechanisms for the early steps in TC-NER have been put forward.

Importantly, TC-NER is not uniform across a single gene, between genes or during different growth phases of the cell. Before RNA polymerase II transcription becomes fully processive, repair around the promoter region of an active gene is performed by GG-NER and thus Rad26-independent in yeast [240]. Highly transcribed genes display mostly Rad26-independent repair [241]. Repair of the TS becomes Rad26-dependent 30-40 bases downstream of the transcription start overlapping with the transition from transcription initiation to elongation of the RNA polymerase [240, 242]. Thus Rad26-dependent repair is specifically required to handle TC-NER of elongation RNA polymerases II. In yeast, DSIF and Spt4 control this mechanism, that when absent results in less processive transcription. By deleting *SPT4*, the RNA polymerase will not transition into fully elongative modus and TC-NER thus becomes completely Rad26-independent [243]. In hindsight *SPT4* deletion was found as a suppressor of *rad26* UV sensitivity, which can now be explained in detail as described here.

In yeast, two sub-pathways of TC-NER based on the action of RNA Pol II subunits Rpb4 and Rpb9 have been described [241]. In yeast cells, Rad26-independent repair can be detected that is dependent on Rpb9 but inhibited by Rpb4 [241]. Rpb9 is a zinc-binding domain protein that has sequence homology to TFIIS. Since TFIIS is known to be able to help RNA Pol II back-track when stalled, Rpb9 could have a similar function in TC-NER also explaining why TFIIS is not required for TC-NER in yeast [241]. However, there is no biochemical evidence available to support this claim.

During the early stages of transcription of the first 40 or so basepairs, TFIIH is still associated with the polymerase thus eliminating the need for active recruitment of TFIIH

by TC-NER factors. Moreover, early transcription complexes are known to be less processive and hence are more easily disrupted. Both of these concepts are thought to bypass the need for Rad26 in yeast TC-NER close to the transcription initiation site or for highly transcribed genes. These data suggest that the rate of transcription, in part determines the faith of the TC-NER process employed.

The early steps in TC-NER that target the RNA polymerase for removal or degradation have been described in more detail for human cells. These data will be described here. RNA Polymerase II stalled at a damage drives TC-NER [242, 244, 245] and the large subunit (LS) of RNA Pol II is subject to DNA damage induced phosphorylation and ubiquitination [242, 246-251]. The Cdk-activating kinase (CAK) subcomplex of TFIIH was shown to phosphorylate the C-terminal tail domain (CTD) of RNA Pol II required for UV induced degradation of the large RNA Pol II subunit [252]. The TC-NER factor Cockayne Syndrome B (CSB) in higher eukaryotes actively associates with a damage stalled polymerase, presumably via interaction with the phosphorylated CTD [242]. CSB then recruits TC-NER factor Cockayne Syndrome A (CSA) and other downstream targets [253]. CSA resides in an E3 ligase complex and associates with RNA Pol II irrespective of UV irradiation [238, 254]. However, the interaction between the ligase and the polymerase increases after DNA damage induction due to the hyperphosphorylated state of Pol II [238]. The CSA complex associates with the COP9/CSN signalosome in response to UV inhibiting the ubiquitin ligase activity. The dissociation of CSN from the complex results in activation of the CSA E3 ligase that is required to resume transcription after DNA repair and is not involved in the TC-NER process [238, 254, 255]. A role for the CSA E3 ligase in RNA Pol II LS ubiquitination and degradation is plausible as the involvement of CSA and CSB in UV induced ubiquitination of RNA Pol II LS has been described [250, 251] but ubiquitinated RNA Pol II LS has not been directly detected in TC-NER protein complexes [238, 253]. Thus it appears that even though ubiquitination of RNA Pol II in response to UV damage is an early response in TC-NER, it can be uncoupled from the repair reaction or complex [238].

Importantly, TC-NER in higher eukaryotes can take place through remodeling and/or displacement the RNA Pol II complex without disrupting it, thus negating the use of ubiquitin directed degradation of the complex under some conditions [242]. Interestingly, CSB shares homology to SWI/SNF chromatin remodelers and it is thought that through this action it can remodel the RNA Pol II DNA-RNA complex. While CSB is not able to fully displace a stalled RNA polymerase *in vitro*, it can stimulate the polymerase to add another nucleotide to the transcript aiding in exposing the damage for the core repair factors [242]. Similarly, recruitment of TFIIS by CSB could drive the more subtle polymerase remodeling mechanism of back-tracking [242, 253]. Remodeling might be the heart of the initial steps during TC-NER when RNA Pol II degradation is not applied.

The multitude of data from both yeast and human cells describes the early stages of TC-NER that act on the RNA polymerase. Importantly, however, a CSA homolog in yeast

does not exist and the equivalent E3 ligase complex in yeast has not been described. Similarly, an active role for the yeast TC-NER factor Rad26 in degradation of Rpb1 or remodeling of the polymerase complex has not been described to date [249]. However, phosphorylation and ubiquitination of Rpb1, the large subunit of RNA Pol II in yeast, and the responsible ligase, Rsp5, have been shown highlighting some important parallels between the dominant model organisms [246-248, 251]. The activity of CSB that remodels the RNA polymerase could be performed by Rad26 in yeast as Rad26 also resembles SWI/SNF chromatin remodelers. However, in yeast RNA Pol II remodeling is not sufficient to bypass the need for Rad4-Rad23, which is the case for XPC-hHR23B in humans. Thus the activity of CSB as opposed to the early steps of TC-NER in yeast mediates damage recognition by processing the RNA Pol II complex into a suitable substrate for NER without the need for XPC. In addition, TFIIS recruitment by CSB could aid in this process. Importantly, TFIIS is not required for TC-NER in yeast a function that could probably be full-filled by Rpb9.

Because the yeast genome consists of short genes that produce smaller transcripts a yeast cell can permit to abort transcription by completely removing and degrading Rpb1 and resume transcription after DNA repair. It is known that in higher eukaryotes more effort is put into maintaining the transcription complex intact. Indeed, TC-NER can take place by back-tracking and remodeling of the transcription complex without the need for disrupting the RNA Pol II complex in higher eukaryotes [242]. TC-NER defects in human cells lead to Cockayne Syndrome and extreme UV sensitivity, while TC-NER deficient yeast cells are not UV sensitive [226]. This underscores the different contribution of TC-NER to cellular survival between yeast and humans.

Summarizing, the active recruitment of downstream NER factors by CSA and CSB is able to drive TC-NER and funnel it into the core NER reaction in humans. The active role for Rad26 and Rad28 in yeast are less well described. The faith of the RNA Pol II complex will differ between organisms but also between different repair loci and difference in rate of transcription or growth phase of the cell. Taken together, the process of damage recognition and remodeling or removal of the transcription complex is at the heart of TC-NER that will fuel NER.

5. rDNA and repair

The rDNA is of interest in the context of NER and this thesis in particular because during evolution *S. cerevisiae* obtained a Rad4-like protein responsible for TC-NER of this locus. This Rad4-like protein, Rad34, is discussed in more detail in the next section and chapter 3 [256, 257].

The higher order structure of the chromatin containing the rDNA genes results in the formation of the nucleolus, a nuclear compartment harboring the rRNA synthesis, processing and ribosome assembly [258-260]. The rDNA locus in yeast resides entirely on chromosome 12. The tandem arrangement of ~150 copies of the 9.6kb rDNA

cassette makes up the entire rDNA. Each cassette contains the 18S, 5.8S and 25S rRNAs that are transcribed concurrently by RNA Pol I to form the 35S pre-rRNA transcript. The intergenic space separating any two copies of the 35S rDNA gene contains the 5S rDNA gene transcribed by RNA Pol III. Roughly 50% of all copies are actively transcribed while the other half is in a heterochromatic state in yeast. The repetitive nature of rDNA makes it a prime target for homologous recombination, a process that is tightly kept in check [261]. Interestingly, sporadic recombination at the rDNA locus results in the formation of Extra-chromosomal rDNA Circles (ERCs) that have been shown to result in replicative aging in yeast [262-265]. This insight provided the first example of a molecular model for aging, that in hindsight appeared to be specific for yeast.

The overall size and transcriptional activity of the yeast genome makes that there is not a lot of heterochromatin as compared to higher eukaryotes. In yeast rDNA, the silent mating type locus HML and telomeres are the most distinct heterochromatic regions. rDNA is therefore an excellent target for research on repair of silent versus transcribed DNA as psoralen crosslinking allows researchers to separate active from inactive rDNA to study their repair kinetics individually [266, 267]. Early reports on DNA repair at the rDNA locus showed that in the active copies the TC-NER driven strand-bias is readily detectable and GG-NER fixes lesions in the inactive fraction in yeast [267]. First and foremost these data showed that the NER machinery has unimpeded access to the nucleolar DNA, which is not as trivial as it seems. Furthermore, yeast appears to be the only model organism to display rDNA TC-NER [266] and, as will be clear in section 6.2, has a special Rad4-like protein exclusively required for this mode of repair [256, 257]. Moreover, these authors were able to show that actually photolyase repair (PR) was very efficient in removal of CPDs at the rDNA locus [267]. Another interesting feature of TC-NER in rDNA links back to the Rad26-dependent TC-NER described in section 4.7. Here we described that Rad26 drives TC-NER of the elongating RNA polymerase II while early repair around the transcription start-site and during the pre-elongating phase, when TFIIH is still associated with the polymerase complex, is independent of Rad26 (Janssen, thesis 2002). Interestingly, data from our lab showed that rDNA repair can be detected in the absence of Rad26, but also in the absence Rad7, Rad16 and Rad4 [268]. The absence of Rad26 in rDNA TC-NER seems unusual but if we recall that the state of transcription modulates the use of Rad26 in a Rpb4-dependent fashion [241] an obvious parallel surfaces. Genes that undergo Rad26-indepedent TC-NER are heavily transcribed, similar to rDNA transcription of RNA Polymerase I that is very proficient [258]. Thus, highly transcribed rDNA does not require Rad26 activity to drive TC-NER.

Interestingly, these data from our lab also hint at Rad7 and Rad16-independent repair of rDNA [268]. It remains to be discerned exactly how rDNA is repaired in the absence of GG-NER.

6. The NER proteins important for this thesis

After a general introduction covering the details of NER and its sub-pathways, I continue with a detailed description of the NER factors that are covered in the research chapters of this thesis. In the context of our research Rad4, Rad23, Rad34 and Rad33 require extra attention and will be described in the following sections.

6.1 The core NER complex Rad4-Rad23

The Rad4-Rad23 proteins make up the core NER complex involved in DNA damage recognition and recruitment of downstream repair proteins in yeast [179]. A novel NER factor Rad33 has been shown to be part of the complex as well [269]. Both TC- and GG-NER funnel into the core repair reaction via Rad4-Rad23 [177]. As soon as Rad4-Rad23 associates with the DNA damage Rad14 and TFIIH can be recruited [186]. The C-terminus of Rad4 is an important protein interaction domain for TFIIH and other NER proteins.

The structure of a truncated Rad4 protein has been solved in complex with partial Rad23, in free form, bound to DNA and bound to a CPD (see figure 5) [184]. Rad4 contains a TransGlutaminase-like Domain (TGD) [270] that together with the Beta Hairpin Domains (BHD) 1 and 2 bind to the DNA phosphate backbone. In doing so these domains provide the affinity for non-damage DNA and position BHD3 to interact with the DNA opposite the lesion [184]. As discussed previously, NER owes its uniquely broad substrate specificity to the affinity for helix distorted DNA. Data from the Rad4 structure confirms this. The protein interacts with the ssDNA opposite the CPD. The CPD is unbound and shows no structure in the crystal data. The beta hairpin of BHD3 inserts into the DNA double helix flipping out the CPD into an extra-helical position [184]. Upon binding of the protein to a lesion the DNA is slightly bend in order to accommodate BHD3 binding opposite the CPD. Mechanistically, this is believed to prevent sporadic binding to non-damaged DNA, because this conformational change cannot occur in normal DNA [184]. The long-standing affinity of Rad4 and XPC for ssDNA, bubble structures and the general propensity to recognize more helix distorting lesions with increasing affinity now materialized with the advent of the Rad4 structure.

As core NER factor, Rad4 cannot be missed. In absence of Rad4, NER is disrupted and yeast cells become extremely UV sensitive. *RAD4* gene expression is not upregulated in response to UV, however, Rad4 is under UV-induced post-translation control via ubiquitination [231, 237]. Association of Rad4 with Rad23 has been described to help stabilize the protein and protect it from proteasomal degradation [271, 272]. However, artificially stabilizing the Rad4 protein levels by overproduction of Rad4 [191, 271] or disrupting Rad4 ubiquitination [231] does not rescue the UV sensitivity or NER defect of a *rad23* mutant. The Rad4 binding domain (R4BD) of Rad23 is uniquely required for the interaction with Rad4 (see chapter 5) and Rad4 is exclusively found in complex with Rad23 when purified from yeast [182, 183]. Similarly, Rad33 has been shown to support

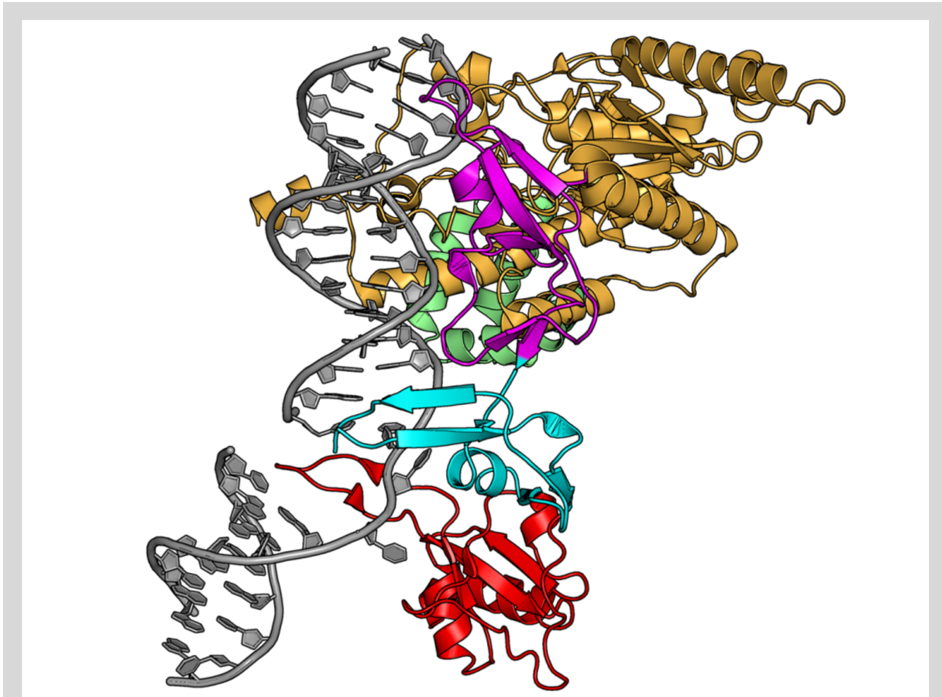

Figure 5 – Cartoon of the Rad4-Rad23 protein structure bound to a short oligonucleotide containing a CPD lesion. A sideview of the protein structure bound to DNA with the protein domains highlighted in their specific color as presented here. In yellow the TGD domain is highlighted, followed by BHD1 (pink), BHD2 (Blue) and BHD3 (red). The Rad23 Rad4 Binding Domain is shown in green bound to the Rad4 TGD. Adopted from Min & Pavletich (2007) [184].

normal Rad4 protein levels via a yet to be identified mechanism. Rad4 protein levels are reduced in *RAD33* deletion cells, but they remain constant in response to UV irradiation [269]. However, in the absence of Rad23, Rad4 protein levels are severely reduced and subject to UV induced degradation.

RAD23 deletion cells are moderately UV sensitive but display hardly any detectable repair activity *in vivo* [273-276]. This contradiction in part underlies the discrepancy between the colony forming ability of UV irradiated cells over the course of 2-3 days in UV survival curve analysis and the repair efficiency of UV treated cells during the first 2 hours after DNA damage. Defects in the NER reaction can be very accurately assessed by strand specific repair assays during the early times after UV irradiation but colony forming ability extends far beyond the first 2 hours of repair and involves the concerted fidelity of replication and cell cycle progression to support UV survival. This implies that mutants displaying absence of repair but moderate UV sensitivity, perhaps have defects in replication or cell cycle arrest, progressing through cell division uninterrupted after UV. Rad23 is known to affect transcription of plethora of UV-inducible genes (chapter 2, [277]), by affecting a change in expression of important DDR and cell cycle checkpoint genes it is possible that *rad23* cells display no detectable repair but still recover from UV irradiation relatively well.

Rad23 is a unique protein that together with Dsk1 and Ddi1 are the only known proteins in yeast that harbor both a Ubiquitin-like (UbL) and Ubiquitin Associating (UBA) domains [278-281]. It is therefore not surprising that these proteins function in shuttling substrates to the 26S proteasome for degradation [282-284]. Rad23 performs this task outside the context of NER, however, some evidence has been put forward describing the participation of Rad4 in protein turnover [285]. The concerted action of binding to ubiquitinated proteins via the UBA domains and association with the proteasome via its UbL domain is thought to underlie the shuttling function of Rad23 and Rad23-like factors in mediating protein delivery to the proteasome. Interestingly, some proteasome mutants are UV sensitive [282, 286], suggesting that even though the function of Rad23 in protein ubiquitination and degradation is mostly separate from NER, some level of interplay between these two functions exists.
The Rad23 protein contains one UbL domain, two UBA domains and a R4BD all separated and connected by flexible linker regions. The UbL domain is crucial for UV survival and can be replaced by the canonical ubiquitin sequence [278]. Rad23 has been shown to be ubiquitinated *in vivo* but is a relatively stable protein [278]. This stability is maintained by the C-terminal UBA2 domain that acts as an intrinsic stabilization domain for UbL containing and/or ubiquitinated proteins and is thought to allow Rad23 to be ubiquitinated and interact with the proteasome without being degraded [287]. Not only does the UBA2 domain elicit an intrinsic stabilization onto Rad23 itself, Rad23 also binds to short ubiquitin chains on proteins inhibiting their multi-ubiquitination and subsequent degradation [280, 283, 286]. The majority of ubiquitinated protein interaction is supported by the more N-terminal UBA1 domain [279, 281, 283, 287]. Importantly, a UBA1,2 double deletion leads to UV sensitivity [279, 280] but residual potential for ubiquitinated protein binding of the mutated Rad23 protein remains [281]. Protecting Rad4 via Rad23 interaction is very much in line with the ascribed function for the UBA domains in interacting with ubiquitinated proteins [280, 283, 288] and the general inhibitory effect of Rad23 on multi-ubiquitin chain formation [286].
The exact role for Rad23 in NER is not fully understood. *RAD23* is UV inducible [274] and contributes to NER [186, 194, 275, 276]. The presence of the UbL domain in Rad23 and its interaction with the 19S proteasome does provide insight into the possible involvement of the proteasome in NER. The role of Rad23 in NER by interacting with Rad4 and its interaction with the proteasome are separable [272]. It is the UbL interaction with the 19S particle that also plays a role in NER [289, 290]. The 19S Regulatory Particle of the proteasome has been suggested to act as a chaperone [282, 290] that could aid in complex assembly or disassembly. However, the action of Rad23 could still be that of a shuttling factor for certain NER proteins (i.e. Rad4) to facilitate their degradation. Further evidence for the role of Rad23 in NER has been described for both yeast and human hHR23B. For both model organisms *in vitro* data has been put forward

that describes a stimulatory role for Rad23 in DNA damage binding of Rad4 and XPC [191, 194].

As will become evident in chapter 2, Rad23 and Rad4 also play an important role in UV induced gene transcriptional regulation of the RNR pathway of genes. Interestingly, the GG-NER E3 ligase in yeast, introduced in section 4.7, plays a pivotal role in this regulation and was shown to ubiquitinate Rad4 in a mode that is independent of active NER [231]. A role for Rad23 in transcription has been described previously [277], however, the implications for DDR signaling and UV induced gene expression via the GG-NER E3 ligase have not been reported previously.

In summary, the Rad4-Rad23 protein complex facilitates NER and the interaction with the 19S RP. At the same time Rad4-Rad23 provides a platform for recruitment of TFIIH and Rad14. More importantly, Rad4-Rad23 also regulate UV-induced gene expression described in more detail in chapter 2. The concerted action of Rad4-Rad23 in these multifaceted processes is a testimony to the importance of this core NER factor in yeast.

6.2 Rad34

NER is highly conserved both genetically as well as mechanistically, however, some exceptions exist between the dominant model organisms. Both Rad4p and XPC share some sequence similarity [291] and reside in protein-complexes of similar makeup, however, yeast contains a second distinct Rad4-like protein called Rad34 which is required exclusively for TC-NER of rDNA [256, 257]. When *RAD34* is knocked out in a *rad4*∆ background, repair of the TS of active rDNA is completely abolished [292, 293]. Vice versa, it is already known for some time that rDNA is still promptly repaired in the absence of Rad4 [294]. Rad34 shares C-terminal homology to Rad4 [291] and associates with Rad23 [295]. These data corroborate the Rad34-Rad4 homology, but at the same time unequivocally underscore the separation of function of these two yeast NER factors.

The role for Rad4 and Rad34 in rDNA repair underscores the TC-NER specificity of Rad34, reminiscent of the Rad26-dependent TC-NER of RNA Polymerase II transcribed genes. TC-NER at active rDNA genes becomes Rad34-dependent ~40 nucleotide downstream of the transcription start site while around the promoter region repair is entirely Rad4-dependent [257]. Highly RNA Pol II transcribed genes do not require Rad26 for TC-NER [296]. rDNA is highly transcribed and 40 basepairs away from the transcription start site, RNA Pol I is in an elongating modus. However, conscientious analysis of the RNA polymerase I initiation complex showed that RNA Pol I switches to elongation around base 12 downstream from the promoter [297]. These data do not perfectly overlap with the Rad34-dependent TC-NER transition 40 basepairs downstream of the promoter. Either the transition from initiation to elongation does not correlate with the transition from Rad4 to Rad34 dependent repair or technical aspects of these study make comparison impossible.

Interestingly, elongating RNA Pol I thus requires Rad34 but not Rad26 [257, 268]. Thus the Rad26 activity suggested for handling an elongating RNA Pol II complex stalled at a damage is not required during rDNA TC-NER but a different Rad4 protein is warranted. This discrepancy will be discussed in further detail in chapter 3, where we try to decipher the conditions that create the exclusive preference for Rad34 over Rad4 during rDNA TC-NER.

6.3 Rad33

Rad33 is a novel NER factor. A *RAD33* deletion results in moderate UV sensitivity and is epistatic with the deletion core NER factors *RAD4* and *RAD14* [298] [299]. In depth DNA repair analysis revealed that TC-NER is moderately affected whereas Rad33 is absolutely required for GG-NER [298]. Similarly, Rad33 was shown to interact with Rad34 [300].

Bioinformatic structure predictions for Rad33 show a weak resemblance to calmodulin-like proteins [269]. Interestingly, in higher eukaryotes a trimeric complex exist around the human Rad4-homolog, XPC consisting of hHR23 and Centrin 2 [301]. Through a series of *in vitro* experiments it became apparent that the calmodulin-like protein CEN2 is a *bona fide* NER factor that in cohort with hHR23B stabilizes XPC and contributes to DNA binding and incision *in vitro* [302]. More detailed analysis of the XPC-CEN2 interaction led to the identification of a conserved WLL binding motif in the C-terminus of XPC [302, 303]. By binding to a conserved motif at the C-terminus of XPC, Centrin 2 molds the unstructured region into an α-helical fold upon binding possibly making the region more amenable for protein-protein interactions required during NER [302-305]. The XPC-CEN2 interaction is of functional significance in NER *in vivo* as XPC-CBM (Centrin Binding Mutant) containing cells are UV-sensitive [306]. Interestingly, the WLL motifs supports the Rad4-Rad33 interaction in yeast as well. Mutating the motif in yeast to three alanines, creating the *rad4AAA* allele, perfectly phenocopies a *RAD33* deletion strain showing that Rad33, like CEN2, functions in NER exclusively through the interaction with Rad4 [269].

The yeast centrin homologue Cdc31 was shown to give rise to low UV sensitivity in conditional deletions and interacts with Rad4 [307]. However, the parallel between Centrin 2 and Rad33 does not hold for Cdc31 [269]. Conversely, there is no evidence for a Rad33-like protein in higher eukaryotes; it thus seems that the centrosome and NER related functions of CEN2 are split between Cdc31 and Rad33 in yeast. This is further supported by the fact that there is no obvious role for Rad33 at the centrosome and the active role of Cdc31 in NER is still debatable. The novelty of a centrosome component at the heart of the XPC-complex is enigmatic and a possible link between centrosome duplication and repair seems plausible but has yet to materialize.

7. Scope of this thesis

In Chapter 2, we describe a novel mechanism for UV induced gene expression controlled by the GG-NER E3 ligase and Rad4-Rad23. This process implies the dissociation of Rad4-Rad23 from promoter regions in response to UV induced ubiquitination of Rad4 by the GG-NER E3 ligase, triggering gene expression of a subset of genes. In Chapter 3 the effect of Histone H1 on repair of the rDNA locus is studied in relation to Rad34. This Rad4-like protein is specifically needed in yeast for TC-NER of rDNA. In the absence of Histone H1, however, Rad4 can replace Rad34 in this repair pathway. A model is presented explaining why for TC-NER of rDNA a different Rad4-like protein is required. In Chapter 4 the homology between human and yeast NER is used to study the phenotype of a *RAD4* mutation, *rad4W496S*. This mutation affects a conserved amino acid in Rad4-like proteins that when present in XPC (W690S) results in a XP disease phenotype. We show that the Rad4W496S is mainly affected in GG-NER. Chapter 5 is dedicated to the detailed study of the Rad4-Rad23 interaction. We provide evidence for presence of two binding domains for Rad23 in Rad4 that are mutually exclusive. The function of these Rad4 binding domains in the two sub-pathways of NER is studied. Research described in Chapter 6 concerns the discovery of a *RAD4* mutant allele in a commonly used yeast background that confers conditional UV sensitivity. Finally, the results are discussed and summarized in Chapter 7.

8. References

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II Nucleotide Excision Repair Factors Directly Regulate dNTP Synthesis in Response to UV Damage in Yeast

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1. Abstract

DNA damage and replication stress can activate a signal transduction pathway called the DNA damage checkpoint. The checkpoint maintains replication fork integrity, promotes the completion of replication and DNA repair and coordinates these processes with progression through the cell cycle. This is achieved by regulating the transcription of DNA repair genes and other genes including those that control the cellular pool of dNTPs, which is necessary for cellular survival. Following DNA damage or replication block, the Dun1 protein kinase is phosphorylated in a Rad53 and Mec1 dependent fashion. The Dun1p controls the expression of a number of DNA damage inducible genes including the ribonucleotide reductase encoding genes, which regulate the cellular dNTP pools. How cellular dNTP levels are regulated in response to DNA damage is not fully understood. Here, we demonstrate that the yeast nucleotide excision repair complex Rad4-Rad23 binds directly to the promoters of several DNA damage response genes including Dun1, regulating their expression in response to UV radiation. We also show that regulation of these genes in response to damage is dependent on ubiquitination of Rad4 by the GG-NER E3 ubiquitin ligase complex. This determines the occupancy of the Rad4-Rad23 complex at the promoters of these genes. This subsequently controls the expression of genes including *RNR1* and *SML1* that collectively regulate the dNTP levels necessary for efficient DNA repair and cellular survival. We propose that the response of these core nucleotide excision repair factors to UV radiation explains how dNTP levels are regulated in response to UV induced damage and defines how the nucleotide excision repair pathway integrates with the DNA damage checkpoint.

2. Introduction

In living cells, genetic information is encoded within the DNA molecule. If left unchecked, DNA damage from a variety of sources would accumulate to levels that would ultimately result in a precipitous loss of the genetic information harnessed within the genome. Such a rapid and continual loss of this information would be incompatible with life. Consequently, mechanisms have evolved in cells to promote genome stability. At the most fundamental level, the removal of genetic damage by DNA repair is central to the maintenance of genome integrity [1]. Malfunction of genes required for the normal processing of DNA damage by any of the major DNA repair pathways including nucleotide excision repair (NER) results in genetic predisposition to cancer [2, 3]. Defective NER is the cause of the hereditary cancer-prone syndrome Xeroderma pigmentosum (XP), and failure to remove DNA damage is the primary cellular phenotype of this disease [2].

Genomic instability is one of the established hallmarks of cancer [4]. In addition to defects in DNA repair, defective DNA replication and recombination are also linked to its causes [5, 6]. Both DNA damage and replication stress can activate a signal transduction pathway known as the checkpoint or DNA damage response [7-9]. In *Saccharomyces cerevisiae* the central spine of this network is characterized by the Mec1/Rad53/Dun1 kinase cascade [7]. In this pathway, damage sensors detect chromatin-associated DNA damage structures [8]. This information is transmitted via signal transducers, which include the diffusible protein kinases mentioned above, to effectors that mediate the physiological response of the cell to damage and promote cell survival [7]. The primary target of the checkpoint kinases is the arrest or slowing of the cell cycle, providing time for DNA repair to take place. However, in addition, they also mediate a transcriptional response to DNA damage involving the regulation of specific genes, many of which directly affect efficient DNA repair and survival of the cell [7]. The checkpoint function, acting in combination with the DNA damage dependent transcriptional response, ensures the integrity of the replication forks whilst promoting DNA repair during the completion of DNA synthesis during replication. It also integrates these processes with cell cycle transitions in coordinated fashion thus promoting genome stability [5, 7].

Failure of the DNA damage response sees progression through the cell cycle with defective or incomplete DNA synthesis and/or the persistence of DNA damage in the genome. The consequences of this include the accumulation of mutations, genome aberrations and chromosomal instability, all of which are associated with many forms of cancer. Defects in the genes that control the response are now known to cause a wide variety of severe human disease syndromes including cancer prone disorders [10]. Mutations in checkpoint genes are also found in cancer cells [4]. Understanding the molecular mechanisms regulating the checkpoint response, and in particular determining how DNA repair pathways are integrated within it, is essential for our deeper understanding of the biology of cancer.

Activation of the DNA damage response in yeast depends on the monoubiquitination of two clamp loader complexes, PCNA and 9-1-1, by the Rad6-Rad18 ubiquitination complex. These clamp loaders are considered to be sensors of DNA damage [8, 11]. Monoubiquitinated PCNA [and possibly 9-1-1], can recruit translesion synthesis polymerases which bypass the replication-blocking lesion [10, 11]. PCNA can also be polyubiquitinated by the Rad5-Ubc13-Mms2 complex which promotes the activation of error-free post replication repair [10, 11]. In a different context monoubiquitination of the Rad17 component of the 9-1-1 complex stimulates the checkpoint pathway leading to phosphorylation of Mec1, Rad53 and subsequently Dun1 [12]. This sequence of events promotes both cell cycle arrest and the regulation of the transcriptional response to DNA damage. Most components of the cascade are shared in activating both end-points. However, although the Dun1 kinase activates the transcriptional response to DNA damage, it apparently does not promote cell cycle arrest [12].

We previously identified an E3 ubiquitin ligase complex of proteins comprised of known components of the yeast Global Genome nucleotide excision repair [GG-NER] pathway [13]. We revealed that this GG-NER E3 ligase protein complex enhanced UV survival following the ubiquitination of the Rad4 protein. Rad4 is a member of the Rad4-Rad23 heterodimer which is involved in DNA damage recognition during the NER process. Remarkably, we noted that the enhanced UV survival observed occurred independently of the proteolytic degradation of Rad4. However, survival was dependent on the UV induced ubiquitination of Rad4 and on *de novo* protein synthesis. These observations suggested that the ubiquitination of the Rad4-Rad23 DNA damage sensor complex by the GG-NER E3 ligase ubiquitination activity may be regulating a component of the transcriptional response to DNA damage. This is reminiscent of the monoubiquitination of the 9-1-1 DNA damage sensor complex by the Rad6-Rad18 E2/E3 ubiquitin ligase activity described above [12]. To investigate this we employed microarray gene expression profiling of *RAD4* and *RAD23* mutants a GG-NER E3 ligase mutant which fails to ubiquitinate Rad4, in order to identify specific misregulated genes that are affected in these strains.

In doing so we identify genes whose regulation depends on Rad4-Rad23 and the UV induced GG-NER E3 ligase dependent ubiquitination of Rad4. Furthermore, using ChIP analysis, we reveal that Rad4-Rad23 and the GG-NER E3 ligase directly regulate gene expression in response to DNA damage by controlling *DUN1* transcription which subsequently regulates key downstream Dun1 targets including the Ribonucleotide Reductase (RNR) dNTP synthesis pathway. We show that this is achieved via the binding of the Rad23/Rad4 complex to elements in the promoter region of *DUN1* and other DNA damage responsive genes including *DDR2*. DNA damage results in changes in the binding of the Rad4-Rad23 complex at the promoter of these genes in a manner dependent on the ubiquitination of Rad4. Collectively, these data describe a novel regulation of UV DNA damage induced gene expression via the well-known NER DNA

damage recognition complex Rad4-Rad23. Finally, we show that the primary role of this novel regulatory pathway is to control the UV induced synthesis of dNTPs to enhance survival following DNA damage. By characterizing the mutant phenotypes, we show that in the absence of the GG-NER E3 ubiquitin ligase regulated gene expression, UV survival is significantly reduced and cell cycle progression is affected. Constitutive upregulation of dNTP synthesis completely rescues both of these phenotypes, confirming the role of the pathway in regulating UV induced dNTP pools. Our results provide insight into a novel regulatory mechanism showing how known NER DNA damage sensors can also control gene expression and describe a point at which the NER pathway is integrated with the transcriptional response to DNA damage.

3. Materials & Methods

3.1 Yeast strains and plasmids

Research Genetics parental strain BY4742, BY4742*rad23*Δ, BY4742*rad4*Δ, BY4742*rad6*Δ, BY4742*rad18*Δ, and BY4742*rad7*Δ strains were obtained from Euroscarf. The double mutant *rad23*Δ*rad4*Δ was derived from BY4742*rad23*Δ by replacing *RAD4* with a *His3* marker fragment. Creation of the Rad7 SOCS box mutation was achieved by site directed mutagenesis of the wild-type *RAD7* gene cloned in pRS314 as described [14]. Two point mutations were made resulting in the amino acid substitutions, L168A and C172A within the conserved SOCS box domain [13]. The *RAD23* gene of BY4742*rad7*Δ was replaced by a *URA3* marker fragment to generate the double mutant *rad7*Δ*rad23*Δ. The triple mutant *rad7*Δ*rad23*Δ*sml1*Δ and *rad7*Δ*rad23*Δ*crt1*Δ were derived from *rad7*Δ*rad23*Δ, respectively. Then pRS314 containing the *RAD7* gene and SOCS box mutated *RAD7* were introduced to *rad7*Δ*rad23*Δ*sml1*Δ respectively to produce the *pRAD7*Δ*rad23*Δ*sml1*Δ and *psocs*Δ*rad23*Δ*sml1*Δ strains. In the same way, the *pRAD7*Δ*rad23*Δ*crt1*Δ and *psocs*Δ*rad23*Δ*crt1*Δ strains were derived from *rad7*Δ*rad23*Δ*crt1*Δ.

3.2 In vivo cross-linking and sonication of chromatin extracts

Cells were grown to a density of 2~4x10^7 cells/ml, and 2.8 ml of 37% formaldehyde was added to 100 ml of the culture medium (containing at least 2x10^9 cells). The mixture was incubated at room temperature for 20 minutes with occasional swirling to allow efficient DNA and protein cross-linking. The cross-linking reaction was ceased by adding 5.5 ml of 2.5M glycine to a final concentration of 0.125M. Cells were collected by centrifugation and then washed with ice-cold TBS buffer and ChIP lysis buffer. Cells were resuspended in 500 µl of ChIP lysis buffer supplemented with 12.5 µl of 20% SDS and 12 µl of 100x protease inhibitors. After 0.5 ml glass beads were added to this solution, the mixture was vortexed (at high speed on a turboMixer) at 4°C for 10-15 minutes. The cell lysate was carefully collected by centrifugation. Next, the cell lysate was sonicated by a Diagenode sonication system at the high output rate for 3-4 minutes (6-8 x 0.5 min on/0.5 min off

cycle). The sonicated cell lysate was spun down at 13,200 rpm for 15 minutes at 4°C. The supernatant (chromatin extract) was finally transferred to a clean tube and stored at -80°C until further use.

3.3 Chromatin immunoprecipitation (ChIP)

Protein A beads were washed twice with ChIP lysis buffer and then equilibrated with ChIP lysis buffer supplemented with 0.1% BSA and 40 µg/ml single strand salmon sperm DNA for 3 hours at 4°C. Next, 50 µl of chromatin extracts were added to 500 µl of ChIP binding buffer (i.e. ChIP lysis buffer supplemented with 0.25% SDS and 1x protease inhibitors), after which the solution was incubated with the equilibrated protein A beads. After removal of the protein A beads by centrifugation, the chromatin immunoprecipitation was carried out by adding 1-5 µl antibody to this cleared solution at 4°C for overnight. In the following step 20-30µl of protein A beads slurry (ChIP lysis buffer washed twice) was added to the solution and incubated for 2-3 hours at 4°C. The protein A beads were quick spun and washed successively with ChIP lysis buffer, ChIP lysis buffer with 500mM NaCl, LiCl solution and TE buffer.

The protein A beads were incubated with 250 µl elution buffer at room temperature for 10 minutes. Then, the supernatant was collected by centrifugation. The pellet was eluted again. The two parts of eluate were pooled together and incubated at 65°C overnight to reverse the cross-linking.

Subsequently, the elution was treated with ribonuclease A and protease K and the DNA was purified by a standard phenol/chloroform purification or a PCR purification kit (QIAGEN). In order to precipitate the DNA, 100 µg glycogen, 1/10 volume of 3M sodium acetate (pH5.2) and 2 volume of ethanol were added to the solution. The precipitated DNA was resuspended in 50-100 µl TE buffer and stored at -20°C.

3.4 The quantitative PCR (qPCR)

Quantitative PCR was carried out with the following primer pairs:
Upper_DDR2-primer-1 TGCTCAAAGGTTTATGCCCGATGTT;
Lower_DDR2-primer-1: TGCATTATTGATGTCCCATAA-GGGG;
Upper_DDR2-primer-2: CCCAGACACGGTTGCCAAGGCCTCG;
Lower_DDR2-primer-2: CGGGCATAAACCTTTGAGCATCATC;
Upper_DDR2-primer-3: AGCCCTCCAAGCAAGCACGC;
Lower_DDR2-primer-3: CGTGCAAAGCAGGAGCAGCG;
Upper-GPG1-primer-1: GCGCCCT-GTATCAAAAAGAAGCTTT;
Lower-GPG1-primer -1: GGAACTTCCTCACACCGCGGTTTGT.
The internal control primers were as follows:
Forward GGTTCTAGTTAGTCACGTGCAG
Reverse CGTTATTTTACTTTTCGGAAGACA

A serial dilution of wild-type genomic DNA strain (Sc507) was used to make a standard DNA concentration curve. Based on this standard curve all raw data acquired from real-time PCR machine was quantified using the iQ5 software (Bio-Rad).

3.5 Northern blot assay

The hot phenol method was applied for RNA isolation and Northern blotting was performed as described previously [13]. The following primers were used in this study:
Actin_NTS: biotin-GCCGGTTTTGCCGGTGACG;
Actin_TS: CCGGCAGATTCCAAACCCAAAA;
DDR2_NTS: biotin- *ACAGAT*TGCTCAAAGGTTTATGCCCGATGTT;
DDR2_TS: GGTATCATCATCGTGGCAGTAA-GCG;
DUN1_NTS: Biotin-*AAAAAA*CAGGATGAATCCAAAGCTCTA;
DUN1_TS: AAACGCTGCA-ATTCTAATGAG;
DDI1_NTS: Biotin-*AAAAAA*GATACAGGGGCTCAAACAACG;
DDI1_TS: ACAGCATCCCTAGGGAAACCT.
RNR1_NTS: bio-TTCAAGGCTTACCAAACGTTCCACA;
RNR1_TS: TCGAACTCGGTTTCCTCATCATCAA.

3.6 Flow cytometric analysis of yeast cell cycle

Cells were grown to log-phase in minimal medium, and then G_1 cells were obtained from the population using the Beckman JE-5.0 centrifugal elutriation system. Cells were adjusted to a concentration of $1x10^7$ cells/ml. From this starter culture 25 ml was treated with 150 J/m^2 UV and 25 ml was left untreated. Samples of 1 ml were removed at different time points and fixed in 70% ethanol. Cells were then resuspended in 50mM Na-Citrate Buffer (pH 7), sonicated on low power for 40 seconds to remove clumps, and treated with RNase. After washing and second sonication, cells were stained with 8 g/ml Propidium Iodide. Flow cytometric analysis was performed to measure DNA content by PI fluorescence. Histograms show gated cell counts based on PI fluorescence height.

4. Results

4.1 The Rad4-Rad23 complex regulates gene expression of UV responsive genes

We previously showed that the ubiquitination of Rad4 in response to UV radiation affected DNA repair and UV survival in a manner dependent on *de novo* protein synthesis [13]. This and other observations made in this report suggested a possible role for the Rad4-Rad23 complex in the regulation of gene transcription in response to DNA damage. Recently, an increasing number of studies have confirmed a role for specific NER factors including yeast Rad23 and the human XPC-hHR23B complex in gene transcription [13, 15-18]. In an effort to determine which genes might be regulated by the Rad4-Rad23 complex, and in particular which genes are regulated by this complex in response to UV radiation, we studied the effect of both the Rad4-Rad23 complex and in particular, the ubiquitination of Rad4 by the GG-NER E3 ligase in response to UV radiation on gene transcription by using microarray gene expression analysis.

Initially, using wild-type yeast or strains deleted for *RAD4* and *RAD23*, either individually or in combination (*rad4Δ*, *rad23Δ* and *rad4Δrad23Δ*), we examined genome wide gene expression using microarray analysis. From these studies we found differentially expressed genes for all three mutant backgrounds tested (Figure 1A and S1A). We expanded this initial list of 139 genes to 205 genes by including all genes exhibiting a greater than 1.5 fold-change in gene expression levels. Cluster analysis of these 205 genes revealed the presence of four hierarchical clusters of genes that display a similar pattern of expression across the three mutant strains analyzed (Figure 1B). Cluster 1 includes genes whose expression is not affected in either of the single mutants, but do require Rad4-Rad23 for their normal level of expression and are repressed in their absence. Cluster 2 represents genes that are repressed by the individual loss of either Rad4 or Rad23, but are upregulated in the absence of both genes. Cluster 3 includes genes that require both Rad4 and Rad23 for their normal expression level, while cluster 4 contains the genes that need both Rad4 and Rad23 for their repression (Figure 1B). Rad23 has previously been noted to have an effect on gene regulation [16], but our studies extend these observations to show that in combination with Rad23, Rad4 also influences gene expression.

In order to determine whether any of these 205 genes were also regulated in response to UV radiation, we UV irradiated wild type cells and examined their gene expression profile at 15 and 60 minutes after exposure. The total number of genes in both the early and late UV response categories amounts to 1360 genes. 309 of these genes are common to both the early and late response categories (Figure S1B). This list of 309 UV responsive genes was then compared with the *rad4Δ, rad23Δ* and *rad4Δrad23Δ* untreated gene expression datasets in an effort to detect the UV responsive genes that are transcriptionally regulated by Rad4-Rad23. 101 of the 205 genes regulated by Rad4-

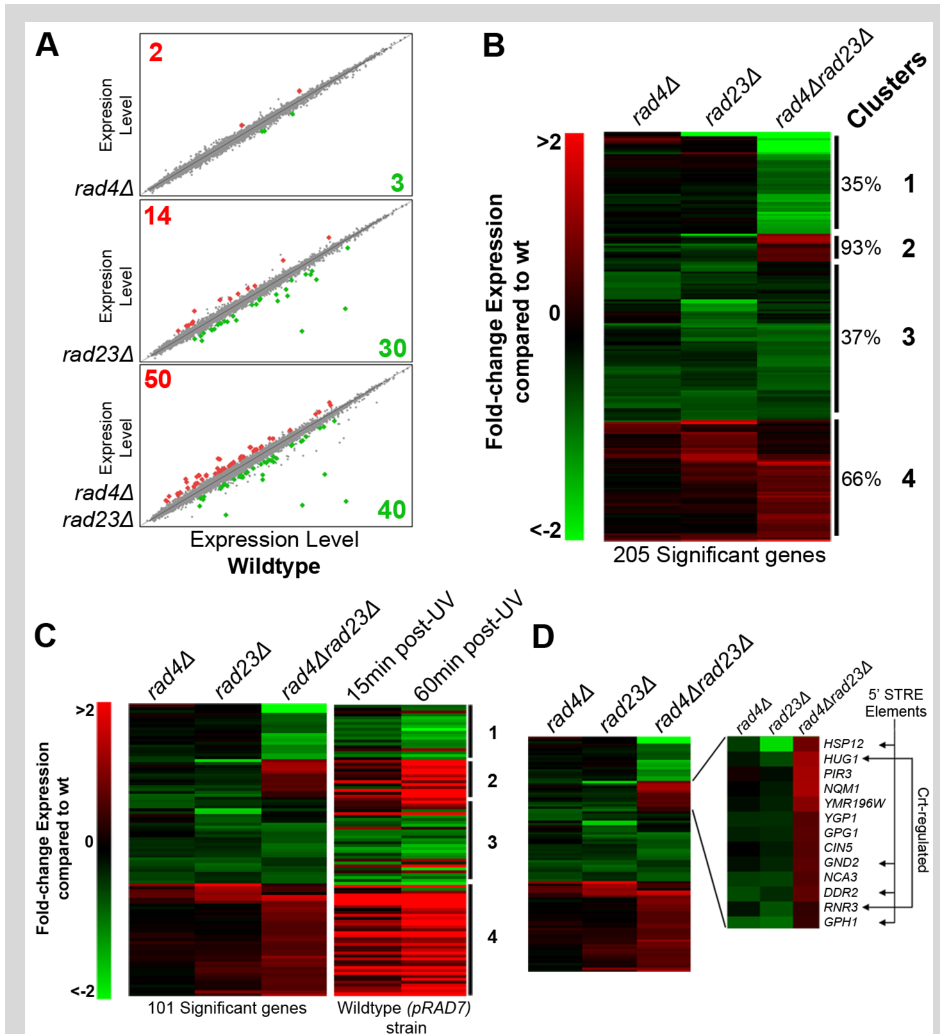

Figure 1 – *The Rad4-Rad23 NER factor affects UV induced gene expression.* (A). Significantly differentially expressed genes in *rad4Δ*, *rad23Δ* and *rad4Δrad23Δ* cells compared to wild-type cells in the absence of UV irradiation is shown here. For each strain gene expression is related to the wild-type control as a fold-change. Genes that did not display changed expression in the mutant backgrounds are in grey on the diagonal (y=x). Significantly upregulated genes are depicted in red while down regulated genes are shown in green. (B) Heat-map of hierarchically clustered genes in the *rad4*/*rad23* double and single mutants. Expression fold-change is relative to the wild-type control. The fraction of UV responsive genes is indicated in percentage per cluster. (C) Heat-map of the hierarchical clusters in B of the UV responsive genes only compared to the UV induced gene expression profile of wild-type cells. Fold-change in expression of the UV-irradiated samples is versus the unirradiated control. (D) Cluster 2 is blown-up showing the STress Responsive Elements containing genes and Crt1 controlled genes being affected in the mutant backgrounds.

Rad23 are also UV responsive, with cluster 2 and 4 having a remarkable 93% and 66% of their constituent genes being UV responsive (Figure 1B and S1C). Significantly, when the hierarchical clusters of these 101 UV responsive and Rad4-Rad23 regulated genes

are compared with the expression data of the UV-irradiated wild-type cells, the expression profile of the *rad4Δrad23Δ* cells is very similar to the post-UV expression profile of wild-type cells (Figure 1C). This indicates that the UV induced gene expression changes observed in wild type cells is mimicked in the *rad4Δrad23Δ* mutant, suggesting a possible role for Rad4-Rad23 in the UV induced regulation of these genes.

4.2 Rad4-Rad23 increases gene expression of STRE containing and CRT1 regulated DDR genes following UV irradiation

We next focused our attention on the identity of genes found in cluster 2 (Figure 1B) since the genes in this cluster require the Rad4-Rad23 heterodimer for their repression in the absence of UV radiation, and 93% of them are also induced in wild type cells after UV irradiation. Close examination of the upstream regulatory regions of the genes in this cluster reveal that they are enriched for genes that contain a Stress Response Element (STRE) within their promoter region (Figure 1D). The STRE is a hexanucleotide regulatory element found in specific DNA damage response genes including *DDR2*, that are targeted by the stress induced transcriptional activators Msn2 and Msn4 (see Figure 4A) [19]. The HUG1 and *RNR3* genes also require Rad4-Rad23 for their repression in the absence of UV irradiation and are also UV inducible. Instead of sharing common transcriptional activators, these genes share a common transcriptional repressor called Crt1, which binds to a regulatory element in their promoters (Figure 1D). Crt1 represses a set of UV induced cell-cycle checkpoint and DNA repair genes, including the *RNR* genes, that become activated following depression of Crt1 by Dun1 phosphorylation in response to DNA damage [20]. The analysis of our data describes two classes of genes whose regulation in response to UV depends on Rad4-Rad23. These observations suggest that the Rad4-Rad23 heterodimer may act directly as a transcriptional regulator of genes involved in the transcriptional response to DNA damage [20, 21], particularly in regulating the RNR pathway which is explored in greater detail later.

4.3 The GG-NER E3 ligase regulates genes involved in the transcriptional response to DNA damage

Next, we specifically wanted to identify the genes controlled by the GG-NER E3 ligase, and in particular those whose regulation is dependent on the ubiquitination of the Rad4 component of the Rad4-Rad23 complex. We have shown previously that a point mutant in the SOCS box domain of the Rad7 component of the GG-NER E3 ligase specifically fails to ubiquitinate Rad4 in response to UV. We demonstrated that this point mutant strain is not UV sensitive, but that it significantly increases the UV sensitivity of a strain that also has the *RAD23* gene deleted [13]. These and other data confirmed that the GG-NER E3 ligase functions in parallel with Rad23 in NER and UV survival, and achieves this by controlling UV induced gene transcription via the ubiquitination of Rad4 [13]. To determine how gene regulation by the GG-NER E3 ligase enhances NER and UV survival, we sought to identify the genes specifically regulated by the E3 ligase complex

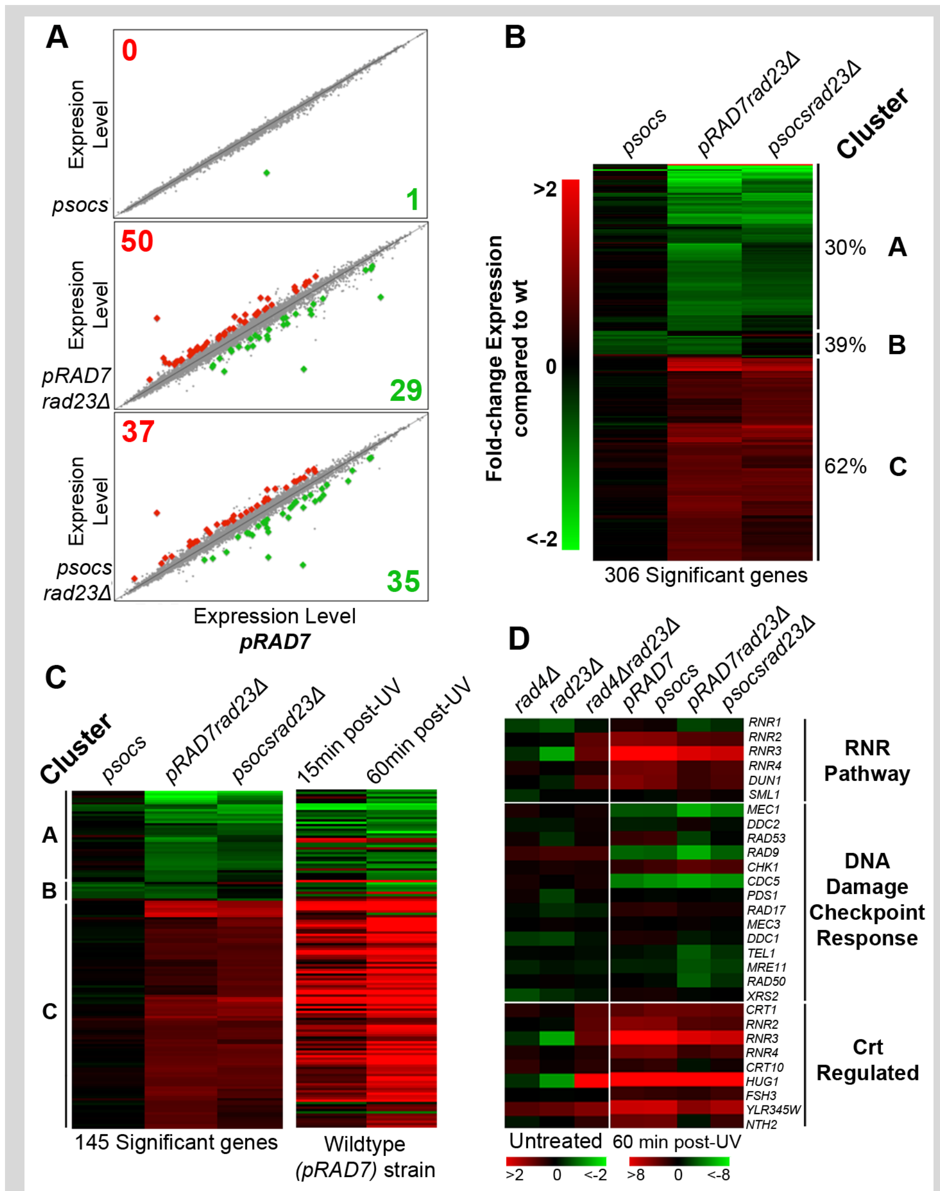

Figure 2 – The GG-NER E3 ligase regulates UV induced gene expression in concert with Rad4-Rad23. (A) Differential expression of the mutant background indicated versus the wild-type *pRAD7* background. Highlighted in red are the genes with increased expression in the mutant backgrounds compared to the wild-type control, while in green genes with reduced expression versus the wild-type strain are indicated. (B) Genes from A that were differentially expressed by 1.5-fc in *psocs*/*rad23* cells were hierarchically clustered resulting in the heat-map displayed here. (C) Heat-map of the hierarchical clusters in B of the UV responsive genes only compared to the UV induced gene expression profile of wild-type cells. Fold-change in expression of the UV-irradiated samples is versus the unirradiated control. (D) Significantly differentially expressed UV responsive genes from the psocs/rad23 dataset combined with the expression data from the rad4/rad23 dataset are shown in this heat-map. The expression of RNR and related genes from the *rad4/rad23* and *psocs*/*rad23* datasets are expressed as the fold-change versus wild-type or untreated controls.

in response to UV radiation. To do this, we made use of a Rad7 strain mutated in the archetypal ECS ligase SOCS-box domain [13, 22]. The point mutated *RAD7* gene was introduced into a strain on a plasmid and is referred to as *psocs*. A strain containing the wild-type *RAD7* gene, referred to as *pRAD7*, served as a control.

The strains bearing wild-type or mutated *RAD7* and/or deleted for *RAD23* were subjected to gene expression microarray analysis, initially in the absence of UV irradiation, in a similar fashion *to* the experiments described earlier in Figure 1. In comparing the untreated mutant strains with the pRAD7 (wild-type) control, we found that the *psocs* mutant did not affect gene transcription in isolation. However, in combination with the *RAD23* deletion, 72 differentially expressed genes were identified (Figure 2A bottom panel), and 79 genes were differentially expressed in the *RAD23* deleted strain (Figure 2A middle panel), approximately a third of these being common to both the *pRAD7rad23Δ* and the *psocsrad23Δ* strains (Figure S1D). Using a similar approach to that described for the *rad4/rad23* deleted strains described in Figure 1, we expanded our initial list of approximately 125 genes to 306 genes by including all genes in the mutant strains exhibiting a greater than 1.5 fold-change in expression levels compared to the wild type. Cluster analysis of these 306 genes revealed the presence of three hierarchical clusters (Figure 2B). Cluster A includes genes that require Rad23 for expression. Cluster B represents genes that are repressed by the individual loss of either functioning *socs* box of Rad7 or Rad23, but whose normal expression level is restored in the *psocsrad23Δ* double mutant. Cluster C includes genes that require Rad23 for their repression (Figure 2B). These results show that there is no significant effect of the E3 ubiquitin ligase on transcription in the absence of UV radiation (Figure 2A and B). In order to determine whether any of the 306 genes, whose expression was altered in the *psocs/rad23* mutant dataset (Figure 2B), were also regulated in response to UV radiation, we compared the expression profiles of these 306 genes to the profile of UV irradiated wild type cells at 15 and 60 minutes after exposure. As described earlier, the total number of genes in both the early and late UV response categories whose expression is altered in response to UV radiation amounts to 1360. 309 of these genes are common to both the early and late response categories (as shown in Figure 1C and Figure S1B). This list of 309 UV responsive genes was then compared with the *psocs/rad23Δ* untreated gene expression dataset to detect UV responsive genes that are transcriptionally regulated in the *psocsrad23Δ* strain. 145 of the 306 genes differentially expressed in the *psocsrad23Δ* background are also UV responsive, with clusters A, B and C having 30%, 39% and 62% of their constituent genes being affected (Figure 2B and C). In summary, of the genes that are differentially expressed in the *psocs/rad23Δ* mutants, around a half were UV responsive (145 of 306) (Figure 2C). Gene ontology [GO] analysis was performed on clusters A-C revealing that the top GO terms for the UV induced genes observed in cluster C were Stress Response, DNA Metabolism and Cell Cycle (Figure S2). Similar to observations made in Figure 1B, the *psocsrad23Δ* mutant

closely mimics the UV induced gene expression response of wild-type cells (Figure 2C). This indicates a possible role for both the E3 ubiquitin ligase and Rad4-Rad23 in the UV induced regulation of the genes in these pathways.

4.4 The GG-NER E3 ligase controls UV induced expression of genes involved in the RNR pathway

The gene expression profiling studies conducted so far suggest that the GG-NER E3 ligase regulates genes involved in the DNA damage checkpoint pathway, particularly genes involved in the RNR pathway that are regulated via STRE elements within their promoters and/or regulated by the Crt1 repressor protein. Having identified the genes misregulated in the *psocsrad23Δ* mutated strain in the absence of UV, we next examined the effect of these mutations on UV induced gene transcription, specifically examining genes involved in the DDR pathways identified above. To do this, we applied microarray analysis for the UV treated *pRAD7, pRAD7rad23Δ* and *psocsrad23Δ* strains. The resulting heat-map is displayed in Figure 2D, with the major DNA damage response genes from the pathways indicated, grouped together.

The most striking feature to emerge from this analysis is that the RNR pathway genes show strong evidence of being regulated by the Rad4-Rad23 complex and the GG-NER E3 ligase activity in response to UV radiation. In contrast, the cluster of genes involved as central players in the DNA damage response, including Mec1, Rad53, Tel1 and Chk1 in Figure 2D does not reveal a significant role for Rad4-Rad23 or the GG-NER E3 ligase in expression of these genes within the central regulatory pathway. Similarly, the expression of other genes such as *RAD9* and *RAD51* which are involved in primary detection and signaling of different types of DNA damage are also not significantly affected by Rad4-Rad23 or the GG-NER E3 ligase.

Quantitative analysis shown in Figure 3A reveals that the regulation of *RNR* pathway genes, in particular of *DUN1* and *RNR1*, does show an important role for Rad4-Rad23 and the GG-NER E3 ligase. The *DUN1* gene is an important activator of *RNR* in response to UV damage, but little is known about regulation of its expression in response to UV. Figure 3A shows that *DUN1* expression is dependent on the GG-NER E3 ligase in response to UV damage. In addition, regulation of *RNR* gene expression is controlled by the *CRT1* repressor. Figure 2D and 3A show that most *CRT1* controlled genes are also regulated by the GG-NER E3 ligase in responsive to UV. To confirm the results obtained from microarray analysis, we performed northern blotting to detect RNA levels in the strains tested. Figure 3B confirms the requirement for the GG-NER E3 ligase in upregulating both *DUN1* and *RNR1* in response to UV radiation. This further underscores the requirement for Rad4-Rad23 and the GG-NER E3 ligase for the regulation of UV induced *RNR* and *DUN1* expression.

A previous report elegantly described the DDR induced gene expression of *MAG1* and *DDI1* by the Rad6-Rad18 ubiquitination complex via the mono-ubiquitination of Rad17

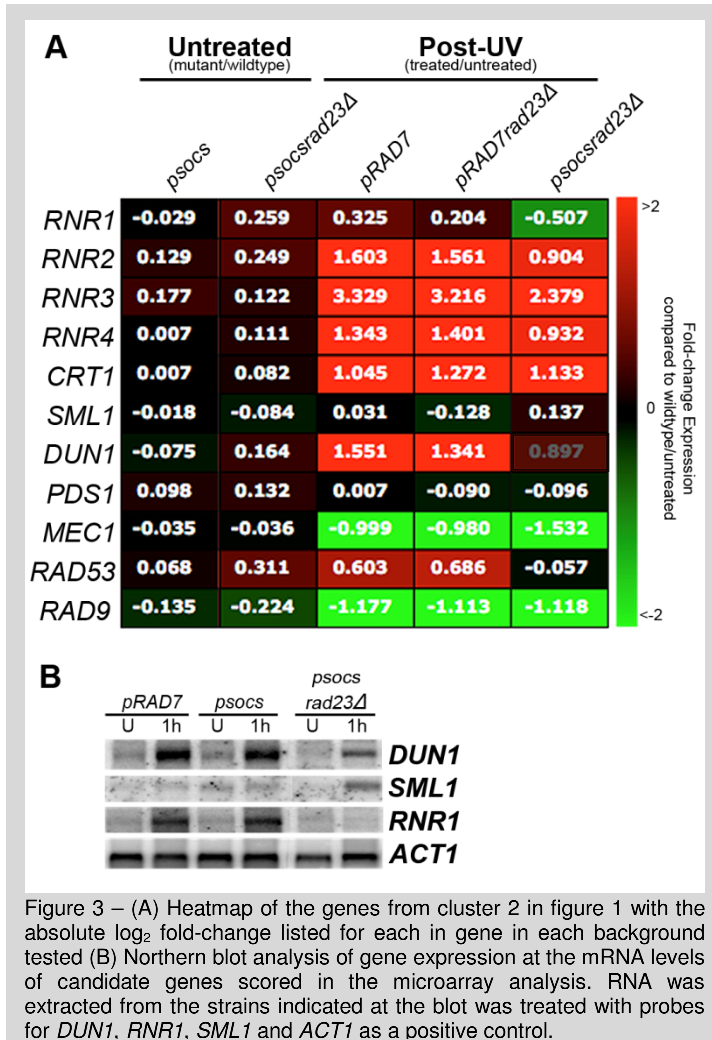

Figure 3 – (A) Heatmap of the genes from cluster 2 in figure 1 with the absolute $\log_2$ fold-change listed for each in gene in each background tested (B) Northern blot analysis of gene expression at the mRNA levels of candidate genes scored in the microarray analysis. RNA was extracted from the strains indicated at the blot was treated with probes for *DUN1*, *RNR1*, *SML1* and *ACT1* as a positive control.

[12]. This report raised the possibility that the GG-NER E3 ligase controlled ubiquitination of Rad4 may be a component of this pathway. However, we did not observe any Rad4-Rad23 regulated gene expression of the *MAG1* and *DDI1* cluster of genes (Figure S3). *MAG1* and *DDI1* are fully up-regulated after UV-irradiation independently of Rad23 or the GG-NER E3 ligase. We conclude that the Rad4-Rad23 controlled and GG-NER E3 ligase coordinated gene expression is not involved in the Rad6-Rad18 mediated DDR transcription response to DNA damage [12]. This is also confirmed when we analyzed the UV induced expression of *DUN1* or *DDR2* in a *rad6Δ* or *rad18Δ* deletion strains (Figure S3).

The combined data on the RNR pathway shows regulation by Rad4-Rad23 and the GG-NER E3 ligase of these genes. *RNR1-4* and *DUN1* all require Rad4-Rad23 and the E3 ligase for repression in the absence of DNA damage. In response to DNA damage both Rad23 and the GG-NER E3 ligase activity are required for full blown expression of these genes (Figure 2D and 3A). A more subtle contribution can be seen for the RNR inhibitor *SML1*. Sml1p is an inhibitor of the RNR enzyme complex, that is degraded in response to DNA damage [23, 24]. We find that in the absence of the GG-NER E3 ligase UV irradiation results in elevated levels of *SML1* gene expression, which further inhibits the RNR pathway function (Figure 3A). We were able to confirm these microarray

expression data for *RNR1*, *DUN1* and *SML1* using northern blot analysis (Figure 3B). It is noteworthy that the upregulation of *SML1* is more evident when measuring mRNA via northern blot (Figure 3B). Interestingly, Dun1 and its downstream targets described here constitute an important end-point for the DDR Mec1-Rad53-Dun1 kinase signaling cascade. To summarize, the RNR pathway is down regulated in the *psocsrad23Δ* background both by reduced *DUN1* and *RNR* gene expression and also via upregulation of its inhibitor *SML1*.

4.5 Rad4-Rad23 complex regulates gene transcription of UV responsive genes via changes in the occupancy of the complex at the promoters of specific DDR genes

The results described in Figure 1 show that the increase in gene expression observed in cluster 2 in a *rad4Δrad23Δ* background, mimics the UV induced increase in gene expression of this cluster of genes observed in wild type cells. Intriguingly, recent evidence in mouse embryonic stem cells has revealed that XPC-hHR23B, the homologues of Rad4-Rad23, can regulate gene expression of specific developmental genes as a result of changes in the binding of the complex to elements in the promoter regions of these genes, suggesting a direct role for XPC-hHR23B in regulating gene transcription [18]. Therefore, we speculated that the Rad4-Rad23 heterodimer may also regulate UV induced gene transcription via direct binding of the complex to elements within the promoter regions of the UV responsive genes identified in cluster 2 (Figure 1D). To examine this, we measured the occupancy of the Rad4-Rad23 heterodimer in the regulatory regions of STRE element containing genes in the cluster including *DDR2* and *DUN1*, using chromatin immunoprecipitation (ChIP) and quantitative PCR. As shown in Figure 4, we found that in unirradiated cells, the Rad4-Rad23 complex occupies the chromatin in the promoter region of *DDR2*. Figure 4A (top panel) indicates the location within the *DDR2* gene amplified by three different sets of PCR primers. The lower panel of Figure 4A shows that the occupancy of Rad23 following ChIP is enriched in the STRE containing promoter element detected by the DDR2-primers-1 PCR primer set compared to two other primer sets located either in the ORF [DDR2-Primers-3] or the upstream region of the promoter [DDR2-primers-2]. We confirmed that Rad23 specifically binds at the promoter region of the *DDR2* gene by examining Rad23 binding in the promoter region of the *GPG1* gene. *GPG1* expression is affected by Rad4-Rad23 (Figure 1D), but does not contain a STRE element in its promoter. No enrichment for Rad23 in the regulatory region of *GPG1* was detected (data not shown). To examine the occupancy of both Rad4 and Rad23 at the promoter of *DDR2*, we performed a double ChIP experiment, first performing chromatin immunoprecipitation using Rad23 antibodies, followed by Rad4 antibodies. This double ChIP procedure was carried out and qPCR performed at the promoter regions of both *DDR2* and *DUN1*. Figure 4B confirms the occupancy of both Rad4 and Rad23 at the promoter regions of the *DDR2* [right panel]

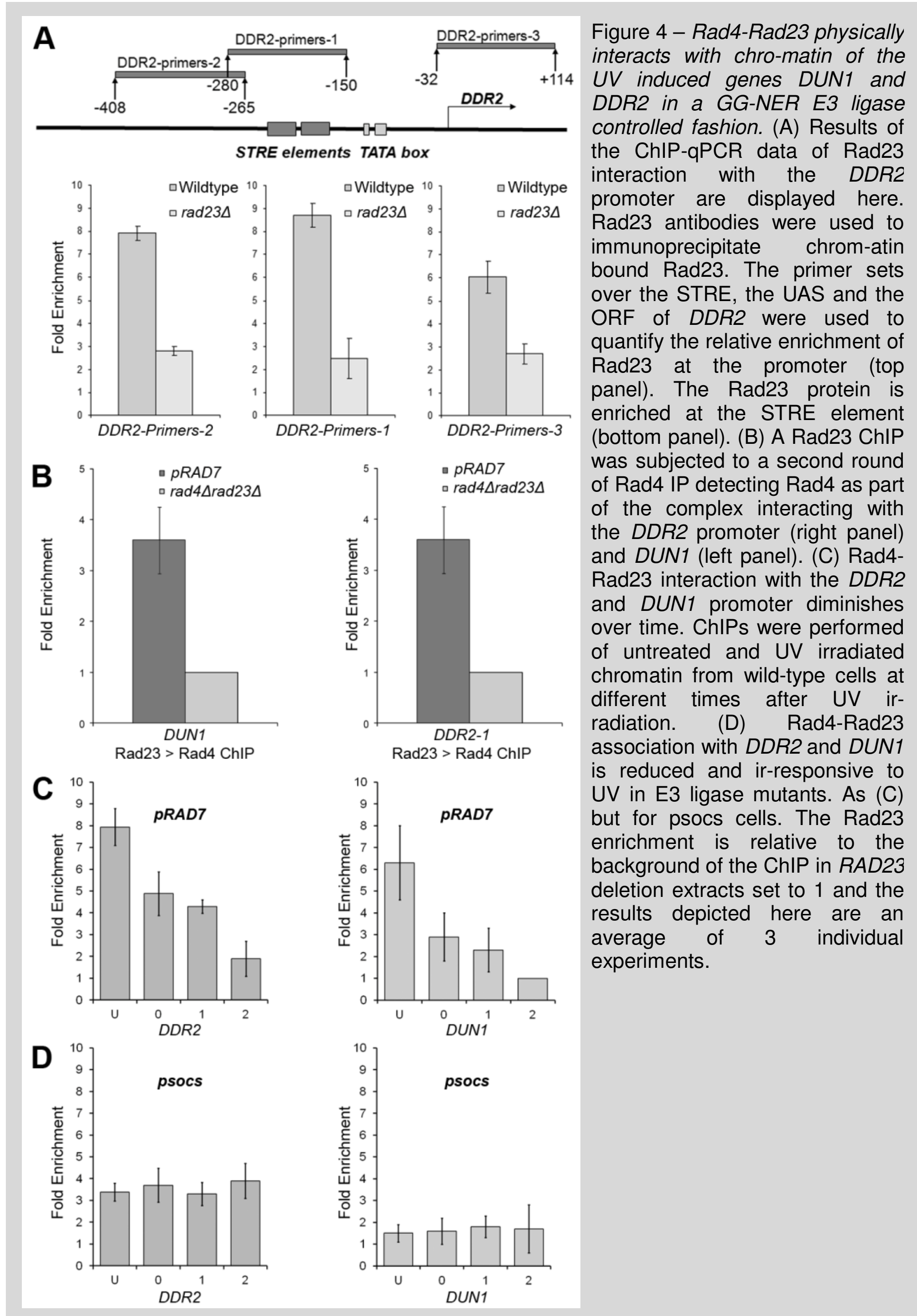

Figure 4 – *Rad4-Rad23 physically interacts with chro-matin of the UV induced genes DUN1 and DDR2 in a GG-NER E3 ligase controlled fashion.* (A) Results of the ChIP-qPCR data of Rad23 interaction with the *DDR2* promoter are displayed here. Rad23 antibodies were used to immunoprecipitate chrom-atin bound Rad23. The primer sets over the STRE, the UAS and the ORF of *DDR2* were used to quantify the relative enrichment of Rad23 at the promoter (top panel). The Rad23 protein is enriched at the STRE element (bottom panel). (B) A Rad23 ChIP was subjected to a second round of Rad4 IP detecting Rad4 as part of the complex interacting with the *DDR2* promoter (right panel) and *DUN1* (left panel). (C) Rad4-Rad23 interaction with the *DDR2* and *DUN1* promoter diminishes over time. ChIPs were performed of untreated and UV irradiated chromatin from wild-type cells at different times after UV ir-radiation. (D) Rad4-Rad23 association with *DDR2* and *DUN1* is reduced and ir-responsive to UV in E3 ligase mutants. As (C) but for psocs cells. The Rad23 enrichment is relative to the background of the ChIP in *RAD23* deletion extracts set to 1 and the results depicted here are an average of 3 individual experiments.

and *DUN1* [left panel] genes in the absence of DNA damage, indicating that they bind to the chromatin in these region as a complex.

Next we examined whether the occupancy of the Rad4-Rad23 complex at the promoter regions of *DDR2* and *DUN1* is altered in response to UV radiation. Figure 4C shows the

loss of occupancy of Rad23 from the promoter region of both *DDR2* and *DUN1* over a 2 hour period following UV irradiation. Taken together, these observations are consistent with a model in which the UV induced loss of occupancy of Rad4-Rad23 from the promoter regions of the UV responsive genes *DDR2* and *DUN1*, results in the increased expression of these genes after exposure of cells to UV radiation.

We then considered how the loss of occupancy of Rad4-Rad23 from the promoter regions of these genes is regulated in response to UV. Previously, we demonstrated that the ubiquitination of Rad4 by the GG-NER E3 ligase plays an important role in NER and UV survival in a manner dependent on *de novo* protein synthesis [13]. In addition, the effect of the GG-NER E3 ligase on gene expression described in figure 2D identifies a role for the E3 ligase in UV induced transcription. We speculated that this E3 ubiquitin ligase regulates the induction of UV responsive genes by controlling the occupancy of the Rad4-Rad23 complex at their promoter regions in response to DNA damage. In order to test this, we measured Rad23 occupancy at the promoters of both the *DDR2* and *DUN1* genes in mutants of the GG-NER E3 ligase. We examined events in strains either deleted for the *ELC1* subunit of the E3 ligase, or mutated in the SOCS-box domain of the Rad7 subunit. We have shown previously that both strains fail to ubiquitinate Rad4 in response to UV radiation [13]. Figure 4D shows no loss of occupancy occurs of the Rad4-Rad23 from the *DDR2* and *DUN1* promoters in response to UV damage in the *psocs* and *elc1* strain (data not shown). We noted that mutating the E3 ligase results in a reduced level of Rad4-Rad23 binding at both the *DDR2* and *DUN1* promoters, even in the absence of UV radiation. However, the reduced level of Rad4-Rad23 binding observed does not induce transcription of either *DDR2* or *DUN1* in the absence of UV damage (data not shown). In summary, our results show that in wild type cells the Rad4-Rad23 complex represses gene expression of the genes in cluster 2 (Figure 1B-C) by directly binding to the chromatin at the promoter region of these UV inducible genes. Following UV irradiation, the Rad4-Rad23 complex dissociates from the promoter region of these genes in a GG-NER E3 ligase dependent fashion facilitating increased gene expression.

4.6 The GG-NER E3 ligase upregulates dNTP pools to enhance survival in response to UV damage

We speculated that the extreme UV sensitivity of *psocsrad23Δ* cells could be caused by the failure to upregulate the expression of the RNR genes and compounded by the increased expression of the RNR inhibitor *SML1*. This failure to respond to DNA damage results in a lack of increased dNTP levels following UV irradiation. To test this hypothesis we examined whether it is possible to suppress UV sensitivity of the *psocsrad23Δ* strain by constitutively increasing the cellular dNTP pools. This was achieved by deletion of two independent inhibitors of the RNR pathway. Sml1 inhibits the RNR enzyme complex, while Crt1 is an inhibitor of transcription of the *RNR* genes. In this way, the Rad4-Rad23

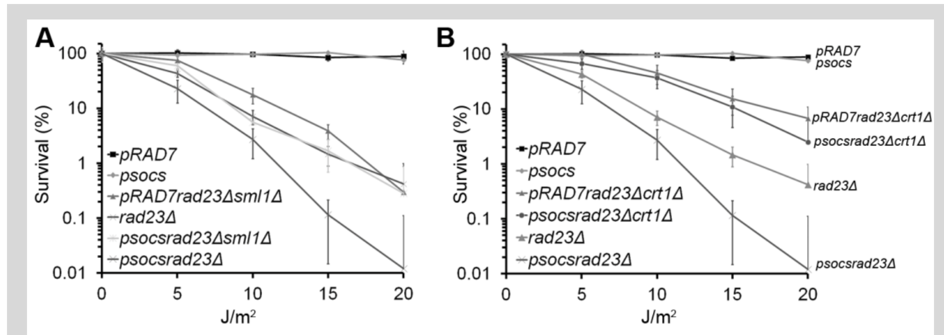

Figure 5 – *The UV sensitivity due to altered gene expression in psocs mutants can be rescued by derepressing dNTP synthesis.* UV-survival curves of *psocsrad23* strains rescued by deleting *SML1* (A) or *CRT1* (B). Cells of the strains indicated were grown to log-phase and treated with increasing doses of UV. Survival is quantified as colony-growth on YPD plates 2-3 days after UV treatment.

dependent transcriptional response we observed following microarray analysis can be explained. Figure 5 shows that upregulation of dNTP pools by two independent processes can indeed be rescue the UV sensitivity of the *psocsrad23Δ* strain by deleting the *RNR* inhibitors *SML1* or *CRT1*. Taken together the triple mutants *psocsrad23Δsml1Δ* and *psocsrad23Δcrt1Δ* lack RNR inhibition that in turn compensates for the absence of Rad4-Rad23 and GG-NER E3 ligase mediated upregulation of *RNR* to restore dNTP levels that can support enhanced UV survival.

4.7 Normal cell-cycle progression requires dNTP upregulation mediated by the GG-NER E3 ligase

Our data shows that the GG-NER E3 ligase regulates dNTP pools in response to UV damage. It is known that normal cell-cycle progression depends on dNTP availability as does survival after DNA damage [25]. Moreover, cell-cycle checkpoint mutants fail to upregulate dNTPs [26], in addition, mutations deregulating dNTP synthesis result in altered cell-cycle progression [27]. These observation predict that the Rad4-Rad23 and E3 ligase guided regulation of dNTP synthesis might be expected to affect cell-cycle progression. Therefore, we analyzed cell cycle distribution in the E3-ligase mutant using flow cytometry.

As anticipated, we detect that the *psocs* mutant progresses through G_1 faster than wild-type cells (Figure 6A). After UV irradiation *psocs* cells again reach S-phase more quickly (Figure S4A). The *psocsrad23Δ* strains show a similar phenotype both in the absence and presence of UV irradiation (data not shown). These data indicate that an E3 ligase mutant fails to properly traverse the cell-cycle. Significantly and in conformation of results shown in Figure 5, the cell cycle progression defect can be restored by derepressing dNTP synthesis via two independent inhibitors of the RNR pathway (Figure 6B and C). In conclusion, our data clearly demonstrate the role of the GG-NER E3 ligase in regulation of dNTP pools promoting cell-cycle progression and UV survival.

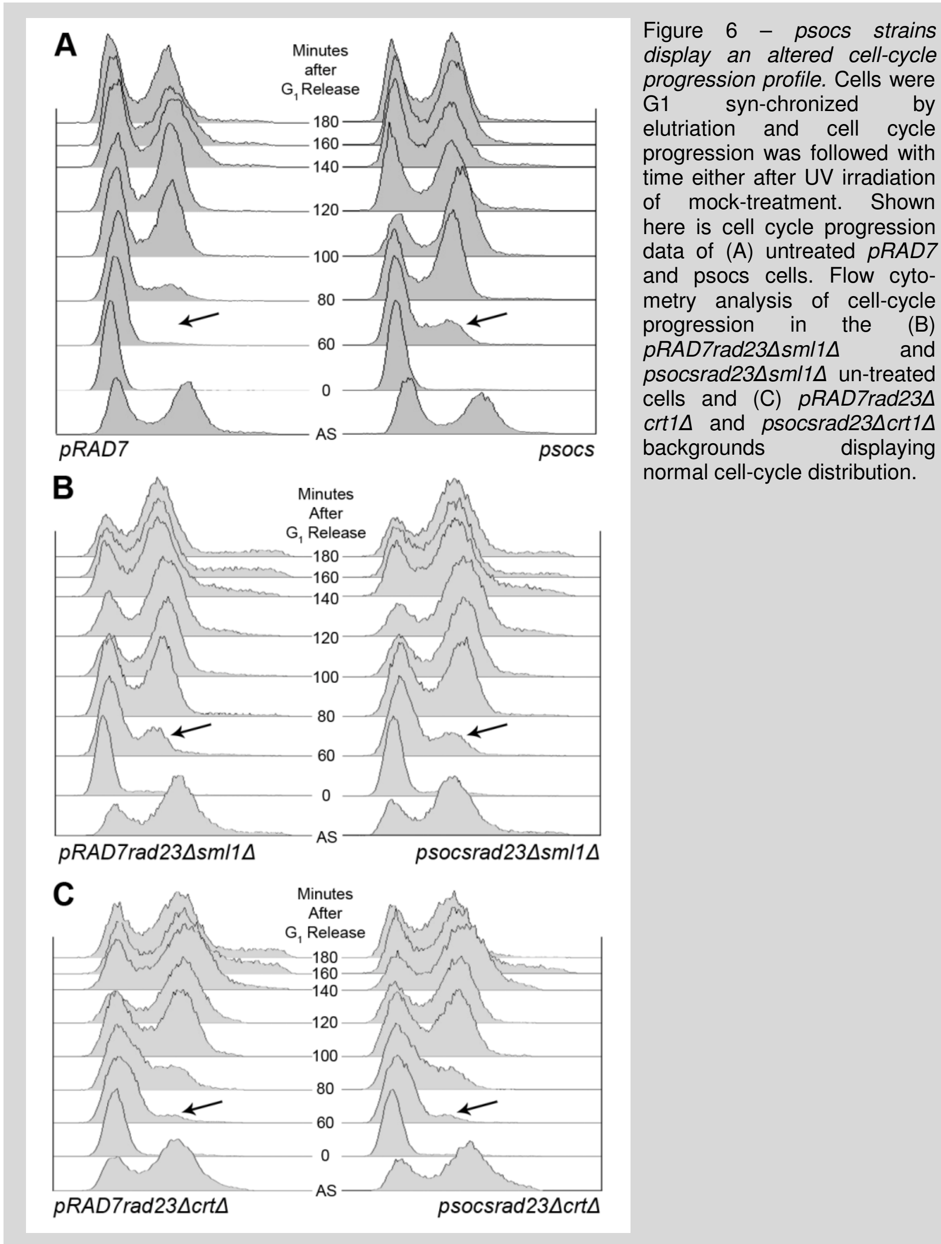

Figure 6 – *psocs strains display an altered cell-cycle progression profile.* Cells were G1 syn-chronized by elutriation and cell cycle progression was followed with time either after UV irradiation of mock-treatment. Shown here is cell cycle progression data of (A) untreated *pRAD7* and psocs cells. Flow cyto-metry analysis of cell-cycle progression in the (B) *pRAD7rad23Δsml1Δ* and *psocsrad23Δsml1Δ* un-treated cells and (C) *pRAD7rad23Δ crt1Δ* and *psocsrad23Δcrt1Δ* backgrounds displaying normal cell-cycle distribution.

5. Discussion

Cells exposed to DNA damaging agents activate a DNA damage response that allows cells to halt cell cycle progression, permitting time to repair the damage. Signaling cascades involving post-translational modifications of key regulatory proteins and an extensive DNA damage-induced gene expression program are processes that underpin this response. It is the intricate interplay between these processes that provide the cell with the opportunity to successfully complete DNA repair and enhance its survival following DNA damage.

In an earlier report we identified a possible link between the transcriptional response to DNA damage and NER, through the action of a novel E3 ubiquitin ligase complex containing two well-known GG-NER factors, Rad7 and Rad16 [12]. We demonstrated that this E3 ligase ubiquitinates Rad4 after UV irradiation, leading to Rad4 degradation. Significantly, we discovered that the effect of this E3 ligase on DNA repair and UV survival depends on the ubiquitination of Rad4, but not its subsequent degradation. Furthermore, we noted that the function of the E3 ligase operates in parallel with a previously described pathway involving Rad23 and the 19S proteasome [28, 29]. Indeed, we showed that the effect of the GG-NER E3 ligase is masked in the presence of Rad23, and its impact on DNA repair and UV survival can only be observed in a strain additionally deleted in the *RAD23* gene. This led us to propose a redundant two pathway system; Pathway I involving an interaction between Rad23 and the 19S proteasome, and Pathway II involving the GG-NER E3 ligase and its ubiquitination of Rad4 (See model described in Figure 7). We demonstrated that the E3 ligase dependent contribution to DNA repair and UV survival is completely inhibited in the presence of the translation inhibitor cycloheximide, indicating a requirement for *de novo* protein synthesis, and therefore its potential function in regulating gene transcription [13]. To investigate this possibility, we used microarray analysis in an effort to determine the effect of Rad4-Rad23 and in particular the GG-NER E3 ligase on gene expression in response to DNA damage. We initially examined the effect of Rad4 and Rad23 on gene transcription and then characterized the genes affected based on their expression profiles using hierarchical clustering. We then compared the genes in these clusters to the UV responsive genes of wild type cells. We found a remarkable 93% of the genes in cluster 2, which contains genes that require Rad4-Rad23 for their inhibition in the absence of DNA damage, to be upregulated in response to UV damage in wild-type cells. This suggests that in the absence of DNA damage, Rad4-Rad23 inhibits expression of these genes which become activated in response to exposure of cells to UV radiation. Examination of the genes in this cluster revealed many involved in the DNA damage response, particularly those in the RNR pathway and many of them regulated by the STRE or by the Crt1 repressor protein. Further gene expression profiling studies involved refinement of this initial analysis in an attempt to identify the genes regulated specifically by the GG-NER E3 ligase via its ubiquitination of Rad4. To do this we used

strains containing targeted point mutations in the SOCS box domain of the Rad7 component of the E3 ligase [22]. The *psocs* mutation has been shown previously to specifically inhibit ubiquitination of Rad4 [13]. The data described here confirmed and extended our initial observations, providing clear evidence for a role of the E3 ligase in regulating transcription of the genes involved in the RNR pathway including *DUN1* and *RNR1-4,* all of which contribute to the control of cellular dNTP synthesis. We confirmed these observations using northern blot analysis. The STRE containing *DUN1* gene was identified as one of the key factors regulated by the GG-NER E3 ligase suggesting the possibility of a direct role for Rad4-Rad23 in upregulating this group of genes in response to UV radiation. We considered whether the Radr-Rad23 complex controls expression of these genes by binding to their promoter regions. To investigate this, we examined the occupancy of the Rad4-Rad23 complex at the promoter of the STRE containing *DUN1* gene and other STRE containing genes including *DDR2* using ChIP. These experiments provide a mechanistic insight into the function of the GG-NER E3 ligase in Rad4-Rad23 controlled gene expression. We found that the Rad4-Rad23 complex is enriched at the STRE of both *DUN1* and *DDR2*. This observation raises the possibility that *DDR2,* a known component of the DDR but currently with unknown function, might play a role in the regulation of dNTP pools. In response to UV irradiation we find that the NER factors dissociate from the promoter element over the course of two hours in a manner dependent on Rad4 ubiquitination by the GG-NER E3 ligase. Thus in the absence of DNA damage Rad4-Rad23 remains bound at the STRE. However, in the absence of the E3 ligase activity we fail to see loss of occupancy of Rad4-Rad23 at the promoters of these genes in response to UV, despite the lower initial binding levels of the complex before UV irradiation. Furthermore, this leads to a failure to induce gene expression of these genes. These results indicate that the active GG-NER E3 ligase mediated dissociation of the complex away from the promoter in response to DNA damage facilitates expression of these genes.

Having established the mechanism by which the GG-NER E3 ligase controlled gene expression of the RNR pathway genes via the UV induced dissociation of Rad4-Rad23 from the promoters of these genes, we set out to examine the significance of this process and its impact on UV survival. To do this we took advantage of our previous findings that demonstrated the severe UV sensitivity of the *psocsrad23Δ* strain [13]. We reasoned that if the increased UV sensitivity observed in this strain compared to the *rad23Δ* mutation was due to failure to upregulate the dNTP pools due to the defect in the E3 ubiquitin ligase in response to DNA damage, that this phenotype might be rescued by the constitutive upregulation of the cellular dNTP pools by deleting the *SML1* and *CRT1* genes, two independent inhibitors of the RNR pathway. Our results show that the effect of the GG-NER E3 ligase mutant on UV survival can be completely rescued by upregulating the cellular dNTP pools confirming the role of the E3 ligase in UV survival.

Upregulation of dNTPs occurs in response to UV radiation and during S-phase and is important for both processes [25]. Based on the mechanism by which the GG-NER E3 ligase controls expression the RNR pathway genes, we predicted that cell cycle progression might also be affected if this mechanism is disrupted, since it is well known that proper regulation of the dNTP pools is necessary for normal transit across the cell cycle. We found that this is indeed the case as we see that the *psocs* mutant progresses through the cell cycle more quickly even in the absence of UV. As we anticipated this particular phenotype can also be rescued by the constitutive upregulation of dNTP synthesis by deleting *SML1* and *CRT1* (Figure 6B and C). This is independent confirmation of the significance of the GG-NER E3 ligase in regulation the dNTP pools in response to DNA damage.

Overall our data shows that Rad4-Rad23 represses a family of UV responsive genes in the absence of UV irradiation by direct interaction with their promoter regions. In response to UV Rad4 is ubiquitinated by the GG-NER E3 ligase and the Rad4-Rad23 complex dissociates from the promoter region. By leaving the promoter ubiquitinated Rad4-Rad23 facilitates the UV induced upregulation of these genes. Amongst these are the *DUN1* and *RNR* pathway genes that control dNTP synthesis during replication and in response to DNA damage. The Rad4-Rad23 and GG-NER E3 ligase controlled gene expression results in UV responsive upregulation of *DUN1* and the *RNR* genes that can drive the increase of dNTP synthesis, which facilitates UV survival and normal progression through the cell cycle. Our findings are consistent with the model shown in Figure 7 in which Pathway II regulates the gene expression of *DUN1*, a key gene in the control of the RNR pathway downstream of the central spine of Mec1-Rad53 signaling. The NER mediated gene transcription response provides downstream factors instrumental for the DDR by facilitating *DUN1* and *RNR1-4* expression in conjunction with the DDR signaling pathway (see Figure 7).

Our results also demonstrate that the pathway described here is distinct from the Rad6-Rad18 mediated control of gene expression described in a previous study [12]. The PCNA and 9-1-1 complexes stimulate the DDR as a results of primary damage detection. Interestingly, these DNA repair factors trigger the DDR via the action of the Rad6-Rad18 E2-E3 ubiquitin ligase complex. This upregulates DNA damage induced gene expression of specific genes including *MAG1* and *DDI1* [12]. We found that disrupting the Rad6-Rad18 pathway does not affect the UV induced gene expression of *DUN1* or *DDR2* (Figure S3B). In a similar vein, *DDI1* expression which is controlled by the Rad6-Rad18 pathway, is not affected in the *psocsrad23Δ* mutant. This clearly indicates that the GG-NER E3 ligase pathway is distinct from the Rad6-Rad18 pathway as illustrated in Figure 7.

The gene expression response regulated by the GG-NER E3 ligase described here, provides a novel mechanism by which the nucleotide excision repair pathway integrates with the DDR. Typically, the hallmark signal for cell-cycle checkpoint activation is

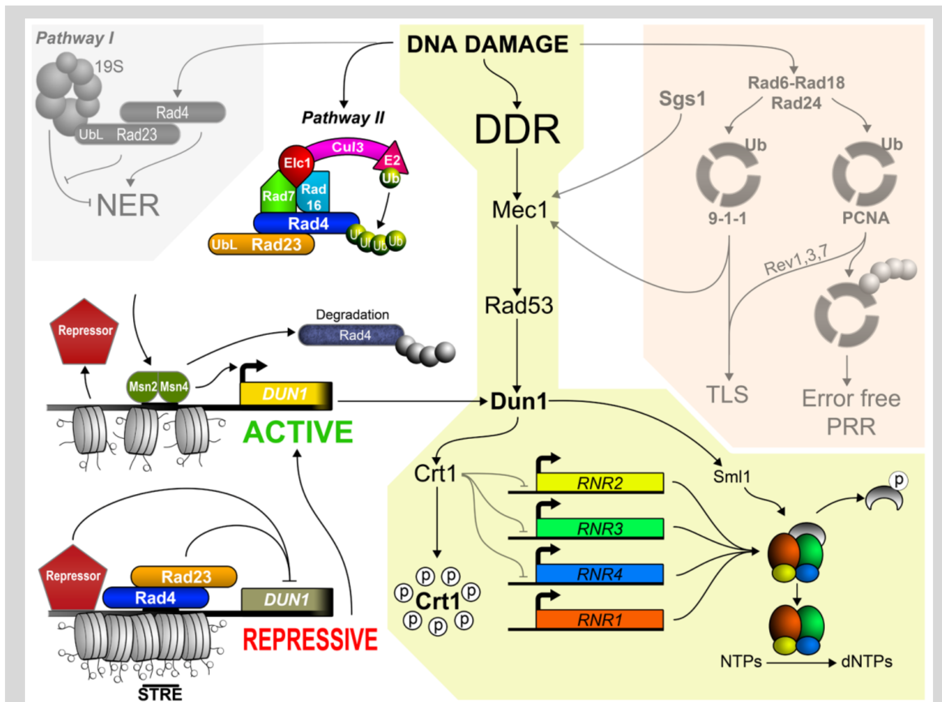

Figure 7 – *A model of the Rad4-Rad23 and GG-NER E3 ligase action in the context of DDR signaling and dNTP synthesis.* On the left side of the figure the redundant NER associated two pathway system is depicted [13]. Pathway I consists of the interplay between Rad23 and the 19S proteasome that controls NER without the need for de novo protein synthesis, while Pathway II involves Rad4 ubiquitination by the GG-NER E3 ligase that regulates the gene expression of *DUN1* and a host of other genes described in this study. Ubiquitination and possible degradation of Rad4 drive its dissociation from the promoter region of STRE containing genes, *DUN1* in this example. This loss of occupancy and the possible concerted action of other repressors and inhibitors changes the gene expression from repressive to active. This example is specific for the STRE containing *DUN1* gene and other targets from cluster in figure 1B. Msn2-Msn4 is shown here as stress induced transcription factor that is known to facilitate transcription for STRE containing genes [19]. The central signaling cascade of Mec1-Rad53-Dun1 signaling is represented in the middle highlighted in orange, including the *RNR* pathway as an important end-point in this context. In response to DNA damage Crt1 is hyperphosphorylated mainly by Dun1. Derepression of the RNR genes by Rad4-Rad23 and Crt1 results in expression of these genes and subsequent upregulation of the cellular dNTP pools. In this way, the gene transcription response of DUN1 and RNR1-4 provides downstream factors vital for the DDR in conjunction with the DDR signaling pathway, similar to the Sgs1 and Rad6-Rad18 DNA damage induced transcription induction, highlighted in pink on the right side of the figure adopted from [12].

stretches of ssDNA either bound by RPA at a DSB [30] or Rad14 as a NER intermediate [31]. Similarly, the PCNA and 9-1-1 complexes trigger the DDR by primary DNA damage detection. The common initiation event is DNA damage sensing that triggers signaling. Whether the same DNA damage signals are sensed in response UV induced DNA damage by the GG-NER E3 ligase remains to be determined.

Future studies will focus on how the GG-NER E3 ligase activity is initiated on in response to UV irradiation. Research into the possible phosphorylation of E3 ligase subunits via the DDR could provide new insight into this mechanism. Alternatively,

association of the COP9/CSN signalosome to the GG-NER E3 ligase could be regulated in an UV dependent fashion, with a possible role for DDR induced kinase activity controlling the E3 ligase signalosome interaction. Interestingly, the E3 ligase activity of the TC- and GG-NER specific ligases CSA-DDB1-CUL4A-ROC1 and DDB2-DDB1-CUL4A-ROC1 can be repressed by interaction with the COP9/CSN signalosome [28, 29]. A similar scenario for the yeast GG-NER E3 ligase could be envisioned, but mounting evidence is not present.

The novel Rad4-Rad23 based transcriptional response raises the question whether or not the change in Rad4-Rad23 occupancy at the promoter region of the genes affected is dependent of DNA damage detection. In other words is Rad4 dissociation driven by ubiquitination alone or in combination with its affinity for UV induced DNA damage? How this does novel pathway links in with the canonical function of Rad4-Rad23 in NER is uncertain. It is still speculation at this stage to state that the Rad4-Rad23 controlled genes expression can be truly independent of damage detection. Interestingly, dNTP upregulation is required for survival in response to UV radiation [25], however whether dNTP levels are required for NER or TLS remains unknown.

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7. Supplementary Figures

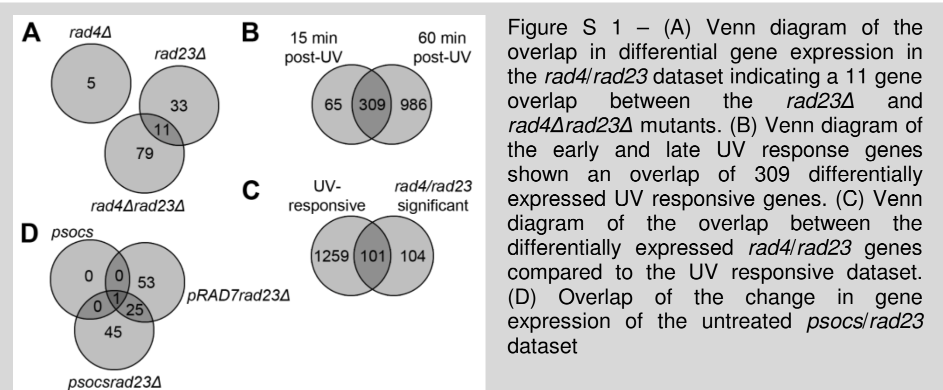
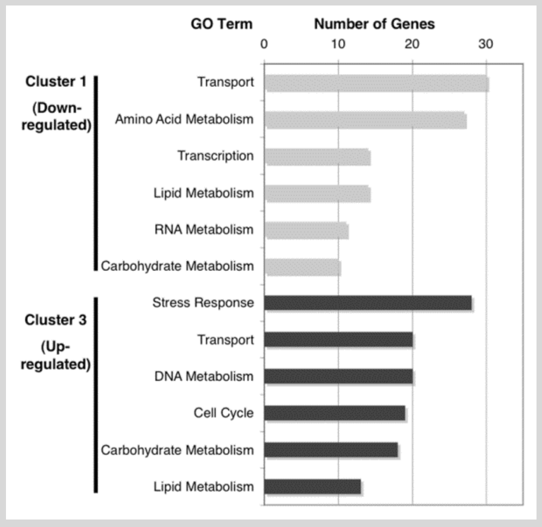


Figure S 1 – (A) Venn diagram of the overlap in differential gene expression in the *rad4*/*rad23* dataset indicating a 11 gene overlap between the *rad23Δ* and *rad4Δrad23Δ* mutants. (B) Venn diagram of the early and late UV response genes shown an overlap of 309 differentially expressed UV responsive genes. (C) Venn diagram of the overlap between the differentially expressed *rad4*/*rad23* genes compared to the UV responsive dataset. (D) Overlap of the change in gene expression of the untreated *psocs*/*rad23* dataset

Figure S 2 – The GO term analysis of UV responsive genes differentially expressed in the *psocs*/*rad23* dataset, highlighting an important role for the GG-NER E3 ligase in regulation of DNA metabolism, cell cycle and stress response pathways.



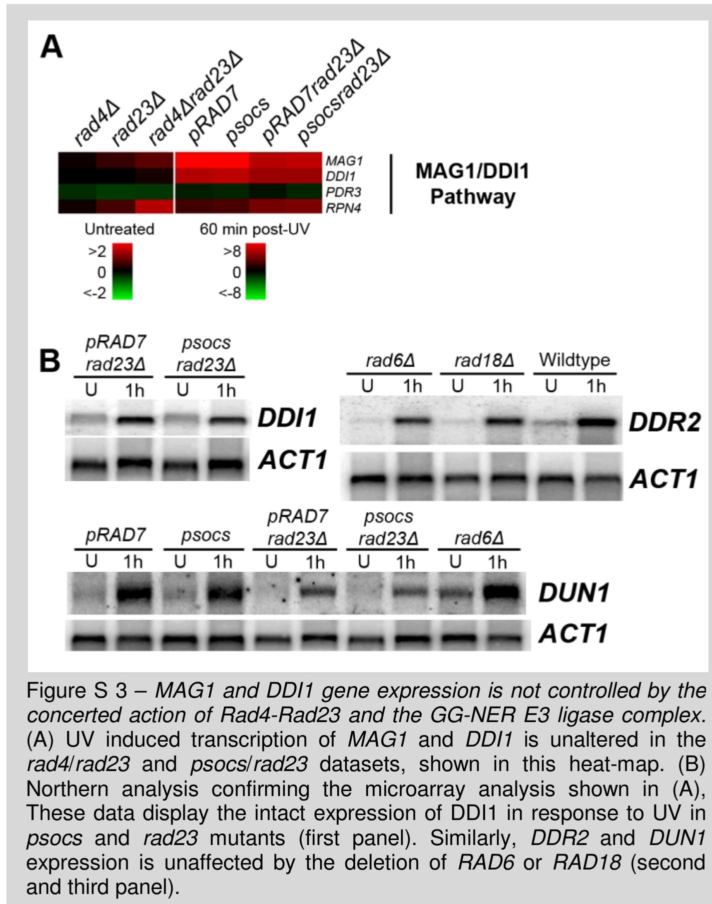


Figure S 3 – *MAG1 and DDI1 gene expression is not controlled by the concerted action of Rad4-Rad23 and the GG-NER E3 ligase complex.* (A) UV induced transcription of *MAG1* and *DDI1* is unaltered in the *rad4*/*rad23* and *psocs*/*rad23* datasets, shown in this heat-map. (B) Northern analysis confirming the microarray analysis shown in (A), These data display the intact expression of DDI1 in response to UV in *psocs* and *rad23* mutants (first panel). Similarly, *DDR2* and *DUN1* expression is unaffected by the deletion of *RAD6* or *RAD18* (second and third panel).

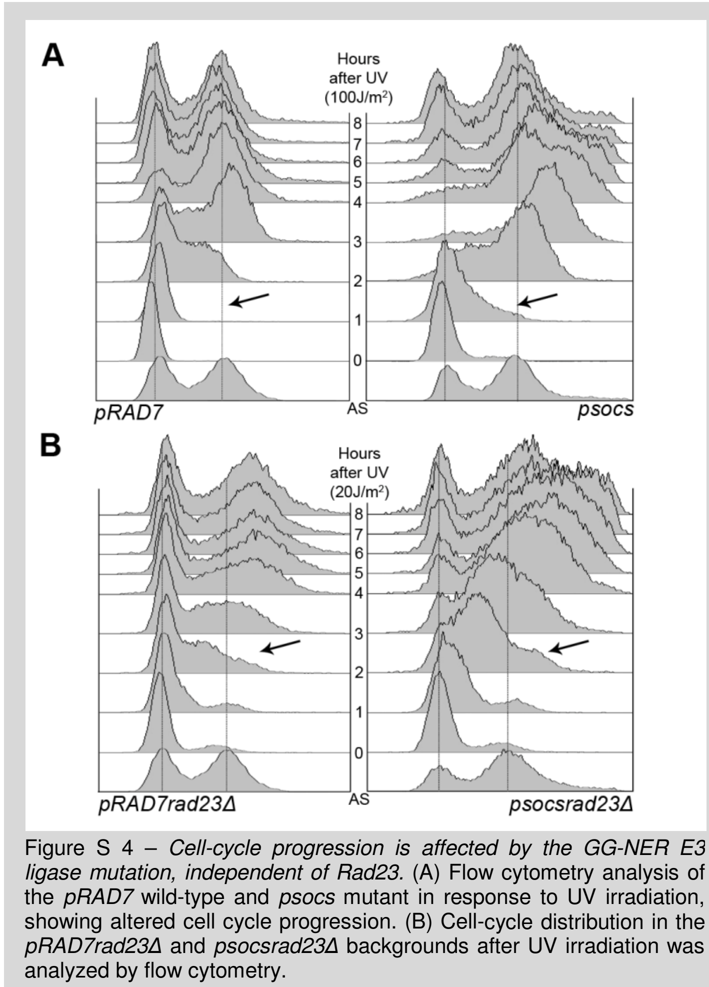


Figure S 4 – *Cell-cycle progression is affected by the GG-NER E3 ligase mutation, independent of Rad23.* (A) Flow cytometry analysis of the *pRAD7* wild-type and *psocs* mutant in response to UV irradiation, showing altered cell cycle progression. (B) Cell-cycle distribution in the *pRAD7rad23Δ* and *psocsrad23Δ* backgrounds after UV irradiation was analyzed by flow cytometry.

III The effect of Histone H1 on repair of rDNA in yeast

P. van Eijk, J. A. Brandsma, M. de Ruijter, J. Brouwer

1. Abstract

In *Saccharomyces cerevisiae* Rad34 is essential for Transcription Coupled Nucleotide Excision Repair (TC-NER) of the transcribed strand of active rDNA [1, 2]. Rad34 is homologous to the core repair factor Rad4, which is required for all other modes of NER. Histone H1 is a chromatin factor in yeast, located in the rDNA region. Here we show that deletion of the *HHO1* gene rescues the requirement for Rad34 in TC-NER of rDNA, an effect shown to be Rad4-dependent. The rescue of the *rad34Δ* repair defect by *HHO1* deletion is not due to increased Global Genome NER (GG-NER). Moreover, the deletion of *HHO1* from wild-type cells does not affect NER. The presence of Histone H1 at the rDNA locus directly or indirectly restricts NER to the use of Rad34 in repair while excluding Rad4.

2. Introduction

Nucleotide Excision Repair (NER) removes a wide variety of helix distorting lesions from the genome [3]. The repair reaction is highly conserved mechanistically and the *in vivo* importance of this mode of repair is evident from the UV sensitivity of NER deficient cells. Moreover, humans and animal models deficient for NER factors display predisposition to cancer [4]. NER deficiency not only results in UV-induced skin cancers but also results in spontaneous tumorigenesis in other organ tissue [4].

Generally, in NER there are two different sub-pathways; Transcription Coupled NER (TC-NER) and Global Genome NER (GG-NER). The Rad7-Rad16 GG-NER complex in yeast is capable of damage recognition throughout the entire genome. TC-NER, on the other hand, is initiated when, at an active gene, RNA polymerase II is blocked at a UV lesion. TC-NER specifically requires Rad26 in concert with the core NER factors to repair DNA damage.

Repair of the transcribed strand (TS) of an active gene is the sum of both GG-NER and TC-NER while the non-transcribed strand (NTS) is repaired by GG-NER. This explains why in general a strand-bias can be observed in active genes, whereby the TS is repaired faster. Conversely, the repair rate of both DNA strands is similar for inactive genes or in a genetic background without active TC-NER.

Rad4 is an essential NER factor in yeast, required for the early steps of the NER reaction in both TC-NER and GG-NER of all RNA polymerase II transcribed genes. However, the TS of active rDNA is still repaired in the absence of Rad4 [5] through the action of Rad34 in yeast [1]. Rad34 shares homology with all Rad4-like proteins [6] and has been shown to interact with both of the known interaction partners of Rad4, Rad23 [7] and Rad33 [1]. When *RAD34* is deleted from wild-type cells TC-NER at the rDNA locus is disrupted (i.e. no strand bias present). Thus, if *RAD34* is knocked out in a *rad4*Δ background, repair of rDNA is completely abolished [1, 2]. These data establish that Rad34 is a genuine Rad4 homologue in yeast, uniquely required for TC-NER at the rDNA locus.

Yeast rDNA exists as a tandem, head-to-tail arrangement of roughly 150 copies of the 35S rRNA gene. This cassette of over 9kb encodes the 18S, 5.8S and 25S rRNAs that are transcribed concurrently by RNA Polymerase I to form the pre-rRNA transcript. Roughly 50% of all copies are actively transcribed while the other half is in an inactive heterochromatic state [8]. Hho1p is a linker histone and thus copurifies with core histones but is non-essential in yeast [9, 10]. The Histone H1 protein has been shown to associate with and function at the rDNA locus [9, 11, 12]. Several functions for Hho1 at the rDNA locus have been proposed: exclusion of RNA Pol II transcription [12], repression of recombination [11, 12], DNA compaction and regulation of RNA Pol I processivity [13]. However, *hho1*Δ mutants in yeast do not have a distinct phenotype [10] and gene regulation at large, is not affected [14]. Here we show that histone H1 is indirectly involved in NER by affecting the damage recognition factor used at the rDNA locus.

3. Materials & Methods

3.1 Strains and Media

The experiments described here were all performed in *Saccharomyces cerevisiae* based on the W1588-4a wild-type strain [15]. All strains used in this study are listed in table 1. Histone H1 was deleted by gene-displacement using the disruption construct from the relevant BY4741 strain from the EuroScarf yeast deletion collection. By means of PCR the disruption was amplified from genomic DNA of the BY4741 deletion strain and used to transform the W1588-4a backgrounds. The short-lived background *sgs1Δ* was constructed similarly. Construction of the *rad34Δ* strain has been described previously [1]. The *RDN1::ADE2* strain was kindly provided by Dr. M. Kaeberlein [16].

3.2 Repair Analysis

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

DNA was isolated using a combination of Yeast Cell Piercing Solution (YCPS, 0.1% SB3-14, 100mM LiCl in TE buffer) and Cell Lysing Solution (CLS, 2mM EDTA, 2% SDS) followed by protein precipitation using 2.5M ammonium acetate and DNA precipitation using isopropanol (3:1 v/v). The cell pellets were thawed and resuspended in 200µl YCPS and incubated at 65°C for 30 minutes. Next, the cells were spun down and resuspended in 200µl CLS and again incubated at 65°C for 45 minutes. After placing the cells on ice for 5 minutes ammonium acetate was added to an end-concentration of 2.5M. At this stage cells were rotated for 10 minutes at room temperature before being centrifuged at 14,000rpm at 16°C for 10 minutes. The supernatant was then transferred and an equal volume of 3 volumes of isopropanol were added. After gentle mixing and incubation at room temperature for 10 minutes the DNA can be precipitated by means of centrifugation at 14,000rpm at 16°C for 12 minutes. The DNA was then washed with 70% Ethanol after which it was dissolved in water. rDNA repair was measured as described previously [1, 5].

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

3.3 Growth curves after UV irradiation

The growth curves after UV irradiation were obtained and data analysis was performed as described [17, 18]. In short cells were grown to exponential phase (OD_{600}~0.5-0.7), transferred to water ($8x10^6$cells/ml) and UV irradiated with 254nm UV at 150Jm^{-2}. Small aliquots of cells (~40.000) were transferred to fresh YPD in a 96-well plate. During 48

hours the AU_{595} was measured every 10 minutes with intermittent shaking at 30°C on a Tecan GENios Microplate Reader.

3.4 Replicative aging

Replicative aging was measured as described [19]. In short cells for study were restreaked on YPG plates containing glycerol as the only carbon-source to eliminate any petits. From the YPG plate a single colony was restreaked on a thin YPD plate for use in the micromanipulator. Using a Sanger Instruments Micromanipulator ~40 dividing cells were picked from and sorted on a YPD plate. After the first division each mother was discarded while the virgin daughter was kept for aging analysis. After incubation at 30°C each 1.5hrs the daughter cell was removed until the mother cell stopped dividing. Plates were incubated at 4°C overnight to slow down cell division. Cells will typically only perform a single division overnight if kept at 4°C.

3.5 rDNA Recombination assay

The strain containing an *ADE2* insert in its rDNA (kindly provided by Dr. M. Kaeberlein) was used to assess the recombination rate in strains deleted for *RAD34*. Cells were grown overnight and diluted in water to accommodate about $1x10^4$-$2x10^4$ cells/mL. This suspension was used to plate 500-1000 cells on YPD plates. After growth at 30°C the plates were stored at 4°C until red sector formation could be detected. If in the early rounds of division recombination and thus loss of the *ADE2* insert occurs sectoring of the resulting colony is an indication of this event. By counting pink sector formation one can get a measure for the frequency of recombination. For each strain 2,500 to 5,000 colonies were counted.

4. Results & Discussion

4.1 Analysis of rDNA repair in Histone H1 mutants

In yeast, Rad4-independent repair of the transcribed strand of rDNA depends on the Rad4-like protein Rad34 [1, 2]. Because Histone H1 is involved in a variety of processes at the rDNA locus [11-13], we deleted *HHO1* to elucidate why rDNA requires Rad34 instead of Rad4. We analyzed this by measuring rDNA repair using a strand-specific repair assay on genomic DNA in several NER deletion backgrounds. In the absence of Rad34, NER at the rDNA locus of both the TS and NTS is performed by GG-NER (Figure 1) [1, 5] and deleting *HHO1* in a *rad34Δ* strain does not change the repair rate of the NTS (Figure 1). However, repair of the TS in *rad34Δhho1Δ* cells is increased compared to that of a *rad34Δ* single mutant (Figure 1). These repair data demonstrate that *hho1Δ* is able to rescue the TS repair defect of a *rad34Δ* mutant.

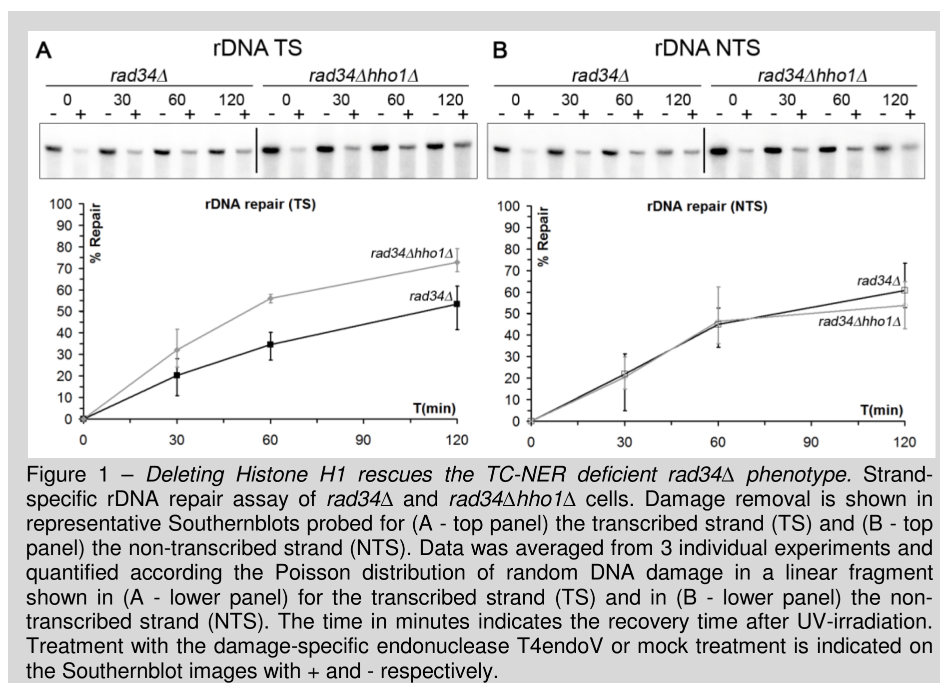


Figure 1 – *Deleting Histone H1 rescues the TC-NER deficient rad34Δ phenotype.* Strand-specific rDNA repair assay of *rad34Δ* and *rad34Δhho1Δ* cells. Damage removal is shown in representative Southernblots probed for (A - top panel) the transcribed strand (TS) and (B - top panel) the non-transcribed strand (NTS). Data was averaged from 3 individual experiments and quantified according the Poisson distribution of random DNA damage in a linear fragment shown in (A - lower panel) for the transcribed strand (TS) and in (B - lower panel) the non-transcribed strand (NTS). The time in minutes indicates the recovery time after UV-irradiation. Treatment with the damage-specific endonuclease T4endoV or mock treatment is indicated on the Southernblot images with + and - respectively.

We next set out to determine what NER factors now contribute to repair in the *rad34Δhho1Δ* double mutant. First, we determined whether the Rad4 protein, that is homologous to Rad34, can replace Rad34 during TS repair in *rad34Δhho1Δ* cells. This was tested by measuring repair in the triple mutant *rad4Δrad34Δhho1Δ* and comparing that to the repair rate of *rad34Δhho1Δ* cells (Figure 2). In the absence of Rad4, Rad34 and Histone H1 no NER activity of either strand can be detected and thus the rescue effect of the *HHO1* deletion is lost. These data show that the TS repair in *rad34Δhho1Δ* cells is Rad4-dependent (Figure 2). Rad4 is now required for TS repair of rDNA but only if, in the absence of Rad34, the *HHO1* gene is also deleted.

The chromatin structure of the rDNA locus becomes decompacted in the absence of the Histone H1 protein [13]. This could allow repair factors to better access the inactive rDNA. In our experiments we make use of whole genomic DNA and this therefore includes both the active and inactive rDNA copies. In case of *rad34Δhho1Δ* cells a more open chromatin structure due to the absence of Histone H1 could lead to an increase in repair of the TS of non-transcribed rDNA copies by GG-NER. In order to test this hypothesis we eliminated GG-NER by deleting *RAD16* in a *rad34Δ* and *rad34Δhho1Δ* background and measured their respective repair rates. If we then compare TS repair of *rad34Δrad16Δ* cells to that of the *rad34Δrad16Δhho1Δ* triple mutant we again find the increase in TS repair in the triple mutant *rad34Δrad16Δhho1Δ* (Figure 3A). This indicates that increased repair in *rad34Δhho1Δ* cells is not due to GG-NER. Interestingly, a mode of GG-NER that is Rad7- and Rad16-independent exists [1, 5] that could also benefit from a more open chromatin structure in *HHO1* deletion cells. However, the contribution

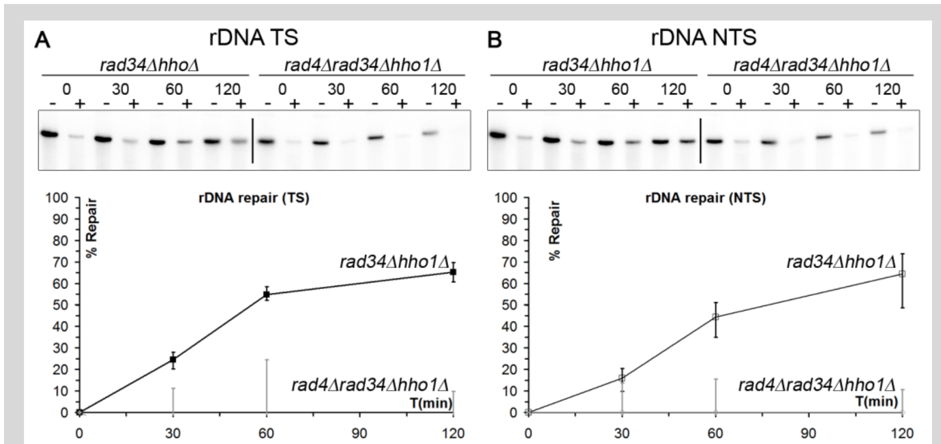


Figure 2 – *The rescue effect of Histone H1 deletion depends on Rad4.* Strand-specific rDNA repair assay of *rad34∆hho1∆* and *rad4∆rad34∆hho1∆* cells as described in Figure 1.

of this unknown repair mechanism occurs at late 2 to 4 hour times points, whereas the rescue effect of the *HHO1* deletion in *rad34∆* and *rad16∆rad34∆* cells takes place during the first 2 hours after UV irradiation, thus making it unlikely that the Rad7- and Rad16 independent repair pathway results in the TS repair we measure.

If Histone H1 merely decompacts rDNA resulting in more active rDNA copies or more efficient overall repair, we expect that the deletion of *HHO1* in the presence of Rad4 and Rad34 would also affect NER. This was tested by measuring strand-specific repair in a single *hho1∆* deletion background (Figure 4). Deleting *HHO1* by itself has no effect on rDNA repair, showing that the absence of Histone H1 does not result in increased accessibility of rDNA for NER factors.

In summary, the data presented here show that Rad4 can contribute to TS repair in the absence of Rad34 only when Histone H1 is absent. The active role of Histone H1 that

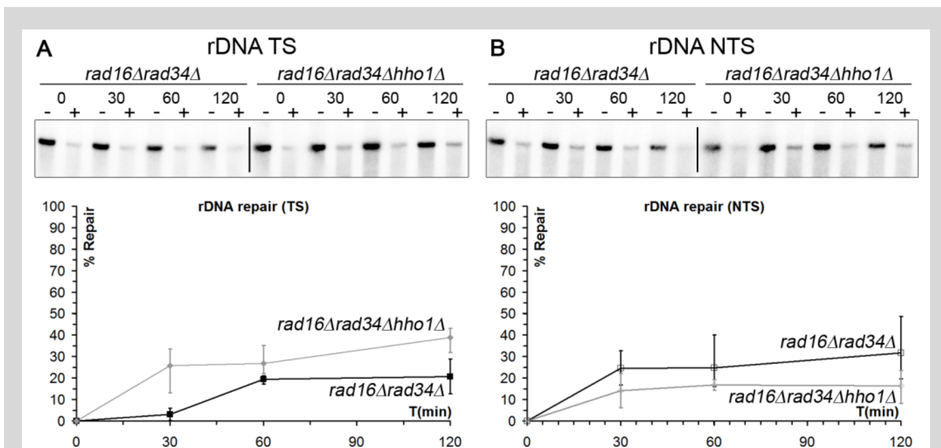


Figure 3 – *The rescue effect of deleting Histone H1 is independent of Rad16.* Strand-specific rDNA repair assay of *rad34∆rad16∆* and *rad34∆rad16∆hho1∆* cells as described in Figure 1.

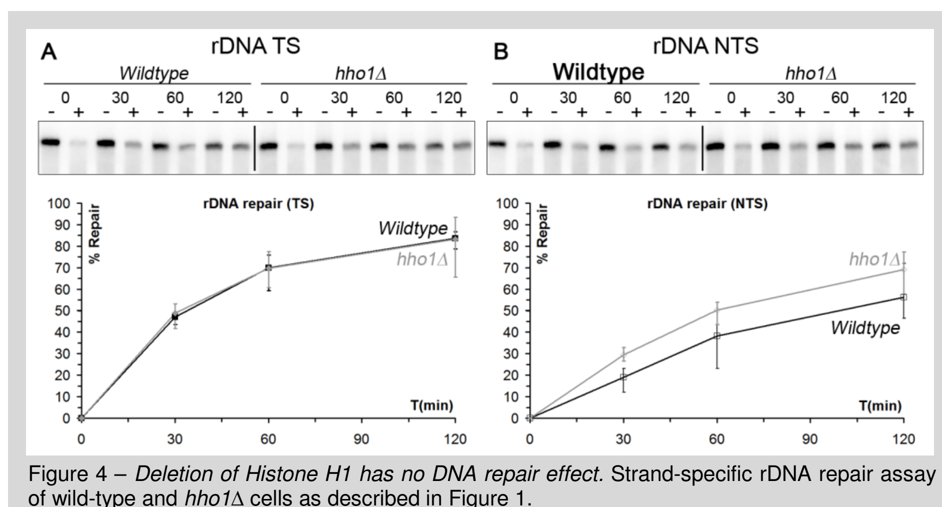


Figure 4 – *Deletion of Histone H1 has no DNA repair effect.* Strand-specific rDNA repair assay of wild-type and *hho1*Δ cells as described in Figure 1.

inhibits RNA Polymerase II transcription at the rDNA locus [12] could help to explain this Rad4-dependency of TS repair in *rad34*Δ*hho1*Δ cells. In other words, in these double deletion cells RNA Pol II might be able to transcribe rDNA. This would require Rad4 for TC-NER leading to increased TS repair in these cells compared to a *rad34*Δ background. If RNA Pol II driven TC-NER occurs at the rDNA locus it could also require Rad26, a factor needed for normal TC-NER of RNA Pol II transcribed genes outside the rDNA locus. However, the repair detected in *rad34*Δ*hho1*Δ cells is independent of Rad26 (data not shown). This means that either the TS repair measured in *rad34*Δ*hho1*Δ cells is not RNA Pol II related or transcription of rDNA by either RNA Pol I or Pol II is so different that it does not require Rad26. This latter assumption is supported by the fact that canonical rDNA TC-NER via Rad34 and RNA Pol I does not depend on Rad26 either [5]. Thus it appears that Rad26 is not required for any type of TS repair at the rDNA locus. However, our results do not elucidate whether RNA Pol II can transcribe rDNA in *rad34*Δ*hho1*Δ cells since Rad26-independent repair of RNA Pol II transcribed genes has also been shown to occur [20].

4.2 Analysis of another possible role of Rad34 at the rDNA locus in yeast

Apparently, the presence of Histone H1 at the rDNA locus makes TC-NER Rad34-dependent. However, this does not help to explain why Rad34 is specifically required for TC-NER of rDNA. Therefore, we studied the phenotype of *RAD34* and/or *HHO1* deleted cells to find other processes in relation to NER and rDNA that are affected by the loss of these genes. A role for Rad34 outside NER but specific for rDNA could aid in our understanding of why Rad34 is specifically required at this locus.

Firstly, we determined the recovery from UV damage of a *rad34*Δ and/or *hho1*Δ strain. This was done by measuring the time it takes a strain to recuperate from DNA damage induced by UV radiation referred to as lag-time (as described by Toussaint et al. [17]).

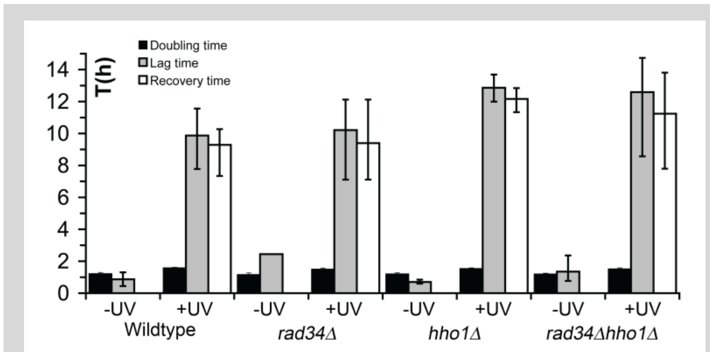


Figure 5 – *UV-phenotypes of RAD34 and HHO1 strains.* Growth curves were obtained with or without UV treatment. Quantification of the growth curve data as described in [17] resulted in the doubling time, lag time and recovery time for all the strains tested displayed here as an average of at least three individual experiments.

Strains deleted for *RAD34, HHO1* or both show no significant UV phenotype, indicating that the repair rates of rDNA in these backgrounds do not hamper overall UV survival.

The rDNA locus is closely linked to aging in yeast and provided the first molecular mechanism to explain aging in a eukaryote [21]. This prompted the idea that Rad34-dependent TC-NER or a separate function of the Rad34 protein could contribute to longevity. Therefore, we tested the replicative aging phenotype of a *rad34*Δ cells compared to wild-type cells and the known short-lived *sgs1*Δ mutant by means of micromanipulation [19]. As shown in figure 6 the mortality rate of *rad34*Δ cells overlaps with two independent wild-type curves while the short-lived *sgs1*Δ cells age much faster as reported previously [22]. Hence the presence of Rad34 or Rad34-dependent TC-NER at the rDNA locus is not a determinant for replicative aging in yeast, thus not explaining the use of Rad34 in the context of rDNA and aging.

The repetitive nature of the rDNA locus makes it a prime target for recombination and it has been shown that the uncontrolled recombination leads to loss of rDNA copies that induces aging [23]. Cells therefore carefully maintain the rDNA copy-number by preventing recombination at the rDNA locus. Again the presence of a specific rDNA TC-NER factor could be warranted by an important contribution of Rad34 or Rad34-dependent TC-NER to prevent recombination. To test the effect of

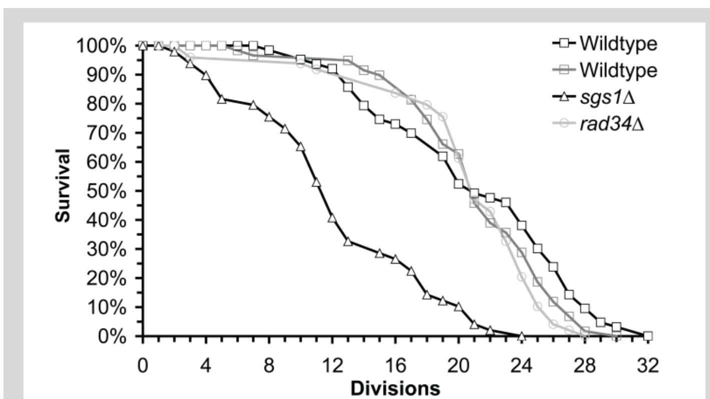


Figure 6 – *RAD34 deletion cells have wild-type mortality rates.* Mortality curves of wild-type, *rad34*Δ and *sgs1*Δ cells. The data are an average of 40 cells monitored over time. The survival is a measure for the amount of cells that continued dividing after a certain number of divisions.

Rad34 on recombination at the rDNA locus we employed an engineered yeast strain kindly provided by M. Kaeberlein [16]. This strain contains an *ADE2* cassette in its rDNA which serves as an indicator for the occurrence of rDNA recombination. For wild-type cells, recombination events are in the order of 1-3 per 1,000 colonies while a hyper-recombinant mutant can display up to 20 red sectored colonies per 1,000 in this type of experiment [16]. For *rad34*Δ cells we found wild-type levels of recombination of around 2.68 per 1,000 versus 2.59 per 1,000 for wild-type cells (data not shown). Therefore, Rad34 has no significant role in preventing rDNA recombination.

5. Concluding remarks

In summary, we have shown that in the absence of Histone H1, Rad34 is no longer required for TS repair of active rDNA but now becomes dependent on Rad4. Most likely, the need for a specific Rad4-like factor in rDNA TC-NER is due to the use of a different RNA polymerase in this DNA region.

Interestingly, in higher eukaryotes, regardless of the polymerase in action, TC-NER takes place in the absence of a Rad4-like protein. The Rad4 homolog XPC is only required for GG-NER in humans and other higher eukaryotes. Thus, transcription and TC-NER in yeast are different in their requirement of two Rad4-like proteins: Rad4 in RNA Pol II related TC-NER and Rad34 in TC-NER of RNA Pol I transcribed rDNA. Apparently, NER at the rDNA locus in yeast is so different from repair of this locus in human cells that it requires a different Rad4-like protein.

It has been proposed that the actively transcribed rDNA is largely devoid of nucleosomes [3, 10]. However, our results show that Histone H1 has an effect on TC-NER of actively transcribed rDNA copies, implicating that at least Histone H1 is associated with active rDNA.

Why is Rad34 only required for rDNA TC-NER when Histone H1 is present? Possibly, the presence of Histone H1 stabilizes RNA Pol I when stalled on a lesion. Rad34 would then be specifically required to remove this stalled RNA polymerase. In the absence of Histone H1 RNA Pol I might be capable of back-tracking from the lesion by itself (without Rad26) allowing access to the lesion by the general recognition factor Rad4. Similarly, RNA Pol II associated Rad26-independent repair has been shown to be facilitated by back-tracking of the RNA polymerase [20].

It remains to be determined why in yeast a specific additional rDNA TC-NER factor is present, while in its absence cells are not UV sensitive and can largely rely on GG-NER for the repair.

Table 1 - Strains and their respective genotype used in this study

Strain	Genotype	Source
W1588-4a	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1*	R. Rothstein [15]
MGSC581	*W1588-4a rad34Δ::loxLEU2lox URA3*	This lab [1]
MGSC982	*W1588-4a hho1Δ::loxHISlox*	This study
MGSC984	*W1588-4a rad34Δ::loxLEUlox hho1Δ::loxHISlox*	This study
MGSC990	*W1588-4a rad4Δ::hisG hho1Δ::loxHISlox*	This study
MGSC991	*W1588-4a rad4Δ::hisG rad34Δ::loxLEUlox hho1Δ::loxHISlox*	This study
MGSC993	*W1588-4a rad16Δ::hisG rad34Δ::loxLEUlox hho1Δ::loxHISlox*	This study
MGSC998	*MATa ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15 RAD5 RDN1::ADE2*	M.Kaeberlein [16]
MGSC1013	*MATa ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15 RAD5 RDN1::ADE2 sir2Δ::KANMX*	This study
MGSC1071	*MATa ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15 RAD5 RDN1::ADE2 rad34Δ::URA3*	This study
MGSC1000	*W1588-4a sgs1Δ::KANMX*	This study

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

P. van Eijk, J.A. Brandsma, M. de Ruijter, J. Brouwer

1. Abstract

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

2. Introduction

Xeroderma pigmentosum (XP) is a rare autosomal, recessive human disorder characterized by defects in Nucleotide Excision Repair (NER). Genetic defects in human NER can also result in other disorders like Cockayne Syndrome (CS) or Trichothiodystrophy (TTD) [2]. XP causes a 2,000-fold increased incidence of sunlight induced skin cancer in patients and, depending on the complementation group, can also result in neurological degeneration [2]. The XP-C complementation group is the most common among XP patients and is defined by mutations in the *XPC* gene homologous to the core NER *RAD4* gene in yeast [3]. The XPC-hHR23A/B complex is, together with the XPC binding protein Centrin 2 involved in the initial recognition of DNA damage.
The yeast *Saccharomyces cerevisiae* is shown to be an excellent model for the study of the conserved mechanism of NER. In yeast a complex similar to that in human cells is responsible for the recognition of DNA damage. The XPC homologue in yeast, Rad4 forms a complex with Rad23, and we showed recently that also a third protein can bind to this recognition complex, Rad33 [4]. Rad33 shows no homology to Centrin2 but the binding site in Rad4 is identical to that in XPC [4]. The homology between the human and yeast recognition complexes in NER allows us to study the role of a conserved tryptophan residue, which was found to be substituted as result of a point mutation in an XPC patient. This XPC mutation is of special interest since it is one of two patient mutations that have been described to lead to an amino acid substitution, leaving the full length protein and its expression unchanged [5, 6]. One of these mutations, XPC-W690S, results in defective repair and UV sensitivity in human cells [1]. It was shown that decreased protein stability, impaired DNA binding and impaired repair all contribute the XP phenotype [9]. Furthermore, mutant XPC protein displayed reduced ssDNA binding *in vitro* while the interaction with Rad23 and Centrin 2 is still intact [10]. The W690S resides in the conserved C-terminus that is shared by Rad4-like proteins. In this region the Rad23 and TFIIH interactions are mapped [5, 7]. The affected tryptophan residue at position 690 in XPC is conserved at position 496 in yeast Rad4 (Figure 1A top panel) [1]. The information from the Rad4 protein structure allows us to pinpoint exactly where the amino acid substitution affects the protein structure [8]. The substituted residue is located in Beta Hairpin Domain 2 (BHD2) (Figure 1A lower panel) in close proximity to the bases opposite the CPD lesion [8]. The neighboring residues R494, Q495 and M498 in Rad4 are all in direct contact with the DNA backbone and bases opposite the CPD lesion [8], suggesting that the mutation might affect DNA damage binding.
To get more insight into the defect caused by this patient mutation we set out to investigate the phenotype of the corresponding *rad4W496S* mutant in *S.cerevisiae*. Surprisingly, we find that the equivalent W496S mutation in Rad4 does not result in UV sensitivity. However, repair is moderately affected in *rad4W496S* cells, with GG-NER being most severely hampered.

3. Material & Methods

3.1 Strains and UV droptest

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

3.2 Strand specific repair assay

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

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

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

4. Results & Discussion

4.1 The rad4W496S mutant is not UV sensitive

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

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

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

4.2 The Rad4W496S mutation results in impaired NER in vivo

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

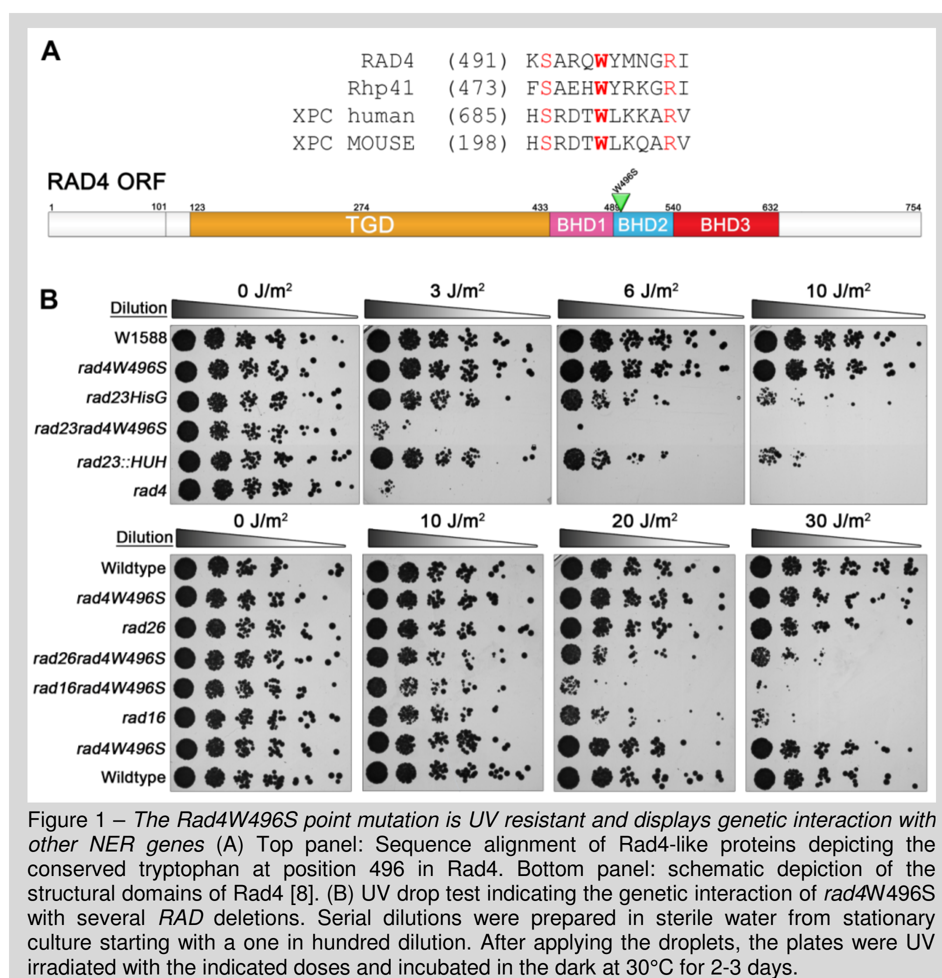

Figure 1 – *The Rad4W496S point mutation is UV resistant and displays genetic interaction with other NER genes* (A) Top panel: Sequence alignment of Rad4-like proteins depicting the conserved tryptophan at position 496 in Rad4. Bottom panel: schematic depiction of the structural domains of Rad4 [8].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

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

Analogous to the survival experiments, strand specific repair was also investigated in GG-NER and TC-NER deficient backgrounds containing the *rad4W496S* mutation. GG-NER in a TC-NER deficient *rad26Δ* single mutant repairs 50-60% of the damages on both strands during the time course of 2 hours (Figure 3B & D). However, GG-NER of both strands in a *rad26Δrad4W496S* double mutant is severely reduced to only 15% after 2 hours (Figure 3B & D). This again shows that GG-NER is severely impaired by the *rad4W496S* mutation.

GG-NER is completely disrupted in a *RAD16* deletion background resulting in the absence of NTS repair in *rad16Δrad4W496S* cells as expected (Figure 3A & C) [15]. In this

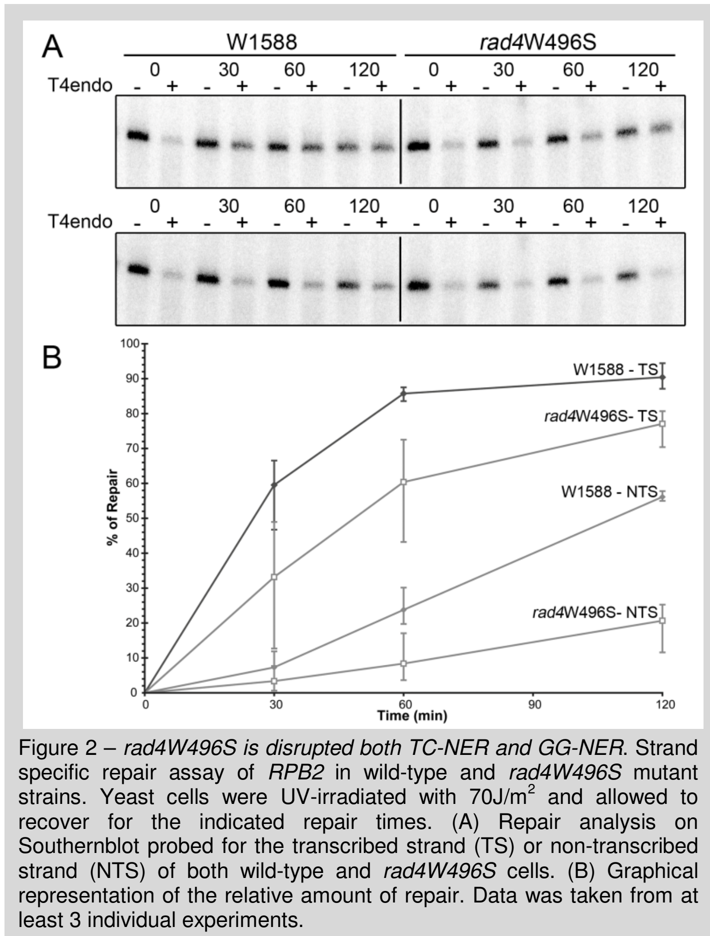

Figure 2 – *rad4W496S is disrupted both TC-NER and GG-NER*. Strand specific repair assay of *RPB2* in wild-type and *rad4W496S* mutant strains. Yeast cells were UV-irradiated with 70J/m^2 and allowed to recover for the indicated repair times. (A) Repair analysis on Southernblot probed for the transcribed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

GG- NER deficient *rad16Δrad4 W496S* background, TC-NER removes ~80% of the damages in the TS two hours after UV irradiation.

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

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

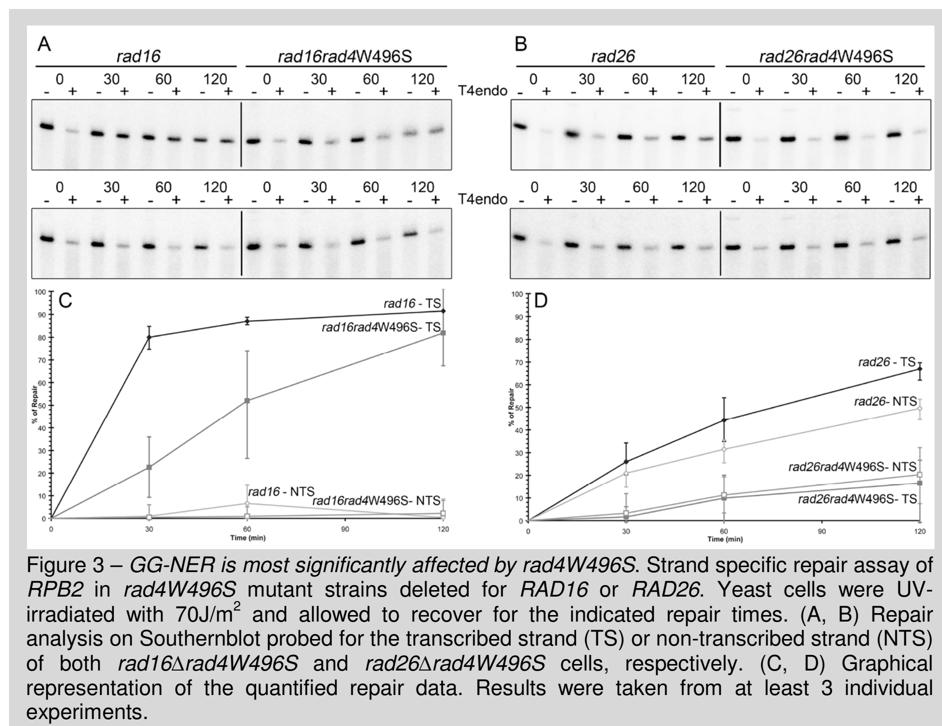

Figure 3 – *GG-NER is most significantly affected by rad4W496S*. Strand specific repair assay of *RPB2* in *rad4W496S* mutant strains deleted for *RAD16* or *RAD26*. Yeast cells were UV-irradiated with 70J/m^2 and allowed to recover for the indicated repair times. (A, B) Repair analysis on Southernblot probed for the transcribed strand (TS) or non-transcribed strand (NTS) of both *rad16Δrad4W496S* and *rad26Δrad4W496S* cells, respectively. (C, D) Graphical representation of the quantified repair data. Results were taken from at least 3 individual experiments.

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

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

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V A novel, functionally distinct, bipartite Rad4-Rad23 interaction in Nucleotide Excision Repair

P. van Eijk, J.A. Brandsma, M. de Ruijter, J. Brouwer

1. Abstract

In the yeast *Saccharomyces cerevisiae* the Rad4-Rad23 complex is implicated in the initial damage recognition step of Nucleotide Excision Repair (NER). In the Rad4-Rad23 heterodimer, as well as in the analogous XPC-hHR23A/B complex in human cells, the C-terminus of Rad4 or XPC is shown to interact with the R4B domain of the Rad23 protein. Elucidation of the structure of Rad4, the homologue of XPC, revealed several interesting domains that might have a role in protein-protein interactions. In this paper we study the Rad4-Rad23 interaction in more detail. We constructed a set of deletions in the Rad4 and Rad23 proteins respectively, and used the yeast two-hybrid technique for extensive analysis.

We show that Rad4 contains two distinct Rad23 binding domains, one located in the TransGlutaminase Domain (TGD) and another in the C-terminal part of the protein. Both interactions, however, require the same Rad4 binding domain (R4BD) of Rad23. We present evidence that the UbL domain controls the interaction of Rad23 with either of the two binding domains on Rad4. We present evidence that the two different Rad4-Rad23 complexes are differentially involved in Global Genome NER (GG-NER) and Transcription-Coupled NER (TC-NER).

2. Introduction

The core damage recognition complex in the Nucleotide Excision Repair (NER) pathway consists of Rad4, Rad23 and Rad33 in yeast [1, 2] and of XPC, hHR23A/B and Centrin 2 in higher eukaryotes [3]. Uniquely to yeast, a rDNA specific Rad4-homolog exists called Rad34 [4, 5]. This protein also forms a complex with Rad33 and Rad23 and is responsible for transcription-coupled NER (TC-NER) in active rDNA [4].
Moderate UV sensitivity is observed when *RAD23* or NER accessory genes are deleted, whereas the deletion of core NER factors, like Rad4, results in extreme UV sensitivity due to complete absence of repair. *RAD23* deletion cells show only moderate UV sensitivity, although no appreciable repair activity can be detected [6]. Currently, Rad23 and hHR23B are believed to stabilize the Rad4 or XPC protein, respectively [7, 8]. This, however, does not fully explain the phenotype of *rad23*Δ cells.
The Rad23 structure and function is interesting as it is one of a few proteins in yeast that contains both Ubiquitin Like (UbL) and Ubiquitin Associating (UBA) domains (Figure 2A bottom panel). Proteins that share these features with Rad23 are Dsk2 and Ddi1 and all three proteins are thought to shuttle ubiquitinated proteins to the proteasome due their affinity for ubiquitinated proteins and ability to interact with the proteasome [9-12]. The UbL domain of Rad23 is ubiquitinated *in vivo* [8] but this does not interfere with protein stability. The domain can also be replaced by the genuine ubiquitin sequence without loss of protein function in NER [13]. The Rad23 UbL domain is partially dispensable for NER both *in vivo* and *in vitro* [13, 15]. Interestingly, the UbL domain of Rad23 was shown to interact with the 19S proteasome subunit, an interaction that seemed to enhance *in vitro* NER [15]. However, the role for the UbL domain in relation to the Rad4-Rad23 interaction is poorly understood. The UBA domains in Rad23 intrinsically stabilize the protein and allow it to be ubiquitinated and to interact with the proteasome without being degraded [14].
The structure of Rad4 bound to a Rad23 fragment and DNA has been solved [16]. The Rad4 protein used to solve the structure does not contain about a 100 amino acids from the N- and C-terminal ends of the protein. These ends of Rad4 are susceptible to proteolytic degradation *in vitro*, suggesting that the Rad4 termini are unstructured or loosely folded [16]. However the truncated recombinant Rad4 protein binds normally to DNA. Rad4 is made up of 4 protein domains (Figure 2A top panel) that interact with the DNA backbone and with the DNA opposit the CPD lesion, but not the CPD itself. The TransGlutaminase Domain (TGD) spans 300 amino acids of the N-terminal part of Rad4 and supports the interaction with the DNA phosphate backbone together with the Beta Hairpin Domain 1 (BHD1). BHDs 2 and 3 interact with a 4 basepair section opposite the CPD lesion. The CPD dimer is solvent exposed and therefore does not exist in a fixed conformation in the structure. BHD3 inserts its Beta Hairpin into the DNA double helix and the domain flips out the two undamaged bases opposite the lesion that interact with both BHD2 and BHD3 [16]. In the crystal structure of the Rad4-Rad23 complex the

partial Rad23 protein containing the Rad4 Binding Domain (R4BD) and UBA2 (Figure 1) interacts with the Rad4 TGD domain.

The Rad4-Rad23 interaction was suggested to occur at the C-terminal domain of Rad4 domain, supported by our previous data [18]. Similarly, the interaction of XPC and hHR23B was also defined to reside in the C-terminal part of XPC [17]. Together these data suggest a possible interaction of Rad23 with a C-terminal part of Rad4, distinct from the TGD interaction shown in the protein structure. We decided to further investigate the Rad4-Rad23 interaction. We show that the Rad4-Rad23 interaction can occur on two sites on Rad4, one with the TGD and one at C-terminal region of Rad4. Interestingly, the Rad23 UbL-domain differently affects the two interactions.

3. Material & Methods

3.1 Strains and plasmids

All strains used in this study are derived from the wild-type W1588-4a [19]. Relevant phenotypes are described in table 1. Full length *RAD4* cannot be propagated in *E.coli* due to lethality. By using the high efficiency of homologous recombination in yeast we constructed the full length *RAD4* plasmid in *Saccharomyces cerevisiae* (described elsewhere).

3.2 Yeast Two-hybrid assay

For yeast twohybrid analysis the Clontech Matchmaker 3 system was used. The respective DNA fragments indicated in figure 2A were put in-frame with the GAL4 activating domain (AD) in pGADT7 or the GAL4 binding domain (BD) in pGBKT7. Subsequently, the resulting fusion constructs were introduced into the selection strains Y187 and AH109, respectively. After mating, cells were spotted onto selection plates. Selection for the presence of both plasmids was maintained by growing on plates lacking tryptophan and leucine while selection for interaction is a result of the histidine and adenine selection markers with a GAL1 UAS and TATA construct in their promoter driving transcription. After growth at 30°C the plates were monitored every day for 3 days.

4. Results & Discussion

4.1 The Rad4-Rad23 interaction has a bipartite binding

We previously reported the two-hybrid interaction of the Rad4 C-terminal part (amino acids 274-667) with Rad23 [18]. The deletions used in this study were based on the homology found between all Rad4 like proteins [4] and included only part of the so-called TGD domain of Rad4 (see figure 1). Structure analysis of Rad4 showed association of the TGD domain of Rad4 with Rad23 [16] with a section of the TGD domain that was not included in the C-terminal construct tested previously (Figure 1) [18]. Therefore we

repeated the two-hybrid experiments using an N-terminal fragment that includes the complete TGD domain (amino acids 123-433). All deletions used are shown in figure 2A. These constructs are the N-terminal Rad4 TGD (amino acids 1-433), the C-terminal Rad4Ct (amino acids 274 to 667) and the Rad4 C-terminal end construct (Rad4end). In addition to truncated *RAD4* constructs, full length *RAD4* was also included in the plasmid collection. The *RAD4* gene cannot be cloned in *E coli* since, due to yet unknown reasons, expression of the *RAD4* gene is lethal in *E.coli*. We succeeded in constructing full length *RAD4* by making use of the very efficient recombination in yeast to create *RAD4* full length clones, and introduced in the same way full length *RAD4* in the two-hybrid vectors.

In the Rad23 protein different functional domains were identified of which UbL and R4BD have been shown to have a function in NER. Therefore constructs with deletions of these domains were included in the two-hybrid experiments described below. The Rad23 full length (FL), Δ*R4B*, Δ*UbL* and Δ*UbL*Δ*R4B* are schematically depicted in figure 2A. Subsequently, different constructs were introduced in two-hybrid vectors and analyzed. The results are shown in figure 2B.

Full length Rad23 interacts with the Rad4Ct as described previously (row 2, columns B, D in figure 2B), showing the established Rad4-Rad23 interaction. In the absence of the R4B domain, Rad23 no longer interacts with Rad4 (row 4, figure 2B) confirming that the R4BD is absolutely required for the interaction with Rad4 [8, 20]. Interestingly, the Rad4 N-terminal TGD construct interacts with Rad23FL as well (C2, figure 2B). These data suggest that two distinct interactions between Rad23 and Rad4 exist: one in the C-terminal part of Rad4 and one with the Rad4 TGD. Strikingly, the R4BD of Rad23 appears to be essential for interaction with the Rad4 TGD as well (C4, figure 2B).

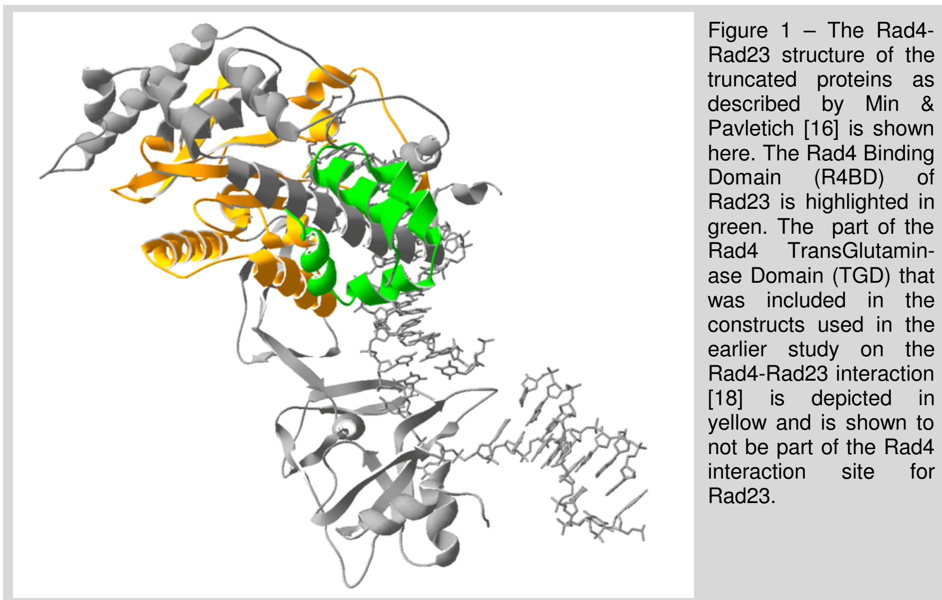

Figure 1 – The Rad4-Rad23 structure of the truncated proteins as described by Min & Pavletich [16] is shown here. The Rad4 Binding Domain (R4BD) of Rad23 is highlighted in green. The part of the Rad4 TransGlutaminase Domain (TGD) that was included in the constructs used in the earlier study on the Rad4-Rad23 interaction [18] is depicted in yellow and is shown to not be part of the Rad4 interaction site for Rad23.

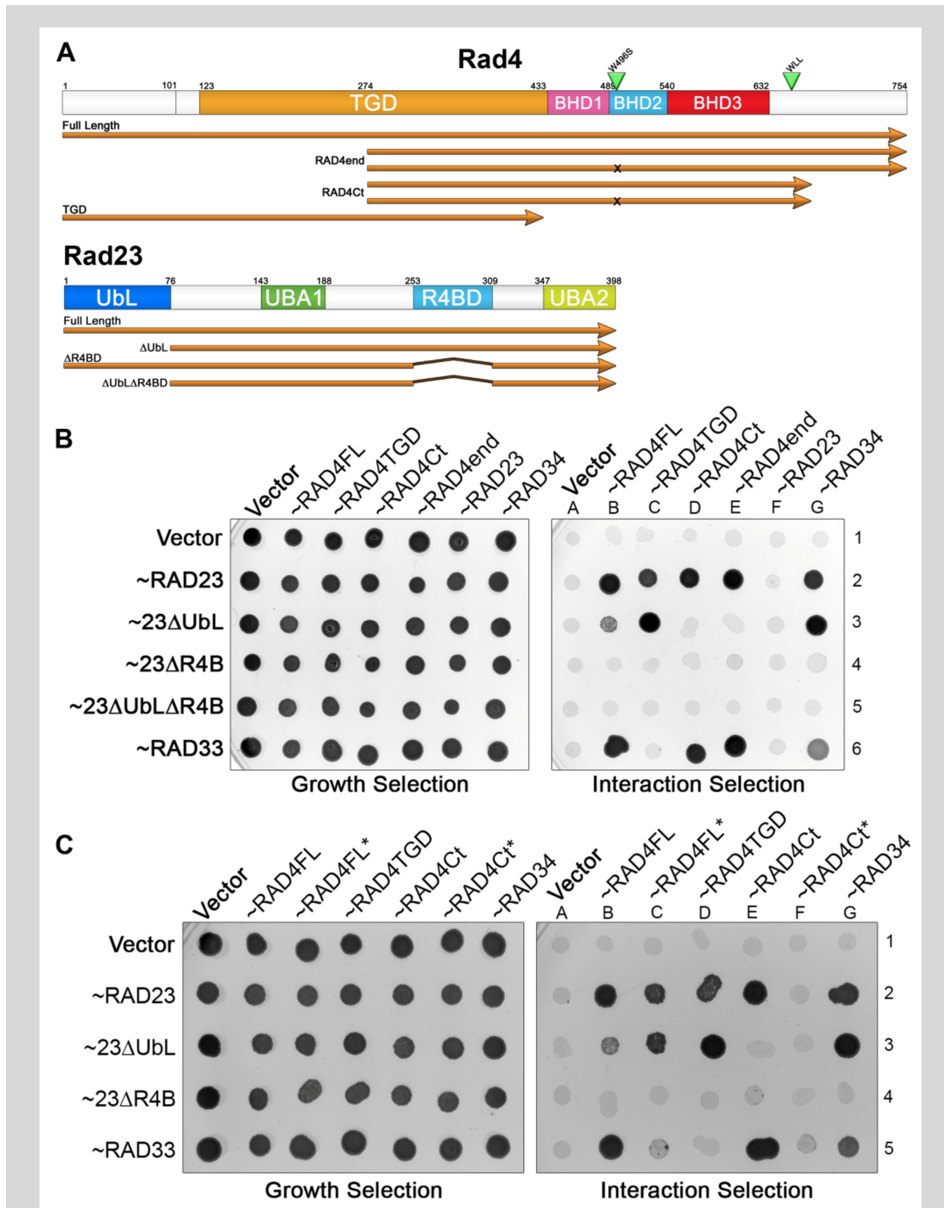

Figure 2 – *Rad23 interacts with both N- and C-terminal constructs of Rad4.* (A) An overview of the Rad4 constructs used in this study. The known domains in Rad4 and Rad23 adopted from Min & Pavletich [16]. TGD – TransGlutaminase-like Domain. BHD – Beta Hairpin Domain. UbL – Ubiquitin Like domain. UBA – Ubiquitin Associating domain. R4BD – Rad4 Binding Domain RAD4FL – RAD4 Full Length. RAD4TGD – Rad4 amino acids 0-433. RAD4Ct – Rad4 amino acids. 274-667. W496S – the location of the mutation corrpesonding to the XPC-W690S patient mutation. (B) Shown here is the result of the Yeast Two-hybrid analysis of full-length and truncated Rad4 and Rad23 constructs. The AD (Activation Domain' fusions of Rad23 on the pGADT7 vector are shown on the left, the Rad4 BD (Binding Domain) fusion-constructs on the pGBKT7 vector are indicated at the top. Growth on the interaction selection plate indicates interaction. (C) As B but now for the point-mutated Rad4 constructs containing W496S Rad4Ct* and Rad4FL*.

4.2 The Rad23 Ubiquitin-like Domain affects the Rad4-Rad23 bipartite interaction

The bipartite Rad4-Rad23 interaction was further confirmed when the Rad23Δ*UbL* construct was included in the analysis. Rad23 without the UbL domain interacts with Rad4FL (B3) and Rad4TGD (C3, figure 2B). However, in the absence of the UbL domain Rad23 can no longer sustain an interaction with the Rad4Ct (D3) and Rad4end (E3) constructs that only contain the C-terminal Rad4 interaction site (Figure 2B). Thus the Rad23 UbL domain is required for Rad23 to interact with the C-terminal end of Rad4 but not for the interaction with the N-terminal region.

Interestingly, the Rad23Δ*UbL*-Rad4TGD interaction (C3) is stronger than the Rad23FL-Rad4TGD interaction (C2, figure 2B). Thus the N-terminal interaction improves when the Rad23 UbL domain is deleted. This differential effect of the UbL domain on the Rad4-Rad23 interactions (stimulation of binding to the C-terminal region and inhibition of binding to the TGD) shows that Rad4 indeed contains two distinct binding sites for Rad23. The Rad23FL interaction with the full-length Rad4 construct (B2) also weakens when the UbL is absent (Figure 2B), suggesting that the C-terminal region is the preferred interaction site *in vivo*.

The R4BD is required for both interactions with Rad4, but in view of the small size of this domain and its position in the co-crystal structure, it is unlikely that Rad23 can simultaneously bind to both domains in Rad4. It is possible that the UbL domain, through the intramolecular interactions with a UBA domain, changes the conformation of Rad23 required to differentially interact with either the N- or C-terminal binding region of Rad4. In the absence of the UbL domain Rad23 might exist as an 'open' conformation (no UbL-UBA interaction) that can only interact with the TGD of Rad4. At the same time this would imply that a 'closed' form of Rad23 due to intramolecular UbL-UBA interaction, preferentially interacts with the C-terminal region of Rad4. It has been described for the hHR23B protein that the interaction between UbL and UBA domains can be disrupted by binding to a proteasomal subunit. Similarly, in Rad23 the UbL and UBA interaction might be disrupted by yeast NER factors, thereby switching Rad23 from one Rad4 binding mode to the other.

4.3 The Rad34-Rad23 interaction behaves similar to the N-terminal Rad4-Rad23 interaction

The Rad34 protein is a Rad4-like NER factor uniquely present in yeast and specifically required for TC-NER of active rDNA. The homology between Rad4 and Rad34 is supported by the sequence conservation and more importantly, by the analogous interaction of Rad4 and Rad34 with both Rad23 and Rad33. To test if this homology includes the novel bipartite interaction we studied the interaction of Rad34 with the same Rad23 deletion constructs (Figure 2B, column G). We find that the Rad23Δ*UbL*-Rad34 interaction (G3) is stronger than the Rad23FL-Rad34 interaction (G2, figure 2B). Thus

the absence of the UbL domain strengthens the Rad23-Rad34 interaction. This pattern is analogous to that of the N-terminal Rad23-Rad4TGD interaction. We therefore speculate that Rad23 might only interact with the TGD-like domain of Rad34 and possibly lacks a C-terminal interaction. The amino acid sequences of Rad4 and Rad34 reveal that Rad4 contains a longer C-terminal region compared to Rad34 [4], perhaps explaining why Rad34 cannot support a C-terminal Rad23 interaction.

Rad34 is specifically involved in TC-NER at the rDNA locus [4, 5]. This could imply that if Rad23 interacts via the Rad34 TGD-like domain that this interaction is sufficient to drive TC-NER at the rDNA. Vice versa, the Rad23 interaction at the C-terminus of Rad4 might be GG-NER specific and could not exist in the Rad34 molecule.

4.4 The C-terminal Rad4-Rad23 interaction is disrupted by a RAD4 mutation analogous to an XPC patient mutation

Interestingly, in the C-terminal region of XPC a patient mutation has been found that still leads to a full-length XPC protein [21]. The tryptophan residue at position 690 in XPC is affected by this mutation and this residue is conserved in Rad4. The XPC-W690S protein has reduced affinity for damaged DNA and results in reduced repair capacity and UV survival in human cells [21-23]. The analogous mutation *rad4W496S* in yeast results in a NER defect, where GG-NER is more severely hampered than TC-NER (Chapter 4). The effect of this mutation on the Rad4 and XPC protein function in NER and the location in the C-terminus of this residue make it an interesting target for study in yeast. Therefore, we measure the effect of the *rad4W496S* mutation in *RAD4* on the Rad4-Rad23 interaction using yeast two-hybrid experiments (Figure 2C).

Interestingly, the W496S mutation completely disrupts the interaction of Rad23 with the Rad4 C-terminal construct (compare E2 to F2, figure 2C) as no interaction between Rad23FL and the mutated Rad4 C-terminal construct (Rad4Ct*) is observed. However, full length Rad4 protein containing the W496S substitution (Rad4FL* in figure 2C) does still interact with Rad23 (B2 and C2, figure 2C). This interaction is reduced compared to the wild-type Rad4 construct but similar to the Rad4TGD construct. Morever, in the absence of the UbL domain the interaction between mutant Rad4 and Rad23 is stronger (C2 and C3). This indicates that in *rad4W496S*, Rad23 can only bind to the TGD.

Extending these observations with the data from chapter 4, it becomes apparent that the GG-NER deficiency as a result of the *rad4W496S* substitution could be linked to the loss of the C-terminal Rad4-Rad23 interaction. In other words, the C-terminal Rad4-Rad23 interaction is more important for GG-NER than TC-NER. We have also shown (Chapter 4) that the *rad4W496S* mutation also affects the activity of Rad4 in the absence of Rad23. In a *rad23Δ* background the *rad4W496S* mutant is more UV sensitive than wild-type *RAD4*. Most likely, the mutation influences the conformation of the Rad4 protein such that on one hand it reduces the affinity of Rad4 for DNA and on the other hand it disrupts the C-terminal interaction with Rad23.

5. Concluding Remarks

In summary, we report that the R4BD of Rad23 can interact with two distinct binding domains on Rad4, one in the TGD and one in the C-terminal part of Rad4. We propose that the switch between the two types of binding is regulated by the UbL domain of Rad23. A mutation In Rad4 that disrupts interaction of Rad23 with the C-terminal domain is mainly affected in GG-NER. On the other hand, the TC-NER specific Rad34 protein seem to mainly interact with Rad23 via a TGD-like domain. Taken together these data suggest that the UbL mediated switching between the different Rad23 binding modes direct the Rad4-Rad23 complex to either GG-NER or TC-NER.

Table 1 - Overview of the strains used in this studied with their relevant genotypes and origin.

Strain	Genotype	Source
W1588-4a	*MATα leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1*	R. Rothstein [19]
MGSC691	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3 : : $MEL1_{UAS}$-$MEL1_{TATA}$-lacZ*	AH109 [24]
MGSC692	*MATa, ura3-52, his3-200, ade2-101, trp1-901 leu2-3, 112, gal4del, met-, gal80del, URA3 : : $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ*	Y187 [24]

6. References

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VI WCG4A, a commonly used *Saccharomyces cerevisiae* yeast strain, contains a *RAD4* mutation affecting UV resistance

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1. Abstract

WCG4A is one of the *Saccharomyces cerevisiae* strains commonly used in DNA repair and transcription studies. Especially when proteasome mutants are required this strain is frequently applied since conditional proteasome mutants are available in this background. Investigating the relation between Nucleotide Excision Repair (NER) and the proteasome we found unexpected, extreme UV sensitivity in different NER deficient derivatives of this strain when compared to the sensitivity in another yeast background, W1588. We identified a mutation in *RAD4* resulting in a C to Y substitution at position 571 of the Rad4 protein in WCG4A. Introducing the mutation responsible for this phenotype into a W1588 wild-type background leads to a comparable phenotype and similar genetic interactions as found for the WCG4A strain.

2. Introduction

Nucleotide Excision Repair (NER) is a general DNA repair process which is conserved in many organisms. The yeast *Saccharomyces cerevisiae* is widely used as a model organism. A recent finding implicates the proteasome in yeast NER in which Rad23 has a pivotal role [1, 2]. In this respect the yeast wild-type strain WCG4A is important because conditional mutants of the 19S and 20S subunits of the proteasome are readily available in this background [3, 4].

We recently identified a novel protein in yeast, Rad33, which is part of the DNA damage recognition complex Rad4-Rad23 and we showed its interaction with Rad4 via a conserved motif [5, 6]. To study the function of Rad33 in NER in relation to the proteasome, we introduced a *RAD33* deletion into the WCG4A strain and studied UV survival. We observed an unusually high UV sensitivity in a WCG4A cells containing a *RAD33* deletion. Furthermore, mutations in other well described *RAD* mutants in WCG4A also resulted in higher than expected UV sensitivity. It appeared that a point mutation in the *RAD4* gene of the WCG4A wild-type strain is the culprit.

3. Materials and Methods

3.1 Survival Curve & UV droptest

Yeast cells were grown to stationary cultures by 3 day incubations at 30°C and diluted in water to an appropriate OD_{600}. Cells were plated on YPD and UV irradiated with the relevant UV dose using a 254nm UV-C lamp (Philips). After a 3 day incubation in the dark at 30°C, colonies were counted and quantified. Data shown are the mean result of 3 individual experiments.

Cells for UV droptests were prepared in a similar fashion, however instead of plating the cells the cell suspension in water (1.5 µL cell suspension in 0.5 mL MilliQ water) was used to dispense 1.5 µL drops on a YPD plate. The YPD plates were irradiated with increasing UV dose relevant to the strains being tested. The UV dose used is indicated in the figures. After UV irradiation YPD plates were incubated in the dark at 30°C for 2-3 days and growth was monitored regularly. Representative scans of these plates are included in the figures described here.

3.2 Mating

The mating of W1588 with WCG4A strains was performed as follows: the two strains are plated together on a YPD plate and incubated at 30°C for 4 hours. Due to the absence of selectable markers zygotes are then selected based on morphology and separated by using a micromanipulator. After two days of growth the size of the colonies and cells can be used to judge the presence of diploid cells. Of each mating 12 zygotes were picked of which 6 were subjected to UV phenotyping.

4. Results & Discussion

The proteasome was shown to regulate the NER reaction by interaction with Rad23 [1, 2]. Rad23 interacts with Rad4 in the damage recognition complex together with Rad33 [5]. In an effort to study Rad33 in relation to the interaction of NER with the proteasome, we introduced a *RAD33* deletion in the WCG4A yeast background. Unexpectedly, a *rad33∆* deletion in the WCG4A strain is as sensitive as a complete NER deficient *rad4∆* strain (Figure 1B - bottom panel) while a *RAD33* deletion leads to only moderate UV sensitivity in a W1588 background (Figure 1B, bottom panel [5, 6]). The UV sensitive phenotype in WCG4A*rad33∆* can be restored by introducing a *RAD33* containing plasmid (Figure 1B - top panel). Furthermore, the results depicted in figure 1B lower panel indicate that a *RAD23* deletion also leads to an unexpectedly high increase in UV sensitivity of a WCG4A background. The WCG4A wild-type strain is as UV resistant as a wild-type W1588 strain (Figure 1A). These data indicate that another repair related mutated allele in WCG4A must exist that only becomes apparent if NER is crippled.

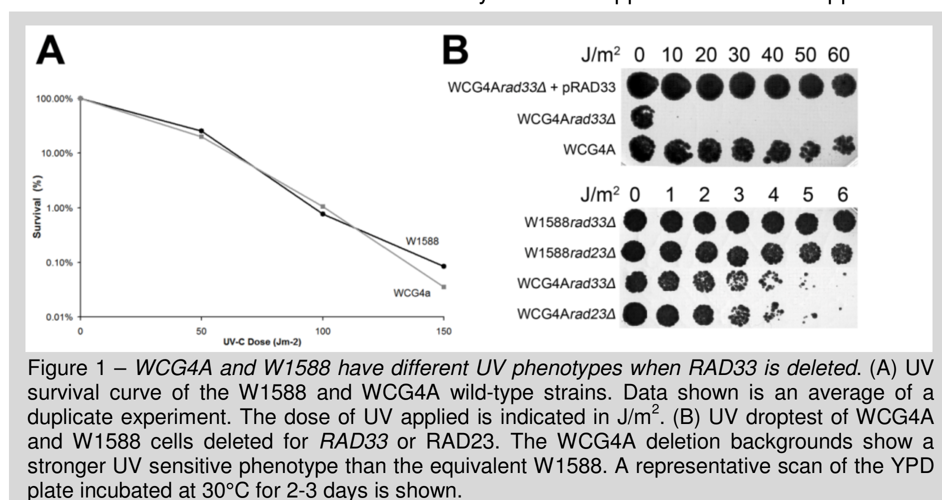


Figure 1 – *WCG4A and W1588 have different UV phenotypes when RAD33 is deleted.* (A) UV survival curve of the W1588 and WCG4A wild-type strains. Data shown is an average of a duplicate experiment. The dose of UV applied is indicated in J/m^2. (B) UV droptest of WCG4A and W1588 cells deleted for *RAD33* or RAD23. The WCG4A deletion backgrounds show a stronger UV sensitive phenotype than the equivalent W1588. A representative scan of the YPD plate incubated at 30°C for 2-3 days is shown.

To identify the gene containing a mutation in the WCG4A strain, two genomic plasmid libraries containing *S.cerevisiae* genes were used to find the complementing gene. However, no complementation was found from either library (data not shown). This raises the possibility that the libraries do not contain the gene of interest, perhaps due to lethality of the gene in *E.coli*.

An efficient method for checking if one of the known NER genes is mutated in the WCG4A strain is mating analysis. We make use of the finding that a WCG4A*rad33Δ* strain is very UV sensitive while W1588*rad33Δ* is only moderately affected in UV survival (figure 1B, bottom panel and figure 2A). Mating of the UV sensitive haploid WCG4A*rad33Δ* stain with a wild-type haploid W1588 allele leads to more UV resistant diploid cells (Figure 2B). To identify the affected NER gene in WCG4A, we mated the haploid WCG4*rad33Δ* with different W1588*rad33Δ* NER deficient backgrounds.

In figure 2A the UV survival of all tested haploid *rad* mutants is depicted. These mating studies indicate that both *RAD14* and *RAD23* alleles in WCG4A do not cause the extreme UV sensitivity in concert with the *RAD33* deletion (Figure 2B & C). However, the extremely UV sensitive haploid W1588*rad33Δrad4Δ* strain fails to rescue the UV phenotype of the WCG4A*rad33Δ* cells; the diploid strain is as sensitive as the haploid cells (Figure 2D). We conclude that the *RAD4* allele in WCG4A carries the mutation that causes extreme UV sensitivity in WCG4A *RAD33* and *RAD23* deletion strains. In hindsight this explains why both genome libraries did not contain the complementing genes as *RAD4* is lethal to *E.coli.*

Indeed, by sequencing we found the mutation to reside in the WCG4A *RAD4* allele at position 1712; G1712A, TGT→TAT, resulting in a C571Y substitution. This mutation is novel and the affected amino acid is conserved. As a control the C571Y mutation was transferred to the W1588 background. In addition a *rad33Δrad4C571Y* double mutant was created. These strains show the same phenotype for UV survival as the comparable

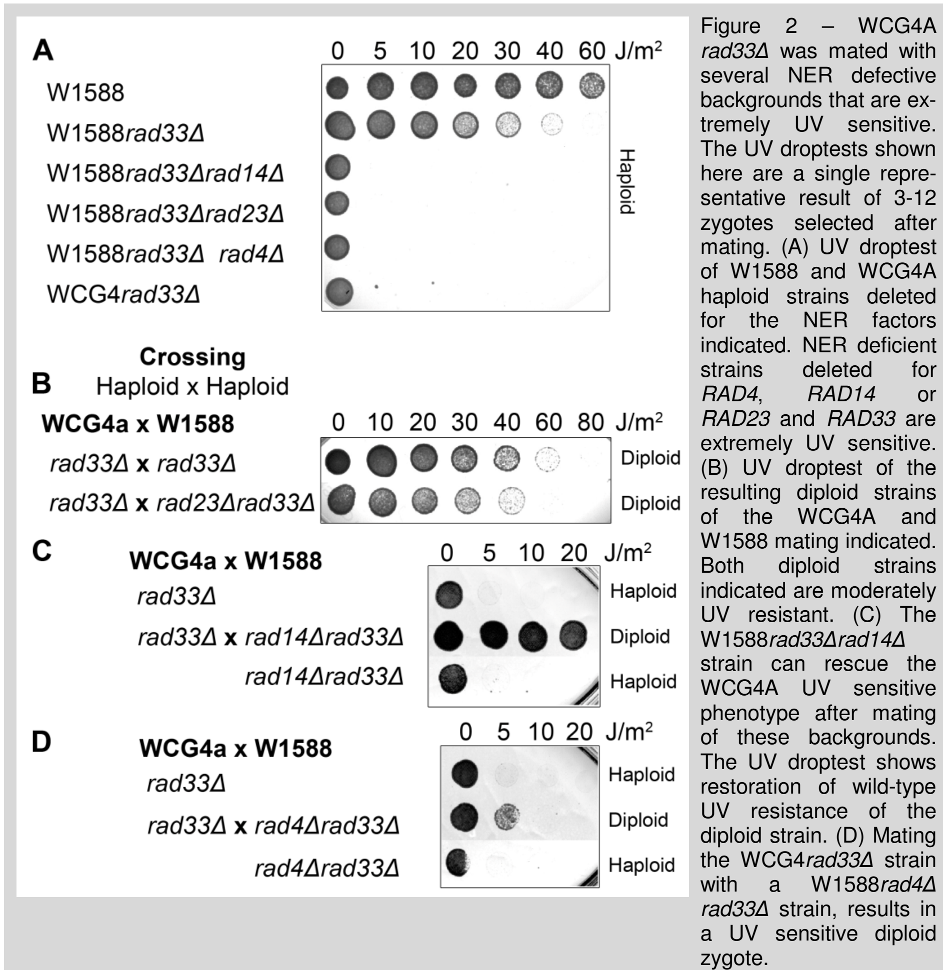
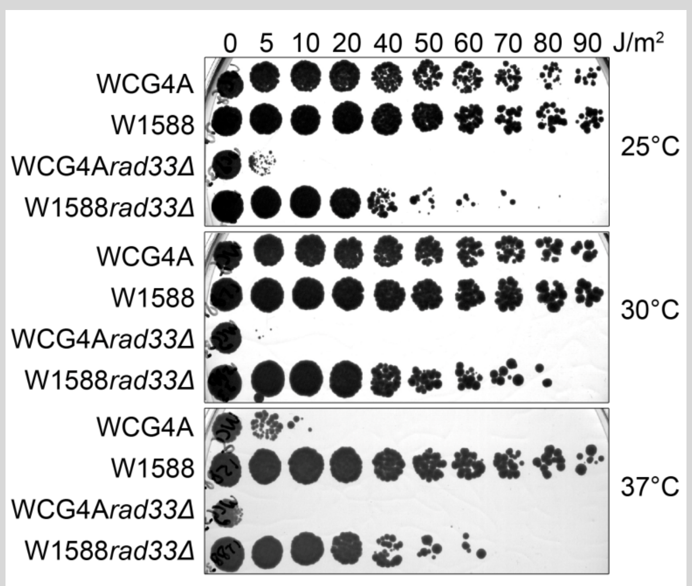


Figure 2 – WCG4A *rad33Δ* was mated with several NER defective backgrounds that are extremely UV sensitive. The UV droptests shown here are a single representative result of 3-12 zygotes selected after mating. (A) UV droptest of W1588 and WCG4A haploid strains deleted for the NER factors indicated. NER deficient strains deleted for *RAD4*, *RAD14* or *RAD23* and *RAD33* are extremely UV sensitive. (B) UV droptest of the resulting diploid strains of the WCG4A and W1588 mating indicated. Both diploid strains indicated are moderately UV resistant. (C) The W1588*rad33Δrad14Δ* strain can rescue the WCG4A UV sensitive phenotype after mating of these backgrounds. The UV droptest shows restoration of wild-type UV resistance of the diploid strain. (D) Mating the WCG4*rad33Δ* strain with a W1588*rad4Δ rad33Δ* strain, results in a UV sensitive diploid zygote.

Figure 3 – The WCG4A strain is more UV sensitive at elevated temperatures. The WCG4A and W1588 strains were subjected to UV treatment and incubated at different temperatures. The top panel shows the results of incubation at 25°C, the middle panel 30°C and the bottom panel 37°C incubation. Shown here is the scan of the YPD plates representative of at least three independent repeats.



WCG4A strains (data not shown). To confirm that the C571Y substitution is indeed present in the WCG4A background used in literature, we requested the strain from the original source [7]. Indeed, also this strain contains the same mutation.

Deletion of *RAD23* and *RAD33* lead to moderate UV sensitivity as single mutants but a double mutant *rad23Δrad33Δ* is extremely UV sensitive. Both proteins have been shown to stabilize Rad4 and are responsible for normal levels of Rad4 [5, 6]. The UV sensitivity caused by the C571Y mutation might be due to an unstable Rad4 protein, leading to a completely defective Rad4 protein in a *rad23Δ* or *rad33Δ* background. The results presented in figure 3 support this assumption since here we show that Rad4C571Y cells are more sensitive to UV at elevated temperatures. The WCG4A strain fails to recover from 5-10 J/m^2 at 37°C whereas the W1588 strain displays UV resistance at 37°C with

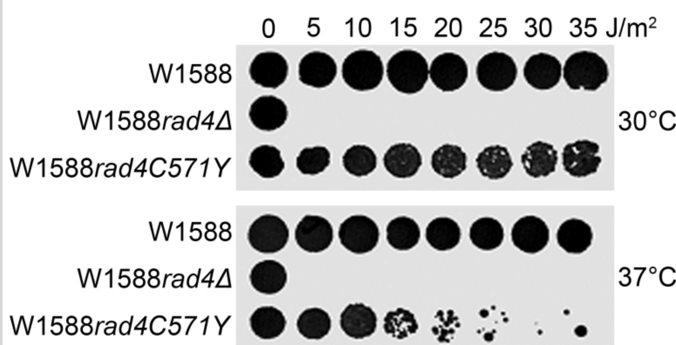


Figure 4 – The *rad4 C571Y* allele causes UV sensitivity in the W1588 background at elevated temperatures. Shown here is a representative scan of the YPD plates used to study the UV sensitivity at elevated temperatures of W1588 *rad4C571Y*. The top panel shows the result of incubation the strains at 30°C and the lower panel shows the same strains incubated at 37°C.

growth up until 90 J/m^2 (Figure 3). As anticipated W1588 cells become sensitive at elevated temperature after the C571Y substitution is introduced into the Rad4 protein, similar to the WCG4A background (Figure 4).

The additive decrease in UV resistance at elevated temperatures or after deleting the direct interaction partners of Rad4C571Y, indicates that the protein is unstable in WCG4A. Indeed, when we measure protein levels using Westernblot analysis we find reduced levels of Rad4 protein in the W1588*rad4C571Y* background (data not shown). Residue 571 is located in β-hairpin domain 3 (BHD3) at the base of one of the strands of the three-stranded β-sheet [8]. The change from a cysteine to a tyrosine could lead to destabilization of the BHD3 domain which in turn might cause the instability of the protein. Apparently, the interactions with Rad23 and Rad33 stabilize this domain.

Table 1 – Overview of the strains used in this study with their relevant genotype and source

Strain	**Genotype**	**Source**
MGSC363 W1588-4a	MATα *leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 LYS2 MET14 RAD5*	Mortensen
MGSC719 WCG4A	MATa his3-11,15 leu2-3,112 ura3-1	Thomas Kodadek
MGSC609	W1588 *rad4Δ::HisG*	This lab
MGSC656	W1588 *rad4::HisG rad33Δ::KANMX*	This lab
MGSC660	W1588 *rad23::HisG rad33Δ::KANMX*	This lab
MGSC662	W1588 *rad33Δ::KANMX*	This lab
MGSC665	W1588 *rad33Δ::KANMX rad14Δ::LEU2*	This lab
MGSC931	WCG4A *rad23Δ::HisGURAHisG*	This study
MGSC932	WCG4A *rad33Δ::KANMX*	This study
MGSC942	W1588 *rad4C571Y*	This study

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VII Summary & Conclusions

Nucleotide Excision Repair (NER) is a conserved DNA repair pathway capable of removing a broad spectrum of DNA damage. In human cells a defect in NER leads to the disorder Xeroderma pigmentosum, increasing the incidence of cancer. The yeast *Saccharomyces cerevisiae* is an excellent model organism to study the mechanism of NER. Two sub-pathways have been described, TC-NER for transcriptionally active genes and GG-NER which removes lesion throughout the entire genome. The yeast proteins Rad4 and Rad23 are important in NER and involved both sub-pathways.
Chapter 2 describes a novel mechanism of gene regulation by the GG-NER E3 ligase. This protein complex can regulate dNTP synthesis via UV induced Rad4-ubiquitination. The Rad4-Rad23 complex interacts with DNA at the promoter region of genes in the absence of DNA damage. The GG-NER E3 ligase regulated dissociation of the Rad4-Rad23 complex facilitates a change in gene expression in response to UV radiation. Amongst genes that are targeted by this novel NER related gene expression mechanism are the genes that regulate dNTP synthesis. The control of dNTP synthesis in response to DNA damage or during S phase is described in more detail in section 2.1. The dNTP synthesis factors are a downstream target of the DNA Damage Response (DDR). The activity of the GG-NER E3 ligase and Rad4-Rad23 DNA binding that drive this pathway are novel and runs independent to the established DDR signaling and DNA damage induced gene expression controlled by Rad6-Rad18. This latter DDR pathway is triggered upon detection and processing of the actually damage. The advantage of this novel Rad4-Rad23 mechanism is that it can adapt gene expression much earlier in response to damage induction. The GG-NER E3 ligase mediated dissociation of Rad4-Rad23 from the promoter regions already alters gene expression before the NER complex associates with UV lesions. In this way the cell can prepare for DDR signaling and UV induced gene expression. In an attempt to expand the data described in Chapter 2, studying the genome-wide association of the Rad4-Rad23 complex using ChIP-on-Chip analysis could be instrumental in deciphering the mechanism behind the association-dissociation process of Rad4-Rad23 in response to DNA damage.
Yeast TC-NER is unique in requiring a specific Rad4-like protein for rDNA repair. Histone H1 is a linker histone specifically associated with the rDNA. In Chapter 3 we describe the effect of this histone on TC-NER at the rDNA. We find that Rad34 is only required when Histone H1 is present. In the absence of both Histone H1 and Rad34 repair of the TS of rDNA becomes dependent on Rad4. We propose a model in which Histone H1 association with RNA Pol I stalled at a lesion holds the complex in place and requires Rad34 for displacement and subsequent TC-NER. This would be similar to the function of Rad26 that is generally required for TC-NER of RNA Pol II transcribed genes. In the absence of Hho1, RNA Pol I is more loosely attached to the DNA when it is stalled at a damage and might thus back-track or dissociate from the lesion by itself. In this process Rad4 can then replace Rad34 and participate in TC-NER.

In Chapter 4 we analyze the phenotype of a yeast strain containing a *RAD4* mutation. The mutation affects a conserved residue that when altered in the human homolog XPC, leads to the cancer prone disorder Xeroderma pigmentosum. The residue is important for the association of XPC with DNA and mutation of this residue leads to UV sensitivity in human cells. Here we determined that this residue is important for *in vivo* repair in yeast as well but does not result in UV sensitivity by itself. The two sub-pathways in NER are both affected, but GG-NER is most severely inhibited and displays a 4-fold reduction in repair in the presence of the mutant *rad4W496S* allele in yeast. We hypothesize that participation of the mutation protein in TC-NER is facilitated by the action of TC-NER specific factors that remodel the RNA Pol II complex at the lesion. In such a way it allows the association of a Rad4 protein that poorly binds to the DNA. Maybe for the same reason the equivalent activity of TC-NER factors in higher eukaryotes completely bypasses the need for XPC in TC-NER. However, the lack of such a remodeling activity during GG-NER in yeast *rad4W496S* cells results in a strong defect in this pathway. The defective GG-NER apparently leads to a very delayed repair that is not detectable in our assays but does contribute to cellular survival as *rad4W496S* mutant cells are UV resistant.

Chapter 5 is dedicated to the Rad4-Rad23 interaction that we describe in more detail. We uncovered a novel N-terminal interaction of Rad23 with Rad4 that is important for TC-NER. We could confirm the established Rad23 C-terminal interaction with Rad4 and were able to show that it is specifically required for GG-NER. Interestingly, the *rad4W496S* mutation, analogous to the mutation in an XP patient described in Chapter 4, is specifically affected in GG-NER and was shown to disrupt the C-terminal Rad4-Rad23 interaction. These data highlight the intricate interaction between Rad4 and Rad23 that can be differentially regulated in response to the specific sub-pathway in which it operates. We hypothesize that the sub-pathways specifically mold Rad4-Rad23 into a different conformation to meet specific requirements needed to drive repair. Moreover, we attribute an active *in vivo* role to the intramolecular UbL-UBA interaction in the differential Rad4-Rad23 interactions.

Finally, in Chapter 6 we reveal an unexpected UV phenotype for the commonly used wild-type yeast strain WCG4A. In this strain we found elevated levels of UV sensitivity in combination with NER gene deletions which on their own lead to only moderate UV sensitivity. Using mating experiments we identified the mutation to reside in the *RAD4* gene. When we transferred the single nucleotide mutation (leading to the C571Y substitution) to another yeast strain background we obtained exactly the same phenotypes. The affected residue is located in the BHD3 domain of Rad4 and results in destabilization of the protein as could be shown by the UV sensitivity at higher temperature. The WCG4A yeast background is commonly used in proteasome related studies as conditional 19S and 20S mutants are readily available. Based on these data

we would like to stress that this WCG4A strain should not be used in DNA repair related studies.

Linking back to the data described in Chapter 4, the W496S mutation in Rad4 behaves fairly similar to the C571Y substitution identified in WCG4A. In both cases the single amino acid substitution in Rad4 does not affect UV survival on its own but does show an effect if NER is crippled by deletion of NER accessory genes. The Rad4-interacting proteins Rad23 and Rad33 both play an important role in maintaining normal Rad4 protein levels. In the absence of these proteins wild-type Rad4 levels are reduced, in part leading to moderate UV sensitivity. This reduction in Rad4 protein levels, however, only has a severe effect on UV survival if also *RAD4* is mutated. The different *RAD4* mutants described in this thesis show that due to the stabilizing effect of Rad23 and Rad33, the Rad4 protein can cope with different mutations without a significant effect on UV survival.

VIII Samenvatting & Conclusies

Nucleotide excisie herstel (NER) is een geconserveerd DNA schadeherstel systeem dat een breed scala aan DNA schades repareert. Een defect in NER in humane cellen leidt tot de ziekte Xeroderma pigmentosum. Deze ziekte wordt gekarakteriseerd door een verhoogd risico op kanker. De gist *Saccharomyces cerevisiae* is een buitengewoon geschikt model organisme om het mechansime van NER te bestuderen. NER kan onderverdeeld worden in twee routes: TC-NER (Transcription-Coupled NER) voor actief getranscribeerde genen en GG-NER (Global Genome NER) dat verantwoordelijk is voor schadeherstel in het hele genoom. De Rad4 en Rad23 eiwitten in gist spelen een belangrijke rol in NER en zijn betrokken bij beide routes.
Hoofdstuk 2 beschrijft een nieuwe vorm van genregulatie door het GG-NER specifieke E3 ligase. Dit eiwitcomplex kan de synthese van dNTP's aansturen via ubiquitinering van Rad4. Het Rad4-Rad23 complex bindt aan het DNA in het promotergebied van sommige genen in de afwezigheid van DNA schade. Het GG-NER E3 ligase zorgt voor dissociatie van dit Rad4-Rad23 complex na UV straling met tot gevolg dat de expressie van sommige genen verandert. Onder de genen die door dit nieuwe NER gerelateerde mechanisme worden beinvloed bevinden zich de genen die zorgen voor de aanmaak van dNTP's. Factoren betrokken bij de dNTP synthese zijn een doelwit van de zogenaamde DNA Damage Respone (DDR). De door ons gevonden activiteit van het GG-NER E3 ligase en Rad4-Rad23 in DDR opereert geheel onafhankelijk van de reeds eerder gevonden DDR (en DNA schade geinduceerde genexpressie) die wordt gecontroleerd door Rad6-Rad18. Voor het aansturen van de DDR door Rad6-Rad18 moet de schade al herkend zijn door schade herstellende eiwitten. Het voordeel van het nieuwe Rad4-Rad23 mechanisme is dat genexpressie veel sneller kan worden aangepast na DNA schade inductie. Het GG-NER E3 ligase zorgt namelijk voor dissociatie van het Rad4-Rad23 complex van de promoter gebieden nog voordat het NER complex de schade gevonden heeft. Op deze manier kan de cel zich tijdig voorbereiden op DDR en UV geinduceerde genexpressie.
Gist TC-NER is uniek omdat het gebruik maakt van een specifiek Rad4 gerelateerd eiwit, Rad34, voor herstel van genen coderend voor rRNA (rDNA). Histon H1 is een linker histon dat specifiek bindt aan rDNA. In hoofdstuk 3 hebben we het effect van dit histon op TC-NER in rDNA beschreven. We hebben laten zien dat Rad34 alleen nodig is wanneer Histon H1 aanwezig is. In afwezigheid van Histon H1 kan Rad4 de rol van Rad34 overnemen bij herstel van de getranscribeerde streng. We postuleren een model waarin Histon H1 ervoor zorgt dat RNA Pol I op het DNA bij de schade blokkeert. Het gevolg is dat Rad34 specifiek nodig is om het RNA polymerase van de schade af te krijgen om zo TC-NER te laten plaats vinden. Deze functie van Rad34 lijkt heel veel op de functie van Rad26 tijdens TC-NER van RNA Pol II getranscribeerde genen. Ook daar is Rad26 nodig om RNA Pol II die vastgelopen is op een schade te verwijderen. In de afwezigheid van Histon H1 is RNA Pol I waarschijnlijk minder stabiel gebonden aan een

schade en kan dus makkelijker uit zichzelf loslaten. Rad4 kan vervolgens direct binden en deelnemen aan TC-NER.

In hoofdstuk 4 analyseren we het fenotype van een gist stam die een mutatie (W496S) heeft in het *RAD4* gen. Dezelfde mutatie in het op Rad4 lijkende humane gen, XPC, leidt tot de ziekte Xeroderma pigmentosum. Het gemuteerde residu is belangrijk voor de DNA binding van XPC en de mutatie leidt tot UV gevoeligheid in humane cellen. We hebben laten zien dat dit residu ook belangrijk is voor herstel *in vivo* in gist, maar de mutatie op zich leidt niet tot UV gevoeligheid. Beide NER routes zijn aangetast, maar het effect op GG-NER blijkt veel ernstiger dan op TC-NER. Mogelijk helpen eiwitten, die specifiek betrokken zijn bij TC-NER, het gemuteerde Rad4 eiwit om toch aan de schade te kunnen binden. Bij GG-NER ontbreken zulke factoren en als gevolg bindt Rad4 veel slechter aan de schade. Dit leidt ertoe dat de schade sterk vertraagd hersteld wordt. Door deze vertraagde werking van GG-NER is er in onze experimenten in de eerste uren na schade inductie nog geen herstel waarneembaar. Uiteindelijk echter weet de gistcel toch nog te overleven aangezien we geen verhoogde UV gevoeligheid kunnen meten.

Hoofdstuk 5 is gewijd aan de interactie tussen Rad4 en Rad23. We laten zien dat Rad23 op twee plekken in Rad4 kan binden: aan het N-terminale TGD (TransGlutaminase) domein en aan het C-terminale deel van het eiwit. Omdat Rad23 voor beide interacties hetzelfde (kleine) eiwitdomein gebruikt, is het niet mogelijk dat Rad23 beide contacten tegelijk maakt. Mogelijk wordt de keuze tussen beide manieren van binden bepaald door een intramoleculaire interactie tussen de UbL en UBA domeinen van Rad23. Wanneer deze interactie plaatsvindt bindt Rad23 aan het C-terminale deel van Rad4. Vindt deze interactie niet plaats dan wordt het TGD domein gebonden. Tenslotte hebben we laten zien dat beide vormen van het Rad4-Rad23 complex een verschillende functie hebben. Wanneer de eiwitten via de TGD met elkaar zijn gebonden zijn ze voornamelijk betrokken bij TC-NER. In de andere vorm lijken ze betrokken bij GG-NER. Het is interessant om te vermelden dat de hierboven beschreven *rad4W496S* mutatie gelegen is in het C-terminale deel van Rad4 en geheel gestoord is in GG-NER. Inderdaad blijkt deze mutant ook niet meer via zijn C-terminale domein aan Rad23 te kunnen binden.

In hoofdstuk 6 wordt het onverwachte UV fenotype besproken van de veelgebruikte giststam, WCG4A. Deze stam laat een sterk verhoogde UV gevoeligheid zien wanneer NER mutaties, die van zichzelf slechts een matige UV gevoeligheid veroorzaken, in deze stam worden ingebracht. Door middel van het kruisen van giststammen hebben we het gemuteerde gen kunnen identificeren als *RAD4*. Als we vervolgens de gevonden punt mutatie (C571Y) overplaatsen in een andere giststam, krijgen we exact dezelfde fenotypes. Het veranderde aminozuur bevindt zich in het BHD3 domein van Rad4 en leidt tot destabilisatie van het eiwit, wat we konden afleiden uit de UV gevoeligheid bij hogere temperatuur. De WCG4A gist stam wordt veel gebruikt in proteasome gerelateerde studies omdat er conditionele 19S en 20S mutanten beschikbaar zijn in

deze achtergrond. Op basis van deze bevindingen willen wij er met klem op wijzen dat deze achtergrond niet geschikt is voor DNA schadeherstel gerelateerde studies.
Terugkomend op de data beschreven in hoofdstuk 4, is het opmerkelijk dat de W496S mutatie zeer veel overeenkomsten laat zien met de C571Y substitutie. In beide gevallen leidt de enkele aminozuur substitutie in Rad4 niet tot verandering in UV overleving, maar vertoont alleen een effect als NER wordt verzwakt door deletie van andere NER genen. De Rad4 bindende eiwitten Rad23 en Rad33 spelen beiden een belangrijke rol in het stabiliseren van het Rad4 eiwit in de cel. In de afwezigheid van deze eiwitten is het Rad4 niveau verlaagd, wat deels bijdraagt aan de UV gevoeligheid. Deze afname in Rad4 eiwit heeft echter alleen een drastisch effect als het Rad4 eiwit zelf ook is gemuteerd. Dankzij de stabliserende werking van Rad23 en Rad33 kan Rad4 veel mutaties verdragen zonder dat dat effect heeft op de UV overleving.

IX Curriculum vitae

EDUCATION

9/2002 – 9/2007	Life Science & Technology, University Leiden & TU Delft Master: Functional Genomics Bachelor: Life Science & Technology
9/1996 – 6/2002	VWO (A-levels), Nova College Montfort, Rotterdam Profile: Nature & Techniek including Biology European Computer Drivers License (ECDL)

WORK & INTERNSHIPS

9-2007 – 2012	PhD Student at the department of Molecular Genetics at the Leiden Institute of Chemistry, University of Leiden on Nucleotide Excision Repair, in yeast. Supervisor: Dr. J.A. Brandsma.
1/2012 – 8/2012	Visiting Scholar at Cardiff University under the auspice of Dr. S.H. Reed. A collaborative project on the research described in Chapter 2 of this thesis was performed.
11/2006 – 7/2007	Master research internship at the department of Molecular Genetics at the Leiden Institute of Chemistry, University of Leiden on Nucleotide Excision Repair, in yeast. Supervisor, Dr. J.A. Brandsma.
8/2006 – 11/2006	Business internship at the Dutch National Museum of Natural History at the department of information services a web theme on 'De Cel' was made. Supervisor: H. Ahrens
4/2005 – 7/2005	Bachelor research internship at the department of Nephrology at the Leiden University Medical Center (LUMC) on 'Complement-mediated nephritis in mice and *in vivo* complement inhibition'. Under supervision of T.W.L. Groeneveld & Dr. A. Roos