Saccharomyces Cerevisiae as a Model Organism to Delineate Initial Lesion Detection Events in Chromatin Repair: A Focus On Ddb2-Mediated GG-NER

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SACCHAROMYCES CEREVISIAE AS A MODEL ORGANISM TO DELINEATE INITIAL LESION DETECTION EVENTS IN CHROMATIN REPAIR: A FOCUS ON DDB2-MEDIATED GG-NER

By

Kristi Lyn Jones

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Doctor of Philosophy

SACCHAROMYCES CEREVISIAE AS A MODEL ORGANISM TO DELINEATE
INITIAL LESION DETECTION EVENTS IN CHROMATIN REPAIR: A FOCUS ON
DDB2-MEDIATED GG-NER

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DNA damage repair is an essential and complex cellular process. Although the basic mechanisms of nucleotide excision repair (NER) have been studied for decades, some mechanistic details remain elusive. The lesion detection step remains one of the most elusive in the process of NER in the contest of chromatin. The work described herein addresses the initial events in the lesion detection step of chromatin repair, also referred to as global genome repair (GG-NER). Both the role of post-translational modifications of lesion identification proteins, and the initial sequence of events in recruitment of repair and remodeling factors are investigated.

First, the controversial role of ubiquitination of DDB2 (a human lesion detection protein) is investigated. Due to documented DDB2 function in alternative physiological processes, its direct role in GG-NER is hard to study in human cells. To overcome this obstacle, we established the budding yeast, *Saccharomyces cerevisiae* as an alternative, simplified model organism to study DDB2-mediated GG-NER. Using this system, we show that inconsistent with the widely accepted model, rapid degradation of DDB2 post-UV irradiation is not an absolute requirement for progression of GG-NER. However,
Interestingly, our data suggest a role for ubiquitination in the release of DDB2 from chromatin. In both UV and mock treated samples, ubiquitin deficient cells had significantly higher amounts of DDB2 remaining bound to the chromatin compared to the isogenic parent cells. The discussion focuses on the possible physiological relevance of these observations.

Additionally, the recruitment of the SWI/SNF chromatin remodeling complex to the silent *HML* (Hidden MAT Left) locus was also investigated. SWI/SNF is known to require recruitment for its role in transcription; therefore we investigate this requirement in GG-NER. Based on previously published data that indicate an UV-stimulated association of SWI/SNF and Rad4 (a lesion detection protein), we hypothesized that Rad4 is involved in recruitment of SWI/SNF to damaged DNA. Interestingly, our data suggest that Rad4 is not an absolute requirement for recruitment of Snf6 to the *HML* locus following UV irradiation. However, Rad16 appears to be. These data present an interesting insight into the lesion detection step in GG-NER and this will be discussed.
Dedication

I would like to dedicate this thesis to both my loving and supportive husband and my wonderfully cheerful and supportive daughter. Without their continued understanding and support I would not have succeeded in this endeavor.
Acknowledgements

I would like to thank my family and friends whose love, support and encouragement has assisted in this process more than they will ever know. I especially want to thank my dear friend Silvia, without whom I would not be here. Also, I would like to thank my “ladies night” friends who helped keep me “normal” through this process.

I would especially like to thank my mentor Dr. Feng Gong for his continued support, patience and encouragement. I also owe much gratitude to Dr. Ling Zhang for her help and discussions. Additionally, I am very grateful to my committee, Drs. Kenneth Rudd, Terace Fletcher, and Antoni Barrientos for going above and beyond to assist me in every aspect of this endeavor and assisting me in becoming a better person.
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1.1 DNA Damage and Cellular Response

Cells are continually exposed to genotoxic stresses. Such stresses include intrinsic replication errors, extrinsic assaults from reactive oxygen species, X-ray exposure and various chemical exposures. Each type of insult results in physical changes to the DNA. These changes include misincorporation of nucleotide(s), double strand breaks, inter-strand cross-linking or double strand helix distortion. Such changes often result in genomic instability. From an organismal standpoint the maintenance of DNA integrity is necessary to prevent deleterious effects. Therefore, the cell has evolved a complex, yet efficient DNA damage response mechanism.

A simplified version of events is as follows. ATM (Ataxia Telangiectasia, Mutated) or ATR (ATM and Rad3-related) is activated upon DNA damage. In turn ATM/ATR then phosphorylates Chk1/Chk2. Following a series of signaling events, cell cycle arrest can occur (Stracker, Usui et al. 2009). This cell cycle arrest induced via the ATM/ATR pathway is thought to provide time for DNA repair pathways to function, thereby preventing the potential propagation of mutations. Cell cycle arrest is not the only outcome of activation of DNA damage response via the ATM/ATR pathway. For example, phosphorylated p53 can either induce cell cycle arrest via p21 or apoptosis via BAX/BAK. How this is regulated is not well understood.
Figure 1.1: Schematic of cell signaling following DNA damage. ATM or ATR activation of various cell cycle regulators via phosphorylation controls cellular response to DNA damage. Taken from http://www.rndsystems.com/mini_review_detail_objectname_MR03_DNADamageResponse.aspx
DNA damage signaling is arguably one of the most important steps to coordinate DNA repair with cell cycle progression and ultimately reduce propagation of mutations. However, these upstream signaling events are not of direct interest to this project. The remainder of our discussion will focus on the mechanisms of DNA repair following the primary damage sensing step. Due to the organismal importance to maintain DNA stability, the cell has evolved several pathways that are responsible for repair of DNA damage. Namely, homologous and non-homologous end joining, base excision repair (BER), mismatch repair (MMR) and nucleotide excision repair (NER). As reviewed by Hoeijmakers (Hoeijmakers 2001), each repair pathway is primarily responsible for repair of a particular type of lesion. For example, recombination (homologous or non-homologous) repairs double strand breaks, BER repairs abasic sites or single strand breaks, MMR repairs replication errors and NER repairs bulky lesion (Figure 1.2). In general, each type of lesion is induced by a unique genotoxic stressor (Figure 1.2). However, there is cross talk between repair pathways, as demonstrated by data indicating that NER, under some conditions, can repair abasic sites which are normally repaired via BER (Kim and Jinks-Robertson 2010). This demonstrated cross talk stresses the importance of DNA damage repair, and suggests there is much more for us to learn about the mechanistic details the pathways involved.
Our lab focuses on the repair of bulky DNA damage that is repaired via the NER pathway. Ultraviolet light (UV) is a well studied genotoxic stress that induces bulky DNA damage. The primary lesions induced by UV irradiation have been characterized, and are described as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). These lesions are depicted in figure 1.3. Both lesions result in the distortion of the DNA double helix. However, there are minor differences between the two types of lesions: (1) 6-4PPs result in a greater helical distortion; (2) CPDs have been consistently shown to have higher incidence than 6-4PPs (Douki and Cadet 2001); (3) CPDs are induced both in nucleosome core and linker DNA, whereas 6-4PPs are formed with 6-
fold greater frequency in linker DNA (Mitchell, Paniker et al. 2007); (4) 6-4PPs are repaired much faster than CPDs, as reviewed by Smerdon (Smerdon 1991).

Nonetheless, these lesions have been shown to be repaired by the NER pathway (Sancar and Reardon 2004). As will be discussed in further detail in subsequent sections, NER lesion recognition is via recognition factor interaction with the structural DNA changes that are induced. Other bulky DNA lesions repaired by NER include those induced by cigarette smoke, cisplatin treatment and a newly identified form of bulky oxidative DNA damage (Zamble, Mu et al. 1996; Setlow 2001; Wang 2008).

Figure 1.3: Schematic of primary DNA damage lesions induced by UV irradiation. (A) cyclobutane pyrimidine dimer (CPD). (B) 6-4 photoproduct (6-4PP)
The NER pathway is conserved from yeast to humans (Table 1.1) (Costa, Chigancas et al. 2003). NER has been extensively studied and the core mechanism is relatively understood. It consists of three main steps: (1) lesion detection, (2) dual incision to remove an oligonucleotide containing the lesion and (3) repair synthesis (Figure 1.4). There are two sub-pathways of NER, termed transcription coupled repair (TC-NER) and global genome repair (GG-NER) (Hanawalt 2003). TC-NER is responsible for repair of damage on the actively transcribed strand; while GG-NER is responsible for repair in the remainder of the genome. GG-NER repairs lesions on the non-transcribed strand of actively transcribed genes, as well as those in repressed or silent chromatin regions. Both TC-NER and GG-NER consist of all three steps, but, they differ in the lesion recognition step. In TC-NER the lesion is thought to be detected by pausing of RNA polymerase I or II (Conconi, Bespalov et al. 2002; Hanawalt 2002; Fousteri and Mullenders 2008). GG-NER, on the other hand, requires a specific lesion recognition heterodimeric protein complex, XPC-hRad23 (Xeroderma Pigmentosum complementation group C- human Rad23) in humans and Rad4-Rad23 (RADiation sensitive) in budding yeast (Guzder, Sung et al. 1998; Jansen, Verhage et al. 1998; Sugasawa 2009; Wood 2010). However, under certain in vivo circumstances as will be discussed later, DDB2 is the pioneering damage recognition factor during GG-NER (Hwang, Ford et al. 1999; Nichols, Itoh et al. 2000; Sugasawa 2009).
<table>
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<tr>
<th>Core Pathway</th>
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<th>S. cerevisiae</th>
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**Table 1.1: Conservation of NER pathway in human and S. cerevisiae.** Modified from Costa (Costa, Chigancas et al. 2003)
Figure 1.4: Schematic of nucleotide excision repair (NER) pathway. There are three primary steps in NER, 1. Lesion identification, 2. Incision, 3. Repair synthesis. Adapted from Auclair (Auclair, Rouget et al. 2009)
Although GG-NER lesion identification proteins have been identified, their mechanism of action remains to be elucidated. As will be discussed, chromatin remodeling is involved in GG-NER lesion recognition via promoting an “open confirmation” of the chromatin. This allows downstream repair proteins to access the lesion. We hypothesize that DDB2 plays a pivotal role in linking chromatin remodeling and subsequent repair, as will be discussed later. Further investigation of these initial steps of GG-NER is necessary to understand the lesion recognition step in intact cells.

Chromatin repair has been discussed in several recent review articles (Gong, Kwon et al. 2005; Nag and Smerdon 2009; Waters, Teng et al. 2009; Zhang, Jones et al. 2009). It remains to be a challenging and relevant research topic because repair defects have clinical relevance. Notably, mutations in NER proteins have been linked to the diseases Xeroderma Pigmentosum (XP), trichothiodystrophy and Cockyne Syndrome (CS) (Cleaver, Thompson et al. 1999; Kraemer, Patronas et al. 2007). The clinical presentation of defects in NER differs, but some XP patients suffer from increased UV sensitivity and a predisposition to skin cancers (Lehmann 2003). Here we will discuss current knowledge of bulky DNA lesion recognition in chromatin. Of particular importance is the role of DDB2. Defects in DDB2 result in the autosomal recessive genetic disorder XPE (Tang and Chu 2002).
1.2 Chromatin Remodeling and GG-NER

Chromatin is a hierarchal structure composed of DNA and protein. It consists of a core nucleosome that is further connected by linker DNA to form “beads on a string”; followed by further compaction to higher order structures like the metaphase chromosome. The role of higher order chromatin structures in NER is largely unknown. Therefore our discussion will be focused at the nucleosome level. The nucleosome is a complex of 147 base pairs of DNA wrapped around the core histone octamer. The core histone octamer consists of four subunits, H2A, H2B, H3 and H4 in a 2:2:2:2 ratio (Luger, Mader et al. 1997; Kornberg and Lorch 1999).

It is this tight association between the DNA and the histone that occludes easy access to DNA binding proteins, including DNA damage recognition proteins. There are several proposed mechanisms for chromatin relaxation in DNA repair. These are presumably analogous to those used in transcription. One proposed mechanism is the “site exposure model”. This model suggests that the transient unwrapping of nucleosomal DNA, i.e., breathing, is enough for necessary proteins to bind DNA appropriately (Polach and Widom 1995; Li and Widom 2004). Another mechanism of remodeling depends on an ATP dependent protein complex. As reviewed by Gong et al., several ATP-dependent chromatin remodeling complexes have been implicated in DNA repair (Gong, Kwon et al. 2005). One such complex is SWI/SNF (mating-type SWItching/Sucrose Non-Fermenting). Although the mechanism of SWI/SNF remodeling is not fully understood, it has been shown to evict or slide the histone to expose the once occluded DNA (Clapier and Cairns 2009; Liu, Balliano et al. 2010). Another mechanism involves histone post-translational modifications. For example, histone acetylation, in which lysine residues of
the histone are acetylated resulting in a change in the net charge, leads to repulsion of the DNA from the histone (Chi, Allis et al. 2010). Each of these mechanisms will be discussed in greater detail in the subsequent sections.

Site Exposure Model

Although the physiological role of chromatin remodeling has focused on transcription, it is becoming more evident that chromatin remodeling plays an important role in DNA repair in vivo. For example, there are interesting in vitro data suggesting that UV damage itself can affect the stability of the nucleosome (Mann, Springer et al. 1997). More specifically, UV-induced DNA damage promotes an unwrapped nucleosome state (Duan and Smerdon 2010). These data suggest that the lesion itself may play a role in the dissociation of the nucleosome core complex and subsequent freeing of DNA to allow necessary protein binding and subsequent repair.

Role of SWI/SNF in GG-NER

However, in vivo data suggest that this lesion stimulated nucleosome breathing is not sufficient for optimal DNA repair. Gong et al. demonstrate a requirement for the ATP-dependent chromatin remodeling complex, SWI/SNF, in repair of CPDs. Specifically, they show that at the budding yeast silent HML (Hidden MAT Left) locus the rate of CPD removal is negatively affected in the absence of Snf6. Furthermore, consistent with Snf6 being a component of the SWI/SNF ATP-dependent remodeling complex, they demonstrated that it facilitates DNA accessibility during GG-NER (Gong,
In this study they also show that SWI/SNF associates with Rad4, a lesion detection protein, in a UV-stimulated manner.

NER stimulation by SWI/SNF appears to be conserved. We show the re-expression of BRG1 (Brahma-Related Gene 1), a component of the human SWI/SNF complex, stimulates GG-NER in human cells (Zhang, Zhang et al. 2009). Additionally, we show that XPC recruitment to CPD sites is reduced when BRG1 is depleted. Similarly, Zhao et al. show a requirement of BRG1 for efficient CPD repair (Zhao, Wang et al. 2009).

As mentioned previously, chromatin is a hierarchal structure, with the nucleosome being at the core. As a silenced locus the next level of compaction at the HML locus involves coating by the Sir (Silent Information Regulator) proteins. Interestingly, Sinha et al. show that SWI/SNF appears to be the only remodeling complex tested that is able to remodel nucleosomes coated by the Sir proteins (Sinha, Watanabe et al. 2009). Taken together with our previous observations that SWI/SNF is required for optimal GG-NER, these data present an interesting hypothesis that SWI/SNF may be involved in modulation of higher order chromatin structure in response to UV irradiation.

Histone Modifications and GG-NER

In addition to ATP-dependent chromatin remodeling, histone modifications have been shown to play a role in NER (Smerdon and Conconi 1999). Recently, Yu et al. demonstrate a global hyperacetylation of histones H3 (at lysine residues K9/K14) and H4 (at lysine residues K5/K8/K12/K16) upon UV irradiation in budding yeast (Yu, Teng et
al. 2005). Teng et al. begin to link UV-dependent histone H3 acetylation (at lysine residues K9 and K14) and GG-NER by showing Rad16 mediates UV-induced acetylation (Teng, Liu et al. 2008). Teng et al. also provide evidence that at loci with constitutively high levels of acetylated H3, Rad16 independent GG-NER is observed. More recent work further links increased acetylation with increased NER activity. Irizar et al. show that deleting the histone deacetylase Sir2 increased UV-induced acetylation of histone H3 and H4 in repressed sub-telomeric regions. Subsequently GG-NER is also increased (Irizar, Yu et al. 2010).

UV-dependent histone acetylation and subsequent stimulation of repair has also been demonstrated in human cells (Ramanathan and Smerdon 1989). Zhao et al. show that UV-dependent acetylation of H3 K14 and overall chromatin accessibility is dependent on p38 MAPK (Mitogen Activated Protein Kinase) activity (Zhao, Barakat et al. 2008). Despite the documented link between UV-induced acetylation of histones and GG-NER activity, the mechanistic role of acetylation remains to be elucidated. It is hypothesized that this acetylation may assist in exposing the chromatin for detection by downstream lesion detection proteins, such as Rad4/XPC. Alternatively, it may be involved in recruiting chromatin remodeling complexes by serving as bromodomain-binding platform (Awad and Hassan 2008). It is plausible that these two hypotheses are not mutually exclusive and may even be additive, as will be discussed in the subsequent section.

In addition to histone acetylation, other post-translational histone modifications have been implicated in GG-NER. For example, there is evidence that histone methylation may play an integral role in GG-NER. Chaudhuri et al. show evidence that
methylation of histone H3 K79 is involved in efficient GG-NER (Chaudhuri, Wyrick et al. 2009). They show increased UV sensitivity and impaired CPD repair at the \textit{HML} locus in H3 K79 mutant cells. Furthermore, they show constitutively reduced chromatin relaxation at the \textit{HML} locus in these mutants.

Yet another histone modification that has been implicated in chromatin remodeling is ubiquitination. As will be discussed in detail in subsequent sections, the ubiquitination of histones post-UV irradiation is DDB2-dependent (Chen, Zhang et al. 2001; Nag, Bondar et al. 2001; Matsuda, Azuma et al. 2005; Sugasawa, Okuda et al. 2005; Kapetanaki, Guerrero-Santoro et al. 2006; Wang, Zhai et al. 2006). Neither the role nor the implications of this modification are understood. It has been suggested that ubiquitination of histones is for both chromatin relaxation and reconstitution (Kapetanaki, Guerrero-Santoro et al. 2006; Zhu, Wani et al. 2009).

Of note, it is becoming increasingly evident that the role of ubiquitination is not only in protein degradation (Sadowski and Sarcevic 2010). Figure 1.5 A illustrates a simplified schematic of the ubiquitination pathway. Additionally, figure 1.5 B illustrates various documented linkages of ubiquitin to its target protein, and the fate of the modified protein (Sadowski and Sarcevic 2010).
Figure 1.5: Ubiquitination and protein fate. (A) Simplified schematic of ubiquitin pathway. ATP-dependent activation of ubiquitin by E1 protein. Followed by transfer to E2 conjugating protein, and finally transfer to target protein in an E3 ubiquitin ligase complex. Taken from http://e3miner.biopathway.org/help_intro.html. (B) Depiction of protein fate dependent on type of ubiquitination linkage. Modified from (Sadowski and Sarcevic 2010)
Interplay between Chromatin Remodeling Mechanisms During GG-NER

As eluted to earlier, the chromatin remodeling mechanisms are probably not exclusive. Indeed, Nag et al. used mutant *Saccharomyces cerevisiae* lacking a portion of the N-terminal domain of histone H2B to show decreased recruitment of Snf5, another component of the SWI/SNF remodeling complex, to the silent *HML* locus (Nag, Kyriss et al. 2010). Additionally, Nag et al. indicate reduced CPD removal and a UV sensitive phenotype for these cells, despite increased micrococcal nuclease accessibility suggestive of increased chromatin relaxation. These data suggest that the N-terminal domain itself, or more likely modifications such as acetylation of the N-terminal domain, are responsible for recruitment of the ATP-dependent chromatin remodeling complex, SWI/SNF. We have data to suggest that Snf6 is recruited to the silent *HML* locus in a *RAD4* knockout strain in a UV-dependent manner (unpublished data). Taken together, we speculate that the initial steps of GG-NER may involve UV-stimulated histone modifications, recruitment of chromatin remodeling activities and subsequent damage binding by DNA damage recognition proteins.

We are currently delineating the sequence of events and molecular mechanisms involved in this model. Interestingly, as will be discussed later, DDB2 has been shown to interact with chromatin remodeling proteins p300 and Brg1 (Datta, Bagchi et al. 2001; Rapic-Otrin, McLenigan et al. 2002; Zhang, Zhang et al. 2009). However, the role of this association remains to be elucidated. Perhaps further investigation of DDB2 function and the role of its association with chromatin remodeling factors will give us critical insight into the initial steps of GG-NER.
1.3 GG-NER Lesion Recognition Proteins

As mentioned in the introduction, GG-NER requires specific lesion detection proteins. Initial studies suggested Rad4-Rad23 and XPC-hRad23 are the initial heterodimers necessary for lesion detection and subsequent recruitment of downstream GG-NER players in yeast and humans, respectively. Later in vivo studies suggest there are upstream players that are required for lesion detection, Rad7-Rad16 in budding yeast and DDB1-DDB2 in humans. We will discuss the properties of each.

Rad4-Rad23/XPC-hRad23

Rad4-Rad23 and its human counterpart XPC-hRad23 are homologs (Masutani, Sugasawa et al. 1994). In vitro data indicate Rad4-Rad23 along with Rad1-Rad10 (nuclease cuts 5’ to the lesion), Rad2 (nuclease cuts 3’ to the lesion), Rad14 (the Rad1-Rad10 nuclease targeting protein), RPA (Replication Protein A) and transcription factor TFIIH (helicase function) are necessary and sufficient for incision (Guzder, Habraken et al. 1995). The human counterparts were shown to act in the same manner as the budding yeast (Mu, Park et al. 1995; Moggs, Yarema et al. 1996).

The role of Rad4-Rad23 as a damaged DNA binding complex was later delineated by in vitro binding assays (Guzder, Sung et al. 1998; Jansen, Verhage et al. 1998). Likewise, human XPC-hRad23 has been shown to have preferential binding to bulky DNA damage (Batty, Rapic‘-Otrin et al. 2000; Sugasawa, Shimizu et al. 2002). Also, like
its budding yeast counterpart, XPC-hRad23 was shown in vitro to be the initial lesion detection complex (Sugasawa, Ng et al. 1998).

Interestingly, crystal structure of Rad4-Rad23 in complex with a 6-4 photo lesion reveals that the Rad4-Rad23 heterodimer binds the DNA strand opposite the lesion (Min and Pavletich 2007). The crystal structure also shows that Rad4 inserts a beta-hairpin between the duplex, this in turn causes the lesion to protrude from the helix. This protrusion may serve as a binding site or signal for Rad14, the protein responsible for targeting the Rad1-Rad10 endonuclease complex to the lesion. Additionally, this crystal structure suggests Rad4-Rad23 has the potential to identify lesions facing toward histones in a nucleosome.

Rad7-Rad16 and DDB1-DDB2

Despite in vitro evidence that Rad4-Rad23/XPC-hRad23 is the initial lesion detection protein complex, in vivo experiments indicate there are additional components necessary for optimal lesion detection and subsequent repair. While both budding yeast and human cells have the need for additional protein complexes in vivo, only DDB1 is conserved in budding yeast; it is reported to be homologous to Mms1 (Methyl Methane Sulfonate sensitivity) (Zaidi, Rabut et al. 2008).

Despite not being necessary for in vitro repair, Rad7 and Rad16 were found to play an in vivo role in GG-NER (Verhage, Zeeman et al. 1994; Mueller and Smerdon 1995). Stimulation of repair in an in vitro system was observed upon addition of purified Rad7-Rad16, verifying a direct role in GG-NER (Guzder, Sung et al. 1997). Rad7-Rad16
form an ATP dependent DNA damaged binding complex (Guzder, Sung et al. 1997). This complex works in concert with Rad4-Rad23 complex (Guzder, Sung et al. 1999). Rad16 has DNA-dependent ATPase activity (Guzder, Sung et al. 1998) and is a member of the SNF2 (Sucrose Non-Fermenting) family (Eisen, Sweder et al. 1995). While the ATPase domain has been shown to be necessary for Rad16 function in GG-NER (Ramsey, Smith et al. 2004), the mechanism remains to be elucidated. It is important to note that although Rad16 is a member of the SNF2 family, it has yet to be shown to have direct role in chromatin remodeling. Additionally, this is a large family and the Rad16 sub-family has some peculiarities such as being the only SNF2 subfamily to have a RING (Really Interesting New Gene) finger domain (Eisen, Sweder et al. 1995). As suggested by the presence of a RING finger domain, Rad7-Rad16 has been identified as an E3 ubiquitin ligase in complex with Elc1 (Elongin C) (Ramsey, Smith et al. 2004). Rad4 has been identified as a UV-dependent target for Rad7-Rad16 mediated ubiquitination (Gillette, Yu et al. 2006). Although mediated by Rad7-Rad16, this Rad4 ubiquitination seems to be controlled by Rad23, via an unknown mechanism (Lommel, Ortolan et al. 2002). Despite biochemical evidence as to functions of Rad7-Rad16, its exact role in GG-NER remains to be elucidated. Notably Rad7-Rad16 has been reported to play a post-incision role in GG-NER (Reed, You et al. 1998).

Like budding yeast, human cells require a protein complex, DDB1-DDB2, upstream of XPC-hRad23 for efficient in vivo GG-NER of CPDs (Tang, Hwang et al. 2000; Wakasugi, Kawashima et al. 2002; Fitch, Nakajima et al. 2003; Pines, Backendorf et al. 2009). Unlike other XP complementation groups, XPE and XPC show defects in GG-NER only, leaving TC-NER fully functional (Hanawalt, Ford et al. 2003). This
emphasizes the role of XPE and XPC specifically in lesion detection in the context of chromatin.

It has been shown that DDB2 is responsible for the lesion detection by directly interacting with the damaged DNA (Tang, Hwang et al. 2000; Scrima, Konickova et al. 2008). Additionally, DDB2 binds the lesion independent of XPC (Wakasugi, Kawashima et al. 2002). DDB2 can co-localize with both CPDs and 6-4 PPs in vivo, while XPC seems to bind 6-4PPs efficiently, but not CPDs. This suggests the necessity of DDB2 in GG-NER is specific for CPD repair (Fitch, Nakajima et al. 2003). Importantly, it has been suggested that the observed high affinity of DDB2 for 6-4PPs aids in the targeting of XPC to 6-4PPs when low levels of damage are present (Nishi, Alekseev et al. 2009).

Additionally, DDB2 is in complex with the E3 ubiquitin ligase complex consisting of DDB1, Cul4 (Cullin 4) and ROC (Ring of Cullins) (Jackson and Xiong 2009). E3 ubiquitin ligases transfer ubiquitin to the target protein (Figure 1.5). DDB2 is thought to be the substrate receptor targeting the E3 ubiquitin ligase complex to DNA lesion sites to facilitate GG-NER. Of note, DDB1 and Cul4 have been shown to be in complex with other proteins, including CSA, a TC-NER specific protein (Jackson and Xiong 2009). Consistent with its classification as an E3 ubiquitin ligase, XPC, histone H2A, H3, H4, and DDB2 itself have been identified as UV-dependent ubiquitination targets of the DDB1-DDB2 E3 ligase complex (Chen, Zhang et al. 2001; Nag, Bondar et al. 2001; Matsuda, Azuma et al. 2005; Sugasawa, Okuda et al. 2005; Kapetanaki, Guerrero-Santoro et al. 2006; Wang, Zhai et al. 2006). The UV-dependent mono-ubiquitination of histone H2A has been suggested to be involved in both chromatin relaxation and restoration (Kapetanaki, Guerrero-Santoro et al. 2006; Zhu, Wani et al.
Additionally it has been shown that the UV-induced ubiquitination of DDB2 results in subsequent protein degradation (Rapic-Otrin, McLenigan et al. 2002; Fitch, Cross et al. 2003; Chen, Zhang et al. 2006). Although the ability of DDB1-DDB2 to act as a UV-dependent E3 ligase on multiple targets is documented, the role this ubiquitination plays in GG-NER remains to be elucidated.

Potentially crucial to its role in GG-NER, DDB2 has been shown to be associated with chromatin remodeling factors. Both in vivo and in vitro data indicate an interaction between DDB2 and the histone acetyltransferase CBP/p300 family proteins (Datta, Bagchi et al. 2001; Rapic-Otrin, McLenigan et al. 2002). Also we have shown that DDB2 associates with BRG1 in a UV-dependent manner (Zhang, Zhang et al. 2009). It is unclear what role this association between UV-DDB and the chromatin remodeling complexes play. Identification of lesions by DDB2 could be upstream of chromatin remodeling. The inverse could also occur. Chromatin remodeling complexes could be recruited intrinsically by the DNA damage or by UV-induced histone modification. This will in turn facilitate DNA damage binding by DDB2 to initiate downstream GG-NER processes. It will be important to further investigate the sequence of events and elucidate the impact of this association on GG-NER.

In addition to direct lesion recognition in GG-NER, there is evidence to suggest additional important roles of DDB2 in the DNA damage response. DDB2 has been shown to indirectly down regulate p21 transcription, by regulating the levels of phosphorylated p53 when cells are exposed to low level UV irradiation (Stoyanova, Yoon et al. 2008). It is interesting to note that DDB2 transcription is p53 dependent in human cells, presenting an interesting UV-dependent regulatory loop (Hwang, Ford et al. 1999; Tan and Chu
Furthermore, there is evidence that DDB2 affects the protein levels of p21 when exposed to high level UV irradiation (Stoyanova, Roy et al. 2009). The role DDB2 plays in regulating p21 levels suggests an alternative mechanism for its involvement in UV damage response, as will be discussed in the next section. Additionally, DDB2 physically interacts with E2F1 (E2F transcription factor 1) \textit{in vitro} and \textit{in vivo}, as well as stimulate E2F1 dependent transcription (Hayes, Shiyanov et al. 1998). Interestingly, a recent study shows that E2F1 is recruited to UV damage site and plays a role in NER independent of its role in transcription (Guo, Chen et al. 2010), shedding more light on the complexity of GG-NER.

Furthermore, DDB2 has been identified in other cellular processes seemingly independent of its role in DNA damage response. DDB2 has been shown to be involved in the regulation of SOD2 transcription in breast cancer cell line MCF7 (Minig, Kattan et al. 2009). Also, DDB2 has been shown to be over-expressed in breast cancer cells, and knock down of DDB2 in these cells results in decreased cell proliferation (Kattan, Marchal et al. 2008). These functions of DDB2 are out of the scope of this review. It is important though to note the complexity of DDB2 both within DNA damage response and in alternative cellular functions.

\section*{1.4 Models for DDB2 role in GG-NER}

The predominant model suggests that DDB2 is ubiquitinated, and subsequently degraded to allow for GG-NER to proceed (Figure 1.6). This model is supported by data indicating a UV-dependent degradation of DDB2 (Rapic-Otrin, McLenigan et al. 2002; Fitch, Cross et al. 2003; Chen, Zhang et al. 2006). It is further supported by subsequent \textit{in vivo} data
linking DDB2 degradation to repair. It has been suggested that the binding of XPC is dependent on the release of DDB2 via its degradation (Wang, Wani et al. 2005; El-Mahdy, Zhu et al. 2006).

There is emerging data to argue against this model. Luijsterburg et al. found that the UV-dependent degradation rate of DDB2 is inconsistent with the previously proposed binding/degradation model (Luijsterburg, Goedhart et al. 2007). Specifically, Luijsterburg et al. show that the degradation of DDB2 is partially dependent on active GG-NER; they also show that neither binding to damaged DNA nor XPC association is the trigger for DDB2 degradation. Additionally using a mouse model, in which DDB2 degradation is prevented, Liu et al. show a positive correlation between DDB2 levels and CPD repair (Liu, Lee et al. 2009).

Additionally, crystal structure data indicate DDB2 and Rad4-Rad23 contact the lesion on opposite strands. DDB2 was shown to contact the damaged strand directly (Scrime, Konickova et al. 2008), while Rad4-Rad23 was shown to contact the strand opposite the lesion (Min and Pavletich 2007). This suggests that possibly both complexes could be bound to the lesion site at the same time. On the basis of these data we developed an illustration depicting a possible confirmation by which both proteins could bind the DNA lesion simultaneously (Figure 1.7). As described in our recent review, this illustration was generated from published structures as to maintain native Rad4-DNA and DDB2-DNA interaction and both proteins could be merged to a 6-4PP DNA lesion without prohibitive Rad4-DDB2 protein-protein contact. This is consistent with a similar co-binding model proposed by Scrime et al. (Scrime, Konickova et al. 2008).
Figure 1.6: Predominate model of DDB2-mediated GG-NER. DDB2 binds CPD lesions as part of an E3 ubiquitin ligase complex. DDB2 is ubiquitinated in a Cul4A dependent manner and there is subsequent degradation. This subsequent degradation has been proposed to be necessary for progression of GG-NER as described in the text. Modified from Ford (Ford 2005).

Figure 1.7: Illustration depicting putative simultaneous binding of Rad4 (XPC) and DDB2 to a DNA lesion. Illustration merged Rad4 aa490-632 (PDB: 2QSG) and DDB2 aa128-455 bound to DNA containing a 6-4 PP (highlighted in yellow; PDB: 3EII), as rendered in Pymol, in a matter consistent with the native protein-DNA interactions. (Jones, Zhang et al. 2010)
The ability of DDB2 and Rad4-Rad23 to interact with opposite strands may facilitate lesion recognition within a nucleosome. For example, if the lesion is facing inward or toward the histones, Rad4-Rad23 (XPC-hRad23) may be able to identify the lesion because it interacts with the opposite, exposed strand. Because DDB2 binds the damaged strand directly with minimal contact, more studies are required to determine how, if at all, it can interact with a lesion in this orientation. However, out-ward facing lesions may be recognized by DDB2, since the histones would presumably occlude the extensive binding sites necessary for Rad4-Rad23. This would suggest that DDB2 may be involved in initiating chromatin remodeling events required for Rad4-Rad23 access to the lesion. While the information obtained from these crystal structures is very useful, further mechanistic studies are necessary to delineate the physiological relevance of these observations.

It is possible that the observed degradation of DDB2 occurs after DDB2 has been released from the lesion. Perhaps the observed UV-dependent degradation of DDB2 is occurring to prevent DDB2 from functioning in a pro-apoptotic pathway. It has been suggested that DDB2 may be acting as a sensor of UV damage levels and thereby determining the fate of the cell (Stoyanova, Roy et al. 2009). In this regard, DDB2 ubiquitination and subsequent degradation may signal cell recovery and prevent entry into the apoptotic pathway. Indeed, DDB2 has been shown to drive apoptosis following DNA damage (Bagchi and Raychaudhuri 2010).

An alternative role for ubiquitination of DDB2 in GG-NER is release of DDB2 from chromatin, independent of degradation. Indeed, in vitro data indicate that DDB2 ubiquitination decrease its binding properties to the lesion (Sugasawa, Okuda et al. 2005).
The experiments described herein are designed to specifically look at the role of ubiquitination in DDB2-mediated GG-NER. To do this we first establish budding yeast as an alternative simplified model system. Then we use ubiquitin deficient mutant budding yeast to show that ubiquitin is involved in the release of DDB2 from chromatin. Additionally, using budding yeast we begin to investigate the initial sequence of events in lesion detection. Specifically, we investigate the recruitment of the ATP-dependent chromosome remodeling complex, SWI/SNF. Our data indicate that Rad4 is not an absolute requirement for SWI/SNF recruitment to the silent HML locus following UV irradiation. The implications of these observations will be discussed.
CHAPTER 2: MATERIALS AND METHODS

2.1 Strains and Vectors

The following Saccharomyces cerevisiae strains were used. Strains rad26Δ, rad16Δ, rad4Δ, rad23Δ, rad14Δ, rad1Δ in BY4741 background were purchased from Open Biosystems. The mms1Δ strain was constructed by replacing the MMS1 locus with the LEU2 gene in the rad26Δ background. A rad16Δrad26Δ double mutant strain was a kind gift from Dr. Shisheng Li and was constructed in the Y452 background as described previously (Li and Smerdon 2002). The E1 temperature sensitive (t^s) strain (RJD3269) and the isogenic parent strain (RJD3268) (Ghaboosi and Deshaies 2007) were a kind gift from Dr. Antoni Barrientos. The Snf6-TAP strain was purchased from Open Biosystems. It is BY4741 background strain with a tandem affinity purification (TAP) tag (Puig, Caspary et al. 2001) at the SNF6 locus. Figure 2.1 A is a schematic to depict the fusion of Snf6 and the TAP tag. Two mutant strains were constructed in the Snf6-TAP background. The RAD4 and RAD16 loci were replaced with G418 resistance gene to construct rad4Δ and rad16Δ Snf6-TAP strains respectively. Strains were confirmed by phenotypic analysis (Figure 2.1 B). Genotypes are listed in table 2.1.

DDB2 cDNA was amplified from purified mRNA of human 293T cells and verified by DNA sequencing, a kind gift from Dr. Ling Zhang. It was sub-cloned into the pYC2/CT (Invitrogen) galactose inducible yeast expression vector in frame with V5His6 tag. Expression of DDB2 was verified by Western blotting using the anti-V5 primary antibody (Sigma). Addition of 4% glucose was used to shut off the nascent DDB2 mRNA production.
Figure 2.1: Snf6-TAP strains. (A) Schematic of TAP tag. Modified from Puig et al. 2001. (B) Phenotypic verification of RAD deletion strains in Snf6-TAP background. Top panel Snf6-TAP wild type and rad16∆ Snf6-TAP. Bottom panel Snf6-TAP wild type, BY4741 rad4∆ and Snf6-TAP rad4∆. TAP tag does not affect UV sensitivity of Rad4 mutant strain. Cells were grown overnight in YPD then back diluted to an OD660=1. 1/10 serial dilutions were made and 3 µl were spotted onto YPD plates. Plates were exposed to the UV dosage indicated and allowed to grow at 30°C in the dark for 2-3 days.
<table>
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<tr>
<th>STRAIN</th>
<th>GENOTYPE</th>
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<td>BY4741</td>
<td>MATa, his3Δleu2Δ0, met15Δ0, ura3Δ0</td>
</tr>
<tr>
<td>Y452</td>
<td>MATa, ura3-52, his3–1, leu2-3, leu2-112, cir&lt;sup&gt;o&lt;/sup&gt;</td>
</tr>
<tr>
<td>Snf6-TAP</td>
<td>BY4741 with TAP tag at SNF6 chromosomal locus</td>
</tr>
<tr>
<td>RJD3268 (E1 t&lt;sup&gt;+&lt;/sup&gt; isogenic parent)</td>
<td>MATa can1-100 ade2-1 his3-1,15 leu2-3, 112 trp1-1 ura3-1 uba1::KANMX [pRS313 - UBA1-HIS]</td>
</tr>
<tr>
<td>RJD3269 (E1 t&lt;sup&gt;+&lt;/sup&gt; mutant)</td>
<td>RJD3268 except pRS313-uba1-204-HIS</td>
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<tr>
<td>rad26Δ</td>
<td>rad26Δ::G418 in BY4741 background</td>
</tr>
<tr>
<td>rad16Δ</td>
<td>rad16Δ::G418 in BY4741 background</td>
</tr>
<tr>
<td>rad4Δ</td>
<td>rad4Δ::G418 in BY4741 background</td>
</tr>
<tr>
<td>rad23Δ</td>
<td>rad23Δ::G418 in BY4741 background</td>
</tr>
<tr>
<td>rad14Δ</td>
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</tr>
<tr>
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</tr>
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<td>rad16Δ::G418 in Snf6-TAP background</td>
</tr>
<tr>
<td>rad4Δ Snf6-TAP</td>
<td>rad4Δ::G418 in Snf6-TAP background</td>
</tr>
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<td>rad26Δ+vector</td>
<td>rad26Δ except pYC2/CT</td>
</tr>
<tr>
<td>rad26Δ+DDB2</td>
<td>rad26Δ except pYC2/CT - DDB2</td>
</tr>
<tr>
<td>rad26Δ+DDB2 K244E</td>
<td>rad26Δ except pYC2/CT - DDB2 K244E</td>
</tr>
<tr>
<td>rad26Δ+DDB2 L350P</td>
<td>rad26Δ except pYC2/CT - DDB2 L350P</td>
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**Table 2.1: Strain list.** Genotypes of strains used in these studies.
<table>
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<th>STRAIN</th>
<th>GENOTYPE</th>
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<td>E1 t&lt;sup&gt;+&lt;/sup&gt; mutant +DDB2</td>
<td>RJD3269 except pYC2/CT -DDB2</td>
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<tr>
<td>E1 t&lt;sup&gt;+&lt;/sup&gt; mutant +DDB2 L350P</td>
<td>RJD3269 except pYC2/CT -DDB2 L350P</td>
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**Table 2.1(cont): Strain list.** Genotypes of strains used in these studies.

<table>
<thead>
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<th>PRIMER</th>
<th>SEQ</th>
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</thead>
<tbody>
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<td>agcttaataatgtctgctcccaagaaacgcc</td>
</tr>
<tr>
<td>DDB2-His reverse</td>
<td>atctcgagcttccgtgctctggc</td>
</tr>
<tr>
<td>Lys244Glu forward</td>
<td>ctcagaatgcacaaaaaggaagtgacgcatgtggcc</td>
</tr>
<tr>
<td>Lys244Glu reverse</td>
<td>ggccacatgcgtcaacctcccttttctttgtgcattctgag</td>
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<tr>
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<td>mms1Δ::LEU forward</td>
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</tr>
<tr>
<td>mms1Δ::LEU reverse</td>
<td>ttgtcagctgacaacgcgtagcacttgatcgagaat</td>
</tr>
<tr>
<td></td>
<td>cagatgttttatgtacaaatatc</td>
</tr>
</tbody>
</table>

**Table 2.2: Primer list.** Primers used for cloning and mutagenesis.
Quickchange mutagenesis kit (Stratagene) was used to construct mutants of DDB2. Two mutations in DDB2 were constructed; lysine was changed to glutamic acid at residue 244 (DDB2 K244E) and a double mutant in which residue 349 was deleted and leucine was changed to proline at position 350 (DDB2 L350P). Mutations were verified by DNA sequencing (data not shown). Primers used for cloning and mutagenesis are listed in table 2.2.

**2.2 UV Sensitivity Spotting Assay**

For spotting assays strains were grown in two ml yeast peptone galactose (YPG) media overnight then back diluted to OD$_{660}$ = 0.7. Ten-fold serial dilutions were made and three µl of each dilution were spotted onto YPG plates. Cells were exposed to various dosages of UV irradiation as indicated and incubated in the dark at 30°C for two days.

For survival curve assay *rad26Δ* cells expressing either empty vector (vector), wild type DDB2 (DDB2), mutant DDB2 K244, or mutant DDB2 L350P were plated onto YPG plates in serial dilutions such that 20 to 500 cells would survive. Cells were exposed to UV irradiation at the dose indicated and allowed to grow in the dark at 30°C for two days. Colonies were counted and percent survival was calculated as number of colonies on UV treated sample over non-UV treated sample.

*E1* t8 mutant UV sensitivity spotting assays were performed as described above, except incubation was at 25°C for three days. RJD3268 and RJD3269 strains expressing wild type or mutant DDB2 were used as indicated.
2.3 CPD Repair

Rate of CPD removal was performed as follows, rad26Δ cells containing vector, wild type DDB2, mutant DDB2 K244E or mutant DDB2 L350P were grown in YPG to OD₆₆₀ = 1. Cells were exposed to 100 J/m² UV irradiation and were allowed to repair in the safe light for times indicated. Cells were lysed using standard glass bead technique. DNA was extracted using standard phenol:chloroform method. DNA concentration was calculated using OD₂₆₀ method. Equal amount of total DNA was denatured using 0.4 N sodium hydroxide (NaOH) followed by boiling. One µg of each sample was blotted onto Amersham HyBond N+ membrane. Each well was washed with 0.4 N NaOH followed by 2X saline-sodium citrate (SSC). The membrane was then dried in a 70°C oven for three to four hours, and washed with 0.5X Tris buffered saline containing Tween-20 (TBST). Western blot was then performed using a CPD-specific monoclonal antibody (Mori, Nakane et al. 1991), a kind gift from Dr. Toshio Mori. Southern blot was performed to verify equal DNA loading.

2.4 Pull-down and Western Blotting

Co-purification of Rad4 and DDB2 was assessed by Western blotting. BY4741 cells expressing DDB2 or empty vector were collected. An equal volume of wet cell pellets was lysed using the standard glass bead method. DDB2 was affinity purified using nickel beads (Qiagen) at 4°C overnight. Empty vector negative control, Snf6-TAP positive control and DDB2 expressing cells were separated using SDS-PAGE
electrophoresis and transferred to a nitrocellulose membrane. Western blot analysis using poly-clonal anti-Rad4 antibody (Sigma) was performed. The reciprocal experiment was also conducted. The Rad4 antibody (Sigma) was pre-bound to protein A agarose beads (Millipore) for one hour at 4°C. Rad4 purification was carried out overnight at 4°C. Whole cell extract (WCE) and purified samples were separated using SDS-PAGE electrophoresis, transferred to nitrocellulose membrane and Western blot was performed using mono-clonal anti-V5 antibody (Sigma).

The ubiquitination state of DDB2 following UV irradiation was also assessed. Cells expressing wild type DDB2 were grown to OD_{660}=1, then treated with 100 J/m^2 UV irradiation and allowed to repair in safe light for time indicated. Equal amounts of cells were lysed using standard glass bead method. Lysis and wash buffers contained imidazole to increase stringency. Western blot analysis was conducted. The membrane was probed with anti-V5 antibody (Sigma), stripped with 0.5N NaOH and re-probed with anti-ubiquitin antibody (Santa Cruz).

Co-purification of Snf6 and Rad4 was performed as above following UV or mock treatment. Human IgG agarose beads were used to purify Snf6-TAP.

2.5 Proteasome Inhibition by MG132

RJD3268 cells expressing wild type DDB2 were grown at 30°C to OD_{660}=0.7, collected, washed with 1M sorbitol and re-suspended in 10 ml zymolyase buffer (1M sorbitol, 0.5 mg/ml zymolyase and 0.1% β-mercaptoethanol). Zymolyase activity proceeded at 30°C with gentle shaking for 20 minutes. Twenty ml of YPG and 20 µM
MG132 (Sigma) was added, and incubation resumed for one hour. Following proteasome inhibition, cells were treated with 100 J/m² UV and allowed to repair in safe light for 30 or 60 minutes, as indicated. Cell lysis and DDB2 pull-down was carried out as described in previous section, except no imidazole was used. Western blot analysis using anti-V5 and anti-ubiquitin antibodies was performed as described in previous section.

2.6 DDB2 Protein Stability

Cells were grown to OD$_{660}$=1 at 30°C in YPG to induce expression of DDB2. Then cells were exposed to 100 J/m² UV irradiation or mock treated. To access protein turnover 4% glucose was added to the culture to stop nascent DDB2 synthesis. Steady state samples were grown only in the presence of galactose, for continual DDB2 synthesis. Samples were collected at times indicated post-UV irradiation and lysed using the standard glass bead method. Total protein concentration was determined using the Bradford method, then adjusted to equal amounts and verified by coomassie blue staining. Western blotting analysis using an anti-V5 antibody was used to visualize DDB2 protein levels as described above.

2.7 Chromatin Fractionation

For chromatin fractionation experiments, the E1 t⁸ mutant strain and its isogenic wild type were used. Additionally, to test if the presence of Rad26 had an effect, rad26Δ +DDB2 strain was also tested. Cells were processed as above with the following modifications. The 25°C samples were grown at 25°C before and after UV irradiation.
The 37°C samples were initially grown at 25°C to OD_{660}=1 then shifted to 37°C for one hour prior to UV irradiation (100 J/m²) and remained at 37°C for the duration of the experiment. For non-steady state experiments, 4% glucose was added immediately post-UV irradiation, or at time 0 for non-UV irradiated samples. Steady state experiments were conducted in the presence of galactose to maintain induction of DDB2. Cells were lysed in buffer containing 50 mM HEPES pH7.5, 140 mM NaCl, 1 mM EDTA pH8.0, 1% Triton X-100, 0.1% sodium deoxycholate, 1mM PMSF and 1X protease inhibitor cocktail (Roche). Debris was removed by low speed spin, 2000 rpm in a table top centrifuge, at 4°C for one minute. Total protein levels were measured using Bradford method and adjusted to 10 mg/ml. Soluble and chromatin bound fractions were then separated by high speed centrifugation, 30,000 xg for 40 minutes at 4°C. Western blots were performed as indicated above with an anti-V5 antibody. Histone H3 antibody (Upstate) was used as a chromatin loading control.

2.8 DNase I Treatment

To ensure the pellet obtained from the fractionation procedure described above represents the chromatin fraction, DNase I digest was performed as follows. The pellet was re-suspended in 1X DNase I buffer (Promega), an aliquot was removed to serve as input (I) and the remainder was divided into 2 equal aliquots. One was treated with DNase I (Promega) and the other was mock treated. Digest was carried out at 37°C for two hours. Samples were spun as above to separate the supernatant and pellet. V5 and histone H3 Western blots were performed as described above. Figure 2.2 shows that treatment of the pellet with DNase1 results in release of both DDB2 and histone H3 into
the soluble fraction. These data indicate that the pellet from the fractionation procedure described above is indeed the chromatin bound fraction.

Figure 2.2: Verification the pellet represents the chromatin bound fraction. Treatment of the pellet with DNase I results in release of both DDB2 and histone H3. I is the input, S is the soluble faction and P is the pellet.

2.9 Chromatin Immunoprecipitation

Cells were grown overnight, diluted and grown to OD₆₆₀=1. Cells were then treated with 100 J/m² UV, allowed to repair in safe light and collected at times indicated. All samples were chilled prior to cross-linking. Samples were cross-linked with 1% formaldehyde for 30 minutes. Cells were lysed using the standard glass bead lysis method in lysis buffer (140 mM NaCl, 50 mM HEPES/KOH pH 7.5, 1 mM EDTA pH8, 0.1% sodium deoxycholate, 1% Triton X, 1 mM PMSF and 1X protease inhibitor cocktail (Promega)). Chromatin was sheared by sonicating samples 15 times 15 seconds each at an output of five watts. Total protein was measured using Bradford method. Total protein for each sample was adjusted to equal amount (either 0.5 or 1 mg/ml). Twenty µl of the adjusted sample was removed and processed for input sample. Human IgG agarose beads
(Sigma) were used to pull-down Snf6-TAP; the protein A of the TAP tag (Figure 2.1 A) interacts with protein A. Beads were washed and complexes were eluted in 60 µl TE containing 1% SDS by incubation at 65°C for 30 minutes. Reverse cross linking was carried out at 65°C overnight. Samples were then treated with proteinase K at 37°C for two hours. DNA was purified by phenol:chloroform extraction followed by ethanol precipitation. DNA was dissolved in 20 µl TE and five µl was used as template for PCR. Primers amplified the nucleosome seven region of the HML locus (Figure 2.3).

**Figure 2.3: Amplification of HML locus in ChIP analysis.** (A) Schematic of HML locus with the region PCR amplified indicated by the box. (B) Primer sequence used in PCR amplification. Figure adapted from Gong et al. (Gong et al. 2006)
CHAPTER 3: RESULTS

3.1. Saccharomyces cerevisiae as a Model System to Study DDB2-Mediated GG-NER

It has been demonstrated that DDB2 is the initial lesion detection factor in GG-NER (Tang, Hwang et al. 2000; Wakasugi, Kawashima et al. 2002; Fitch, Nakajima et al. 2003; Pines, Backendorf et al. 2009). Although it has been implicated in the recruitment of XPC to CPD sites (Fitch, Nakajima et al. 2003); how DDB2 transfers these identified lesions to XPC remains controversial. As indicated in the introduction, it is believed that ubiquitination of DDB2 leads to its degradation at damage sites and this degradation is required for progression of CPD repair via GG-NER. However, there are several lines of evidence to dispute this model; (1) inhibition of ubiquitination-mediated DDB2 degradation in mouse via Cul4a ablation enhances CPD repair (Liu, Lee et al. 2009), (2) DDB2 degradation is not stimulated by either DNA binding or XPC association (Luijsterburg, Goedhart et al. 2007), and (3) crystal structure data suggest DDB2 and XPC can co-localize on the lesion (Min and Pavletich 2007; Scrima, Konickova et al. 2008). We establish and utilize budding yeast as a simplified, alternative model system to begin to dissect the role(s) of ubiquitination in DDB2-mediated GG-NER.

Key Findings

1. DDB2 expression suppresses UV sensitivity of rad26Δ S. cerevisiae cells
2. Clinically relevant mutations in DDB2 abrogate the DDB2-dependent suppression of UV sensitivity in rad26Δ S. cerevisiae cells
3. DDB2 associates with Rad4
4. DDB2 expression stimulates CPD removal in rad26Δ S. cerevisiae cells
3.1.1 Galactose-Induced Expression of DDB2 in *S. cerevisiae*

As discussed in the introduction, DDB2 has no homolog in budding yeast. However, conservation of the GG-NER pathway and interacting partners such as DDB1 are known (Zaidi, Rabut et al. 2008). Therefore, we hypothesized that DDB2 would act in a physiological relevant manner in budding yeast GG-NER. We first cloned the DDB2 gene into a low copy number, galactose inducible yeast expression vector as described in materials and methods. The cloning results in a fusion protein; DDB2 fused with V5His6.
tag (Figure 3.1 A). Both the empty plasmid vector and the DDB2 containing plasmid were transformed into *S. cerevisiae* as described in materials and methods. As expected, when cells were grown in the presence of galactose, DDB2 protein was produced as identified by Western blot using both anti-V5 or anti-DDB2 antibody (Figure 3.1 B and data not shown). No protein was detectable at the calculated molecular weight of DDB2 in the empty vector control using the same Western blot technique (Figure 3.1 B and data not shown).

To access the efficacy of the galactose induction 4% glucose was added to the media. Rapid shut down of the galactose inducible promoter is presumed due to the significant decrease in DDB2 protein levels 30 minutes post addition of glucose (Figure 3.1 C). This observed decrease in DDB2 protein levels is likely due to normal protein turnover in the absence of nascent DDB2 transcription and subsequent translation. These data confirm DDB2 is expressed in *S. cerevisiae* cells under the control of the galactose promoter.

### 3.1.2 DDB2 Suppresses UV Sensitivity of *rad26Δ* Cells.

Next we identified a genetic background in which a DDB2-dependent phenotype could be observed. We screened several strains in which various NER proteins were deleted. The strains tested were *rad4Δ*, *rad23Δ* and *rad16Δ* in which only TC-NER is active, *rad26Δ* in which only GG-NER is active, and *rad1Δ* in which the core pathway is defective and therefore there is no NER. Additionally the *rad16Δrad26Δ* double mutant was tested. The spotting assay as described in materials and methods was used to determine DDB2-dependent suppression of UV sensitivity. DDB2 expression suppresses
the UV sensitive phenotype of rad26Δ cells (Figure 3.2 A). Survival curve experiments verified these findings (Figure 3.4 B).

As discussed in the introduction, both DDB2 and Rad16 are necessary for lesion identification in vivo and are part of E3 ubiquitin ligase complexes (Verhage, Zeeman et al. 1994; Mueller and Smerdon 1995; Shiyanov, Nag et al. 1999; Tang, Hwang et al. 2000; Wakasugi, Kawashima et al. 2002; Fitch, Nakajima et al. 2003; Groisman, Polanowska et al. 2003; Ramsey, Smith et al. 2004; Pines, Backendorf et al. 2009). Therefore, it was surprising that DDB2 is unable to suppress the rad16Δ UV sensitive (Figure 3.2 B). Our data suggest that despite similarities in their biochemical properties, on a gross functional level DDB2 and Rad16 are not analogs. It should be noted that Rad16 has also been implicated in post-incision processes (Reed, You et al. 1998) while DDB2 has not. It is therefore plausible that DDB2 and Rad16 have analogous functions in the lesion identification step of GG-NER, but this post-incision function of Rad16 is unable to be rescued by DDB2 expression.

DDB2 was also unable to suppress UV sensitivity of the rad16Δrad26Δ double mutant (Figure 3.2 C). Also, DDB2 was not able to significantly suppress UV sensitivity of any other knockout strain (data not shown). These data are consistent with no known DDB2 homolog in budding yeast. The observed DDB2-dependent suppression of TC-NER deficient UV sensitivity is consistent with reported DDB2 stimulation of GG-NER (Wakasugi, Shimizu et al. 2001; Wakasugi, Kawashima et al. 2002). Taken together, these data suggest that DDB2 is acting as a second site suppressor to stimulate the active GG-NER in rad26Δ cells.
Figure 3.2: DDB2 expression suppresses UV sensitivity of rad26Δ mutant, but not rad16Δ mutant. BY4741 (WT) cells expressing DDB2 or empty vector were diluted 1/10 and plated on galactose media. Cells were exposed to UV irradiation at dose indicated and grown in dark at 30°C for 48 hours. rad26Δ (A). rad16Δ (B). rad16Δ rad26Δ (C).
3.1.3 DDB2 Mutations Abrogate its Ability to Suppress \textit{rad26}\Delta UV Sensitivity

To assess if DDB2 is functioning in a physiologically relevant manner we first examined the phenotypic effects of mutant DDB2 on DDB2-dependent suppression of \textit{rad26}\Delta UV sensitive phenotype. Several DDB2 mutations identified in XPE patients are known to interfere with its ability to function properly in GG-NER. It has been reported that a point mutation changing lysine 244 to glutamic acid (DDB2 K244E) results in inability of DDB2 to make contact with DNA lesions (Scrimsa, Konickova et al. 2008) (Figure 3.3 A). However, this mutation does not alter the ability of DDB2 to interact with DDB1 in the Cul4a E3 ubiquitin ligase complex; therefore its role in ubiquitination is not altered. When this damage recognition deficient mutant DDB2 was introduced into \textit{rad26}\Delta cells, it cannot suppress \textit{rad26}\Delta UV sensitivity (Figure 3.4 A and B). This suggests that the observed DDB2-conferred UV resistance is linked to its function in DNA damage detection.

The second mutation tested (DDB2 L350P) has been reported to prevent DDB2’s function via interruption of the interaction with its \textit{in vivo} partner DDB1 (Nichols, Itoh et al. 2000). This mutation is a complex mutation, consisting of both a deletion of amino acid 349 and a proline substitution for leucine at amino acid 350 (DDB2 L350P) (Figure 3.3 B). Like DDB2 K244E, this mutation also abrogates DDB2’s ability to suppress UV sensitivity in \textit{rad26}\Delta cells (Figure 3.2). These data suggest DDB2-conferred UV resistance is dependent on a conserved interacting partner.
Although Mms1 has been identified as the budding yeast DDB1 homolog (Zaidi, Rabut et al. 2008), there are no reports of it being involved in NER. Our previous observation suggesting DDB2 function requires a conserved interacting partner prompted us to test DDB2 function in the absence of Mms1. To test this, wild type DDB2 was expressed in the rad26Δmms1Δ double mutant and UV sensitivity was accessed by spotting assays as described in materials and methods. Indeed, this reciprocal experiment verified that Mms1 is necessary for DDB2-dependent suppression of UV sensitivity (Figure 3.4 B). Taken together, these findings provide genetic evidence that DDB2 is functioning in a conserved physiological manner in budding yeast GG-NER. This

Figure 3.3: Crystal structure of DDB2 mutations modified from crystal structure solved by Scrima et al. (A) Lysine to glutamic acid substitution at aa 244 predicted to effect DDB2 DNA interaction. Red residue indicates site of mutation. Yellow indicates damaged DNA strand. (B) Deletion of aa 349 and substitution of proline for leucine at aa 350. This mutation is predicted to effect the DDB2-DDB1 interaction. Red indicates site of mutation. Mutants were constructed by site directed mutagenesis as described in materials and methods.
observation requires further investigation to determine if Mms1 is involved in UV-dependent DDB2-mediated ubiquitination of XPC, histones and itself.

Despite this genetic evidence, it was plausible that the observed abrogation of suppression of UV sensitive phenotype was due to rapid protein degradation. To rule this out we examined the protein stability of the mutant proteins following UV irradiation. Western blot analysis indicated that the protein stability of DDB2 and its mutants was similar following UV irradiation (Figure 3.4 C and data not shown). Thus, the inability of mutant DDB2 to suppress UV sensitivity is not due to significant differential protein stability.

Taken together, these data suggest that exogenously expressed DDB2 is acting in a physiologically relevant manner in rad26Δ S. cerevisiae cells. Additionally, our data indicate that the DNA damage recognition function of DDB2 is essential for the observed suppression of UV sensitivity. We also found that DDB2 function is dependent on interaction with Mms1 (the yeast homolog of DDB1), a subunit of an E3 ubiquitin ligase. These observations are consistent with what is reported for DDB2 function in human cells.
Figure 3.4: DDB2 mutations abrogate suppression of rad26Δ UV sensitivity. (A) DDB2 mutations abrogate suppression of UV sensitivity. Spotting assays were performed as described in materials and methods. (B) Quantitative plate count assay as described in materials and methods. Graph represents an average of three independent experiments. (C) Mutant DDB2 does not affect protein stability. Wild type and mutant DDB2 were expressed in rad26Δ cells, exposed to 100 J/m² UV and samples were collected at the time points indicated. Cells were lysed and Western blot of whole cell extract (WCE) was carried out as described in materials and methods.
3.1.4 DDB2 Associates with Rad4

DDB2 associates with XPC, the homolog of Rad4 in human cells (Sugasawa, Okuda et al. 2005). To test if this interaction was conserved, co-purification assays were performed as described in materials and methods. Pull-down of DDB2 via nickel beads resulted in the co-purification of Rad4 (Figure 3.5 A). Likewise, the reciprocal experiment, pull-down of Rad4, resulted in the co-purification of DDB2 (Figure 3.5 B). These data indicate this association is conserved, further suggesting DDB2 acts in a physiologically relevant manner in budding yeast.

Figure 3.5: Rad4 associates with DDB2. (A) Rad4 co-purifies with DDB2. Nickel beads were used to pull-down DDB2 as described in materials and methods. Snf6 has been shown to co-purify with Rad4, therefore Snf6-TAP pull-down was performed as a positive control. Western blot using Rad4 antibody was performed as described in materials and methods. (B) DDB2 co-purifies with Rad4. Rad4 pull-down was performed as described in materials and methods. DDB2 was detected in whole cell extract (WCE) and Rad4 pull-down samples using V5 antibody as described in materials and methods.
3.1.5 DDB2 Expression Stimulates CPD Removal in \textit{S. cerevisiae}

DDB2 has been shown to stimulate GG-NER both \textit{in vivo} and \textit{in vitro} (Tang, Hwang et al. 2000; Wakasugi, Shimizu et al. 2001; Wakasugi, Kawashima et al. 2002; Fitch, Nakajima et al. 2003; Pines, Backendorf et al. 2009). Based on this we hypothesized that the observed suppression of UV sensitivity in \textit{rad26}Δ cells expressing DDB2 is due to stimulation of DNA repair. To test this we compared the rate of CPD removal in yeast cells expressing empty vector, wild type DDB2, DDB2 K244E mutant or DDB2 L350P mutant. As expected, the expression of wild type DDB2 in \textit{rad26}Δ cells enhances the rate of CPD removal from genomic DNA (Figure 3.6 A and B). Additionally, consistent with spotting assays the two DDB2 mutants bearing mutations found in XPE patients failed to stimulate repair of CPDs. These data strongly suggest that the increased UV resistance observed in the spotting assays (Figure 3.2 A) is a result of increased DDB2-mediated DNA repair.

In conclusion, these data suggest \textit{S. cerevisiae} is an effective model system to study the mechanistic details of DDB2-mediated GG-NER. Use of this simplified system will enable studies to focus on DDB2’s direct role in GG-NER. Consistent with what is reported in human cells, DDB2 (1) stimulates GG-NER, (2) decreases UV sensitivity, (3) clinically relevant mutations abrogate these functions, and (3) associates with conserved binding partners.
Figure 3.6: Wild type DDB2 expression stimulates the repair of CPD. (A) rad26Δ cells expressing empty vector, wild type, or mutant DDB2 were exposed to 100 J/m² UV and samples were collected at the time points indicated. DNA was extracted and blotted as described in materials and methods. Western blot was carried out using anti-CPD antibody as described in materials and methods. Southern blot serves as loading control. (B) Quantitation representative of 4 independent trials.
3.2. Role of Ubiquitination in DDB2-Mediated GG-NER

As discussed in the introduction, the mechanistic role of DDB2 ubiquitination in GG-NER remains to be elucidated. Having successfully recapitulated DDB2-mediated GG-NER in budding yeast we hypothesized that using this simplified model system we could gain more insight into the role of ubiquitination in this process.

Key Findings

1. UV exposure increases the association of ubiquitin and DDB2 in *S. cerevisiae*

2. DDB2 degradation is not stimulated following UV exposure in *S. cerevisiae* cells

3. UV sensitivity is increased in ubiquitination deficient *S. cerevisiae* cells expressing DDB2

4. Ubiquitination is involved in the release of DDB2 from chromatin, however, this is not UV-dependent

3.2.1 UV Stimulates DDB2 Association with Ubiquitin

There are several reports that ubiquitination of DDB2 is stimulated upon UV irradiation (Chen, Zhang et al. 2001; Nag, Bondar et al. 2001; Matsuda, Azuma et al. 2005). We tested if DDB2 is ubiquitinated in our system in a UV-dependent manner. DDB2 was purified from UV-irradiated cells with nickel beads; and Western blots with both anti-ubiquitin and anti-V5 antibodies were performed as described in materials and
methods. Consistent with what was previously observed in human cells, we observed UV-stimulated DDB2 ubiquitination (Figure 3.7 A). Although detectable, the ubiquitination levels of DDB2 were low. This low level of ubiquitinated DDB2 is possibly due to rapid DDB2 turnover. To test this hypothesis, we repeated the experiment in the absence of an active proteasome. As expected, when the proteasome was inhibited by MG132 treatment the detectable levels of ubiquitinated DDB2 increased. Importantly, the UV-stimulation of DDB2 ubiquitination is still observed (Figure 3.7 B). Interestingly, when the proteasome was inhibited, a band is detected at 55 kDa, the expected size for mono-ubiquitinated DDB2, 60 minutes post-UV irradiation. The significance of this observation requires further investigation.

It is possible that the ubiquitin antibody is recognized ubiquitinated proteins that may have co-purified with DDB2, such as Rad4. To minimize this possibility, we increased the stringency of the purification conditions. Additionally, as expected, the ubiquitin signal is a smear. Therefore, it can be concluded that, consistent with what is reported in human cells, the majority of the ubiquitinated protein being detected is DDB2.
Figure 3.7: DDB2 is ubiquitinated following UV irradiation. (A) Ubiquitination of DDB2 increases in a time dependent manner following UV irradiation. Cells were treated as described in materials and methods. DDB2 was purified by pull-down and ubiquitination was determined by Western blot using anti-ubiquitin antibody. DDB2 levels were determined by Western blot using anti-V5 antibody. (B) Inhibition of proteasome by addition of MG132 increases detectable levels of ubiquitinated DDB2. Cells were treated with MG132 as described in materials and methods. Purification and Western blotting were performed as described for A. All samples are BY4741 rad26Δ +DDB2. Cells were treated with 100 J/m² UV irradiation, allowed to repair in safe light for the time indicated.
3.2.2 UV Does Not Stimulate DDB2 Degradation

There are data to indicate that in human cells, UV irradiation induces rapid ubiquitin dependent DDB2 degradation (Rapic-Otrin, McLenigan et al. 2002; Fitch, Cross et al. 2003; Chen, Zhang et al. 2006; El-Mahdy, Zhu et al. 2006). Therefore, we examined if the observed DDB2 ubiquitination in our yeast system was associated with UV-stimulated protein degradation.

The steady state levels of DDB2 do not change following UV irradiation (Figure 3.8). Next, DDB2 protein turnover was examined in UV and mock treated cells as described in materials and methods. Nascent DDB2 production was inhibited by the addition of 4% glucose immediately post-UV exposure. No significant alterations in DDB2 turnover are observed following UV irradiation (Figure 3.8 B). These data suggest that, unlike what is reported in human cells, UV irradiation does not induce rapid DDB2 degradation in budding yeast. However, under the same conditions, DDB2 enhanced GG-NER of CPDs (Figure 3.6) and rendered rad26Δ cells more resistant to UV irradiation (Figure 3.2 A). Taken together, it appears that degradation of DDB2 is not absolutely required for the observed DNA repair function. This conclusion is consistent with what is reported in the mouse model, DDB2 degradation was inhibited and increased CPD repair was observed (Liu, Lee et al. 2009).
Figure 3.8: UV does not stimulate DDB2 degradation. (A) UV does not affect steady state levels of DDB2. Whole cell extracts (WCE) were collected post UV irradiation and total DDB2 levels were determined by Western blot using anti-V5 antibody. Equal loading was verified by coomassie staining. (B) UV irradiation does not augment DDB2 turnover. Nascent protein synthesis was inhibited by addition of glucose following UV or mock treatment as described in materials and methods. WCE Western blot were performed as described for A. Strain used here is BY4741 rad26Δ+DDB2. Cells were treated with 100 J/m² UV, allowed to repair in safe light for the time indicated, and lysed as described in materials and methods.
3.2.3 DDB2 Degradation is Ubiquitin Dependent

These observations prompted the question, “what is the role of the observed UV-stimulated DDB2 ubiquitination?” To further investigate the potential role of the ubiquitination pathway in DDB2-mediated stimulation of GG-NER, we utilized a yeast E1 t\(^\text{s}\) mutant (Ghaboosi and Deshaies 2007). Like the NER pathway, the ubiquitin pathway is conserved in yeast. Unlike human cells, yeast has only one E1 gene, UBA1, therefore inactivation of the UBA1 gene product will effectively inactivate the entire ubiquitin pathway.

First, DDB2 was transformed into E1 t\(^\text{s}\) mutant cells. DDB2 expression in this strain does not alter its ubiquitination deficiency as assessed by temperature sensitivity. Both E1 t\(^\text{s}\) mutant and E1 t\(^\text{s}\) mutant expressing DDB2 remain sensitive to high temperature (Figure 3.9).

![Figure 3.9: DDB2 expression does not affect E1 t\(^\text{s}\) mutant temperature sensitivity phenotype. DDB2 was transformed into E1 t\(^\text{s}\) mutant cells as described in materials and methods. Cells were grown for three days at the permissive or non-permissive temperature as indicated.](image)
In addition to complete inactivation of the ubiquitin pathway at the non-permissive temperature, it was demonstrated previously by Ghaboosi et al. (Ghaboosi and Deshaies 2007) that this E1 t⁰ mutant has increased sensitivity to cadmium chloride at the permissive temperature indicating that the ubiquitination pathway is only partially active even under permissive conditions.

The effect of ubiquitination on degradation was examined. DDB2 degradation seems to be dependent on an active ubiquitin pathway (Figure 3.10). This observation is consistent with increased levels of ubiquitinated DDB2 in the absence of active proteasome (Figure 3.7). The decreased DDB2 levels at 120 minutes post addition of glucose in the E1 t⁰ mutant strain may be a result of non-specific protein degradation following cell death. Alternatively, since DDB2 is an exogenous protein it maybe being degraded in a less efficient, non-specific manner in the absence of ubiquitin.

![Coomassie staining verified equal loading. Wild type (WT) is RJD3268 and E1 t⁰ mutant is RJD3269 expressing DDB2.](image)

**Figure 3.10: DDB2 degradation is ubiquitin dependent.** Cells were grown as described in materials and methods, shifted to 37°C to inactivate the ubiquitin pathway for one hour. Glucose was added to stop nascent DDB2 production and samples were collected at the time points indicated. Cell lysis and Western blots of whole cell extract (WCE) using anti-V5 antibody were carried out as described in materials and methods. Coomassie staining verified equal loading. Wild type (WT) is RJD3268 and E1 t⁰ mutant is RJD3269 expressing DDB2.
3.2.4 Ubiquitination is Involved in DDB2 Release from the Chromatin

These previous experiments suggest that DDB2 turnover is dependent on ubiquitination, but independent of UV irradiation. However, the question remains, what is the role of the observed UV-stimulated DDB2 ubiquitination. Based on a previous *in vitro* study indicating ubiquitinated DDB2 has lower affinity for damaged DNA (Sugasawa, Okuda et al. 2005), we hypothesized that ubiquitination of DDB2 results in release from the chromatin.

Initially, we accessed the UV sensitivity phenotype of E1 t\(^e\) mutant cells expressing DDB2. Surprisingly, expression of DDB2 in the E1 t\(^e\) mutant renders cells slightly more sensitive to UV irradiation at 25 °C (Figure 3.11 A). Importantly, the E1 t\(^e\) mutant expressing mutant DDB2 L350P behaved the same as the E1 t\(^e\) mutant itself (Figure 3.11). This indicates that the observed increase in UV sensitivity is a result of functional DDB2 instead of an artifact of exogenous protein production. When temperatures were increased to 35°C (semi-permissive temperature for this mutant), no cells survived identical exposure to UV irradiation (Figure 3.11 B). This lack of cell survival at a semi-permissive temperature is presumably due to the multiple stresses of temperature, UV irradiation and an impaired ubiquitin pathway. Taken together, these data suggest that DDB2 requires a fully functional ubiquitination pathway to function in DNA repair.
Figure 3.11: DDB2 expression in E1 mutant cells renders them more sensitivity to UV irradiation. (A) Wild type and mutant DDB2 L350P were transformed into E1 t" mutant cells. The spotting assay was conducted as described in materials and methods. Cells were exposed to UV irradiation at the dose indicated. Cells were grown at 25°C for 3 days in dark. (B) UV sensitivity was tested at the semi-permissive temperature, 35°C. There was no growth in either E1 t" mutant strain. Strains used in these experiments are as indicated. Wild type strain is RJD 3268. UV dosage is as indicated.

This observed phenotype combined with no observable UV-stimulated degradation of DDB2 (Figure 3.7 A and B) suggests an alternative role for ubiquitination in DDB2-mediated GG-NER. To investigate what this role might be, we monitored soluble and chromatin bound levels of DDB2. We hypothesized the ubiquitin pathway may be involved in DDB2 localization. Indeed, our data suggest that in the absence of a fully functional ubiquitin pathway, DDB2 remains bound to the chromatin. At both 60 and 120 minutes post-UV irradiation approximately 20% more DDB2 remained bound to the chromatin in ubiquitin deficient cells compared to wild type cells (Figure 3.12 A and D). To assess if this is a strain specific phenomenon the localization of DDB2 following
UV irradiation was evaluated in BY4741 \textit{rad26}\textDelta cells. Figure 3.12 D shows that this strain behaves as the E1 t\textsuperscript{E} isogenic wild type. To ensure this was an ubiquitin dependent phenomenon, we also did the experiment at the non-permissive temperature. A slight increase in chromatin bound DDB2 was observed in E1 t\textsuperscript{E} mutant cells; unlike the wild type cells in which DDB2 is released (Figure 3.12 B and E). These observations suggest that the ubiquitination pathway controls the release of DDB2 from the damaged chromatin following UV irradiation. Steady state DDB2 expression does not alter the ubiquitin dependent release of DDB2 from chromatin (Figure 3.13).

Next, we tested if this was a UV-dependent phenomenon. Experiments were conducted as before with complete inactivation of the ubiquitin pathway in the absence of UV irradiation. There is no significant difference in the release of DDB2 in wild type cells treated with UV or mock treated (Figure 3.12). However, even in the non-UV treated samples, more DDB2 remains associated with the chromatin in E1 t\textsuperscript{E} mutant cells. These data suggest release of DDB2 from chromatin is dependent on ubiquitination independent of damage. Since DDB2 is a DNA binding protein it will bind DNA non-specifically, therefore release is necessary. These data suggest that this release is dependent on ubiquitination both in the presence and absence of UV-induced DNA damage. Because DDB2 has been shown to preferentially bind damaged DNA, it was surprising that there was no observable increase in DDB2 association with chromatin in wild type cells treated with UV. On the contrary, in E1 t\textsuperscript{E} mutant cells UV irradiation seems to stabilize binding (Figure 3.12). These data are consistent with our hypothesis that ubiquitination of DDB2 is necessary for release from chromatin.
In conclusion, these data suggest a possible mechanism by which DDB2 binds DNA and is released by ubiquitination. The ubiquitination and release of DDB2 from chromatin is independent of UV irradiation, but UV-stimulated ubiquitination is observed. Perhaps this increase in ubiquitinated DDB2 is due to increased association of DDB2 with DNA. Additionally, the role of ubiquitinated release of DDB2 from chromatin in non-irradiated cells needs to be further investigated.
Figure 3.12: DDB2 release from chromatin is dependent on ubiquitin. (A). Impaired ubiquitin pathway suppresses release of DDB2 from chromatin. E1 t\(^{a}\) mutant and WT cells were grown at 25\(^{\circ}\)C and treated with 100 J/m\(^2\) UV. (B) Inhibited ubiquitin pathway prevents release of DDB2 from chromatin. E1 t\(^{a}\) mutant and WT cells were incubated at 37\(^{\circ}\)C and treated with 100 J/m\(^2\) UV. (C) Absence of UV exposure does not alter ubiquitin mediated release of DDB2 from chromatin. E1 t\(^{a}\) mutant and WT cells were incubated at 37\(^{\circ}\)C. No exposure to UV. Time 0 represents time at which glucose was added. Cells were treated as indicated in materials and methods and collected at time points indicated. (D) Quantitative data of impaired ubiquitin pathway (25\(^{\circ}\)C). (E) Quantitative data of inhibited ubiquitin pathway (37\(^{\circ}\)C). Data was produced using Image J. % represents amount of DDB2 bound at time x / amount of DDB2 bound at time 0, normalized to histone H3. Points represent the average of 3 independent trials for E1 t\(^{a}\) mutant +UV 25 and 37\(^{\circ}\)C, WT +UV 37\(^{\circ}\)C; two trials for WT +UV 25\(^{\circ}\)C and all –UV samples. rad26\(^{\Delta}\) 25\(^{\circ}\)C was done only one time. Error bars represent the standard deviation for each sample. Strains used in these experiments are BY4741rad26\(^{\Delta}\), RJD3268 (WT) and RJD3269 (E1 t\(^{a}\) mutant) all expressing WT DDB2.
Figure 3.13: Steady state levels of DDB2 do not alter ubiquitin dependent release. (A) Inhibited ubiquitin pathway prevents release of DDB2 from chromatin in presence of nascent protein production. (B) Impaired ubiquitin pathway suppresses release of DDB2 from chromatin in presence of nascent protein production.
3.3. Recruitment of SWI/SNF to Silent HML Locus Following UV Irradiation

In addition to histone modifications, chromatin can be remodeled in an ATP-dependent manner. ATP-dependent chromatin remodeling has been implicated in GG-NER as discussed in the introduction. However, the mechanistic details of how ATP-dependent chromatin remodeling complexes contribute to GG-NER remain to be elucidated. We begin to investigate this by examining the recruitment steps of a SWI/SNF component to the silent HML locus in the absence of various NER proteins.

Key Findings:

1. Snf6 Localization to HML is Dependent on Rad16, but not Rad4

3.3.1 Rad4 Co-Purifies with Snf6

It was demonstrated initially by Gong et al. that Rad4 co-purifies with components of the SWI/SNF complex (Gong, Fahy et al. 2006). In their study, both Snf6 and Snf5 were found to associate with Rad4 in a UV-dependent manner. It is theoretically possible that the association observed by Gong et al. (Gong, Fahy et al. 2006) is due to the ectopic expression of Snf6. Therefore, we reproduced these findings using the chromosomally expressed Snf6-TAP strain. Consistent with previous observations Rad4 associates with Snf6 in a UV-dependent manner (Figure 3.15).
3.3.2 Snf6 Localization to HML is Dependent on Rad16, but not Rad4

Recruitment of the SWI/SNF remodeling complex to chromatin during transcription via transcription factors and histone acetylation is established. However, despite its documented role in GG-NER how SWI/SNF is recruited to a DNA lesion is unknown. Gong et al. propose a model in which SWI/SNF may either be recruited by Rad4, or vice versa (Gong, Fahy et al. 2006). We use chromatin immunoprecipitation to investigate the role of Rad4 and Rad16 in recruitment of Snf6 to the silent HML locus following UV irradiation.

Consistent with previous reports (Nag, Kyriss et al. 2010), our data indicate Snf6 binds to the HML locus following UV irradiation (Figure 3.16). Despite the observed association of Snf6 and Rad4, Snf6 does not appear to require Rad4 for recruitment to damaged chromatin. Interestingly, our data suggest not only does Snf6 not require Rad4, it may be inhibitory. In the absence of Rad4 Snf6 binds to HML immediately following UV irradiation.
UV irradiation, and remains bound. However, in wild type cells, Snf6 appears to localize to HML 30 minutes post-UV irradiation and return to non-treated levels at 60 minutes post exposure (Figure 3.16). This apparent delay of Snf6 localization in the presence of Rad4 requires further investigation. Additionally, the binding of Rad4 to the HML locus in the absence of SWI/SNF also needs to be investigated. Although more detailed studies are necessary, these data suggest Rad4 is not an absolute requirement for SWI/SNF association with damaged chromatin.

Rad16 has also been implicated in the lesion detection step of GG-NER as discussed in the introduction. Therefore, despite no previously observed association of SWI/SNF and Rad16, we tested if Rad16 is required for Snf6 binding to the HML locus following UV exposure. Indeed it is. In the absence of Rad16 Snf6 does not localize to the HML locus (Figure 3.16).

Taken together these data suggest a possible sequence of events as follows: Rad16 recruitment to and immediate release from the damage site, SWI/SNF recruitment and subsequent remodeling followed by Rad4-Rad23 recruitment. Rad4-Rad23 then recruits subsequent downstream repair factors and NER progresses. This possible model is depicted in figure 3.17, modified from Gong et al. (Gong, Fahy et al. 2006). The role of histone modifications in this sequence of events is a key component that needs to be investigated as well. These data provide suggestive evidence that SWI/SNF activity maybe involved in recruitment of Rad4 as initially suggested by Gong et al. (Gong, Fahy et al. 2006). However, the recruitment of Rad4 in the absence of SWI/SNF needs to be evaluated first.
Figure 3.16: SWI/SNF recruitment to chromatin is dependent on Rad16, but not Rad4. Snf6 ChIP analysis of silent HML locus. (A) Representative gels of PCR amplification of HML region in Snf6-TAP, rad4Δ and rad16Δ and BY4741 wild type (WT) strains following 100 J/m² UV irradiation. Mutations were in Snf6-TAP background. Samples were collected at time points indicated and ChIP was carried out as described in materials and methods. (B) Quantitative data is average of 3 independent experiments, except WT.
Figure 3.17: Model for sequence of events in SWI/SNF-mediated GG-NER. DNA damage promotes histone modifications and initial Rad7-Rad16 binding. The Rad7-Rad16 complex is released and either SWI/SNF binds and remodels chromatin or Rad4-Rad23 binds. This is followed by subsequent recruitment of necessary proteins and progression of NER.
CHAPTER 4: DISCUSSION

As more research is conducted, the complexity of GG-NER is being highlighted. Of particular interest is the lesion detection step in the context of chromatin. It is becoming more evident that the role of chromatin remodeling in GG-NER is important (Gong, Fahy et al. 2006; Zhang, Zhang et al. 2009; Zhao, Wang et al. 2009). Both post-translational modifications and ATP-dependent remodeling have been implicated in this process. However, the mechanism by which this occurs remains elusive. The work presented here contributes to our further understanding of how these processes affect the lesion detection step of GG-NER. We established and utilized budding yeast as an alternative model system to study the role of ubiquitination in DDB2-mediated GG-NER. Additionally, we utilized budding yeast to investigate the initial binding sequence of events in SWI/SNF-mediated GG-NER.

4.1 DDB2-Mediated GG-NER

The role of DDB2 in DNA damage response is complex. While published data agree DDB2 is involved, what role it is playing is under debate. DDB2 has been shown to be involved in DNA damage response both indirectly and directly. There is a body of research that indicates DDB2 protects cells from deleterious effects of DNA damage indirectly via its role in cell cycle DNA damage response checkpoints, and stimulation of pro-apoptotic events (Itoh, O'Shea et al. 2003; Stoyanova, Yoon et al. 2008; Stoyanova, Roy et al. 2009; Bagchi and Raychaudhuri 2010). Several lines of evidence, both in vitro and in vivo, support this hypothesis. For example, in vivo data using DDB2 deficient cell lines suggest that there is no physiologically significant defect in removal of UV-induced
lesions compared to cells expressing DDB2 (Itoh, Linn et al. 2000). Additionally, XPE cells (cells isolated from XPE patients, in which DDB2 is known to be deficient) have decreased expression of p53 (Itoh, O'Shea et al. 2003). Consistent with the reduced p53 levels UV-induced apoptosis is suppressed in these cells (Itoh, O'Shea et al. 2003). Various *in vitro* data suggest that DDB2 does not stimulate excision of damaged DNA (Kulaksiz, Reardon et al. 2005).

However, on the other hand, there is data to indicate DDB2 is directly involved in repair. For instance, XPE cells are sensitive to UV. This sensitivity has been shown to be due to decreased CPD removal specifically via the GG-NER pathway in these cells (Tang, Hwang et al. 2000; Wakasugi, Kawashima et al. 2002; Fitch, Nakajima et al. 2003; Pines, Backendorf et al. 2009). Additionally, *in vivo* data suggest DDB2 is the first protein to form foci at DNA damage sites (Wakasugi, Kawashima et al. 2002; Fitch, Nakajima et al. 2003). Consistent with the co-localization data, and a plausible explanation, is evidence that DDB2 directly binds damaged DNA (Scrima, Konickova et al. 2008). It is also reported that DDB2 has an increased affinity for damaged DNA (Chu and Chang 1988; Kulaksiz, Reardon et al. 2005).

The observed indirect and direct involvement of DDB2 in the DNA damage response does not have to be mutually exclusive. In fact it has been proposed that DDB2 acts as a sensory protein that detects the level of damage and controls the cellular response (Stoyanova, Roy et al. 2009). This needs to be further investigated. However, it will be critical to first determine the molecular mechanisms of DDB2-mediated GG-NER. Deciphering these mechanistic details will allow inactivation of this function of DDB2 so the indirect effects of DDB2 on DNA damage response can be studied more
effectively. The establishment of budding yeast as an alternative, simplified model system described herein provides a tool to begin to elucidate these details.

Although DDB2 has been implicated in recruitment of XPC to CPD sites, how this occurs is not understood. The predominate model to explain this suggests that DDB2 is ubiquitinated and subsequently degraded to allow binding of XPC and progression of GG-NER (Wang, Wani et al. 2005; Chen, Zhang et al. 2006; El-Mahdy, Zhu et al. 2006). However, as indicated in the introduction, there are lines of evidence that contradict this model (Luijsterburg, Goedhart et al. 2007; Liu, Lee et al. 2009). As previously indicated, DDB2 is the targeting component of an E3 ubiquitin ligase (Jackson and Xiong 2009). It has been shown to be responsible for the UV-dependent ubiquitination of XPC, histones and itself (Chen, Zhang et al. 2001; Nag, Bondar et al. 2001; Matsuda, Azuma et al. 2005; Sugasawa, Okuda et al. 2005; Kapetanaki, Guerrero-Santoro et al. 2006; Wang, Zhai et al. 2006). However, the physiological importance of these ubiquitination events is not understood. Therefore, we utilize an alternative, simplified budding yeast model system to investigate the role of ubiquitination in DDB2-mediated GG-NER.

Consistent with previous reports (Liu, Lee et al. 2009), our data suggest UV-induced DDB2 degradation is not a requirement for GG-NER progression. Also consistent with what has been demonstrated in human cells, DDB2 degradation is ubiquitin dependent and DDB2 is ubiquitinated in a UV-dependent manner. Although there was no observed UV-stimulated DDB2 degradation, our data suggest ubiquitination is important in DDB2-mediated DNA damage response as indicated by the increased UV sensitivity of ubiquitin deficient cells (Figure 3.11). This observation, in conjunction with previous in vitro data to suggest ubiquitinated DDB2 has a reduced affinity for DNA
(Sugasawa, Okuda et al. 2005), prompted us to examine the effect of ubiquitination on DDB2 association with chromatin. Indeed, our data suggest ubiquitination is involved in DDB2 release from chromatin (Figure 3.12). However, further investigation is required to determine if this ubiquitin dependent release is the result of mere release followed by degradation or direct degradation from the chromatin, as is discussed further in appendix A1.1. Both are theoretically plausible, as ubiquitination has been demonstrated to be involved in both protein localization and degradation (Sadowski and Sarcevic 2010). Additionally, it is well established that the proteasome can degrade proteins from the chromatin.

Based on the data reported here and previously published data to indicate DDB2 associates with chromatin remodeling factors, we propose the following model: in the presence of UV-induced DNA damage, the DDB1-DDB2 complex identifies the lesion, ubiquitinates histones which contribute to chromosome remodeling. DDB complex then recruits XPC-hRad23 to UV damage sites, presumably via the reported physical interaction between DDB2 and XPC (Sugasawa, Okuda et al. 2005). Subsequent ubiquitination of DDB2 and XPC results in the release of DDB2 from the lesion (or direct degradation). NER then proceeds to eventually repair the lesion (Figure 4.1). In the absence of UV-induced DNA damage, DDB1-DDB2 complex will bind DNA, but is then ubiquitinated and released immediately. The exact sequence of events needs to be further investigated to determine if the observed association of DDB2 and chromatin remodeling proteins is due to DDB2 recruitment of these complexes or vice versa. Of note, mono-ubiquitination has been implicated in DNA repair. When the proteasome was inhibited, a band correlating with the expected size of mono-ubiquitinated DDB2 was
detected 60 minutes post-UV irradiation. Because additional ubiquitin species were identified, it is plausible that this is just an intermediate species that was captured with no physiological relevance. Nonetheless, this is an interesting observation that requires more investigation.

Figure 4.1: Schematic to illustrate proposed model for DDB2-mediated lesion detection in chromatin. DNA damage is recognized by DDB2 which is associated with chromatin remodeling complexes either before binding lesion or soon after. Chromatin is then remodeled to expose the damaged DNA. DDB2 then recruits XPC-hRad23 to the lesion site. The DDB2 containing E3 ligase ubiquitinates histones, XPC and DDB2 itself. Histone ubiquitination may destabilize nucleosomes and facilitate chromatin remodeling. Ubiquitinated DDB2 is released and GG-NER proceeds.
Interestingly, in wild type cells this ubiquitin dependent release from chromatin does not appear to be UV-dependent. This observation suggests that perhaps auto ubiquitination of DDB2 is not physiologically relevant for GG-NER progression, but rather is necessary for normal turnover of DDB2 from the chromatin. DDB2 is a DNA binding protein, and it has been shown to bind DNA in the presence and absence of damage (Kulaksiz, Reardon et al. 2005; Minig, Kattan et al. 2009). Therefore, it would logically follow that it will require release. Our data suggest this release is dependent on an active ubiquitination pathway. Alternatively, DDB2 is recognizing abnormal DNA structures in DNA that are intrinsic.

Despite no observed UV effect on the ubiquitin dependent release of DDB2 from chromatin, we observed UV-stimulated ubiquitination of DDB2. There are two plausible explanations for these observations. The simplest is that this increased ubiquitination post-UV irradiation is due to increased DDB2 association with the chromatin. Although no UV-stimulated DDB2 chromatin association was observed in wild type cells, the increased affinity of DDB2 for damaged DNA has been established. Additionally, in the absence of ubiquitination, an increase of chromatin bound DDB2 was observed following UV irradiation. This increased association of DDB2 with chromatin post-UV irradiation, suggests that indeed in this model system DDB2 association with damaged DNA is increased. Therefore, perhaps the inability to observe UV-stimulated binding in wild type cells is masked by the background binding. Of course this hypothesis requires experimental validation.

Alternatively, perhaps the increased ubiquitination signal we and others observe is not due to physiologically relevant, direct modifications of DDB2, but rather, its role in
ubiquitination of other proteins. Indeed it has been demonstrated that ubiquitination is necessary for DDB2 stimulation of NER \textit{in vitro} (Sugasawa, Okuda et al. 2005). This hypothesis requires further investigation to delineate the cause of this increase in ubiquitination of DDB2 post-UV irradiation. First it will need to be determined that DDB2 is functioning as the targeting component of an E3 ubiquitin ligase complex in budding yeast. Once this is verified, various DDB2 lysine mutations can be constructed and screened for their effect on the observed UV-stimulated ubiquitination. Additionally, these mutants could be screened for their ability to suppress the UV sensitivity phenotype of \textit{rad26$\Delta$} cells.

Another interesting observation from this work is the finding that Mms1 is required for effective DDB2 function (Figure 3.4). This observation provokes an interesting possibility that Mms1 may play a role in the endogenous yeast GG-NER pathway. It also prompts the speculation of an unidentified DDB2 homolog in budding yeast. Mms1 acts in complex with Rtt109 and forms a DDB1-Cul4a-like E3 ubiquitin ligase complex (Zaidi, Rabut et al. 2008). This complex was shown to promote replication through damaged DNA (Zaidi, Rabut et al. 2008). Investigation of a potential role for this complex in GG-NER will be an interesting avenue for future study.

Additionally, our data suggest that on a gross functional level, DDB2 and Rad16 are not functional analogs. Despite reported similarities in biochemical properties as described in the introduction, DDB2 expression was unable to suppress the UV sensitivity of \textit{rad16$\Delta$} cells (Figure 3.2). Rad16 has been implicated in post-incision events in GG-NER (Reed, You et al. 1998), while DDB2 has not. Perhaps DDB2 and
Rad16 perform analogous functions in the lesion detection step, but not the post-incision function of Rad16. This is yet another hypothesis that requires experimental validation.

It is necessary that DDB2 function in DNA repair continue to be studied as mutations in it have been implemented in both the genetic disease XP, and breast cancer (Tang and Chu 2002; Kattan, Marchal et al. 2008). Because DDB2’s documented physiological roles are diverse; delineating its specific role in GG-NER becomes more complex. The budding yeast model system described herein provides an alternative, simplified system to further investigate the mechanistic means by which DDB2 facilitates GG-NER.

4.2 UV-Stimulated Recruitment of SWI/SNF to Silent HML Locus

The importance of ATP-dependent chromatin remodeling complexes in GG-NER is becoming more evident. We began to investigate the sequence of events in initial lesion detection in the context of chromatin. Using chromatin immunoprecipitation (ChIP) analysis our data suggest that SWI/SNF can localize to damaged chromatin independent of the lesion detection protein complex, Rad4-Rad23. Interestingly, Snf6 localized to the HML locus immediately following UV-exposure in the absence of Rad4 and remained bound one hour post-UV irradiation. SWI/SNF is known to bind complex structures (Quinn, Fyrberg et al. 1996; Zlatanova and van Holde 1998). Perhaps this is why it is binding damaged DNA in the absence of Rad4. Additionally, the prolonged binding maybe a result of retarded or lack of recruitment of downstream factors as RAD4 mutant cells are known to have a moderate to severe UV sensitive phenotype. This will need further investigation. It is interesting that the temporal recruitment of Snf6 in the presence
and absence of Rad4 differs. This result requires further investigation, but perhaps Rad4 has higher affinity for damaged DNA, therefore it is recruited to damage more efficiently, and occludes the damaged site from SWI/SNF binding. The maximal binding of Snf6 at 30 minutes post-UV irradiation in WT cells may be a result of binding to lesions that are “buried” therefore required initial chromatin remodeling prior to Rad4 lesion identification. Further studies need to be conducted to investigate the ability of SWI/SNF to identify and bind lesions. Perhaps, since Rad4-Rad23 binds the lesion on the opposite strand, it is possible that it is recognizing lesions that face toward the nucleosome core, and then recruits SWI/SNF to “open” the area.

Interestingly, despite lack of co-localization data in budding yeast of Rad16 and SWI/SNF (Gong, Fahy et al. 2006), Rad16 seems to be necessary for Snf6 recruitment to the HML locus following UV irradiation (Figure 3.16). These data argue that perhaps SWI/SNF is not able to directly identify DNA damage in the context of chromatin; rather it suggests Rad7-Rad16 is upstream of SWI/SNF dependent chromatin remodeling. More experiments need to be conducted. Taken together with the previous published lack of association between Rad16 and Snf6, these data suggest Rad7-Rad16 may be involved in initial preparation of the lesion area for subsequent detection and remodeling that is necessary for repair. The mechanism by which this could happen requires further investigation as it could be a result of post-translational modifications induced by the Rad7-Rad16 complex, as Rad16 has been shown to be involved in E3 ubiquitin ligase activity. Alternatively, it could be acting to remodel chromatin in a more direct way; it is a member of the SNF2 family (Eisen, Sweder et al. 1995), however, no direct remodeling activity has been shown to date.
As initially proposed by Gong et al. (Gong, Fahy et al. 2006), histone modifications such as acetylation, may be important in the recruitment of SWI/SNF to UV damaged sites. It has been shown in transcriptional regulation that acetylation of histone lysine tails plays a role in SWI/SNF recruitment. This recruitment via binding of SWI/SNF bromo-domains could be playing a critical role in UV-dependent SWI/SNF mediated chromatin remodeling. This is an important component that was not tested here.

The lesion identification step is arguably the most critical step in GG-NER without it there will be no repair. However, it remains the least understood step in GG-NER. Chromatin structure complicates elucidation of this step. Although progress is being made, this step of GG-NER requires further study.
REFERENCES:


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APPENDIX 1: EXPERIMENTAL APPROACHES FOR FUTURE EXPERIMENTS

A1.1 Delineating release of chromatin bound degradation of DDB2

To do this, experiments that examine the localization of DDB2 in the absence of an active proteasome would need to be conducted. This could be done utilizing the method for chromatin fractionation described herein, combined with the proteasome inhibition method also utilized here. I speculate that in the absence of an active proteasome but in the presence of functional ubiquitination pathway, DDB2 will be released from the chromatin and accumulate in the soluble fraction. This speculation is based on previous in vivo work by Luijsterburg et al. (Luijsterburg, Goedhart et al. 2007) indicating the binding of DDB2 to damaged DNA does not trigger degradation. Additionally, our data also shows no UV-stimulated DDB2 degradation. These would be informative experiments as the results may begin to shed light on the direct and indirect roles of DDB2 in DNA damage response.

A1.2 Relevance of observed mono-ubiquitination of DDB2 60 minutes post-UV irradiation

First, it would need to be determined by mass spec analysis that the band migrating at the calculated size of mono-ubiquitinated DDB2 is indeed DDB2. Additionally, various other bands should also be tested by mass spec to unequivocally determine the majority of identified ubiquitinated proteins are indeed DDB2. I suspect that this band is an intermediate of DDB2 poly-ubiquitination. This speculation is based
on the fact that it was only identified in the absence of an active proteasome. Previous reports indicated that poly-ubiquitination generally results in ubiquitin dependent degradation. Therefore if this is an independent species, it should have been observable in the presence of an active proteasome. With that said it has also been shown that mono-ubiquitination is involved in DNA damage response, therefore, this species may be responsible for release and then subsequent ubiquitination steps may occur that result in degradation.

A1.3 The UV-induced association of DDB2 and chromatin in WT cells is masked by background levels

This could be addressed by conducting dose-dependent experiments at early time points. By increasing the level of DNA damage via increased UV irradiation, there should be a point at which the chromatin associated DDB2 levels are induced above background in the presence of ubiquitination mediated release.

A1.4 Observed UV-stimulated increase in ubiquitin DDB2 is result of its role in an E3 ubiquitin ligase complex.

To address this hypothesis several things could be done. The role of DDB2 as a functional component of an E3 ubiquitin ligase in budding yeast would need to be established. Based on the genetic evidence that Mms1, a known component of an E3 ligase, is required for DDB2 function, and a mutation that may potentially abrogate this interaction also abrogates DDB2 function, I suspect indeed it is. First, the association of
DDB2 and Mms1 would need to be established, this would be done via co-purification assays followed by Western blotting. This would establish DDB2 as a component of an E3 ubiquitin ligase complex. Then known targets would need to be verified, again this could be done by Western blotting in various knockout strains to see if there is an effect on the ubiquitin state of the target protein. Alternatively, Western blot analysis of DDB2 ubiquitination levels in the absence of Mms1 should also be investigated to determine if DDB2 ubiquitination is dependent on Mms1. Also, both the co-purification and ubiquitination state of the mutant DDB2 L350P should be investigated.

It is currently not known what lysine is ubiquitinated in DDB2 post-UV irradiation, therefore a systematic lysine mutation analysis to determine what, if any will abrogate the observed UV-stimulated ubiquitination could also be performed. The release of DDB2 from chromatin should also be determined in these DDB2 mutants. It is theoretically plausible that different lysines are necessary for different ubiquitination events and protein fate. These experiments could be done in the alternative, simplified yeast system described. The rapid growth rate, compared to human cells offers an advantage for these types of screening experiments.

**A1.5 Role Mms1 in endogenous yeast GG-NER**

As indicated previously, the potential role of Mms1 in endogenous yeast GG-NER is interesting. To test this, pull-down of Mms1 post-UV irradiation followed by Western blot for known GG-NER proteins could provide a starting point. If it is found that Mms1 associates with known GG-NER proteins in a UV-dependent manner, the implications of Mms1 deletion could be investigated using DNA repair assays at the
If no association between Mms1 and known GG-NER proteins are observed, perhaps it is still involved in endogenous GG-NER via unknown protein (a homolog/analog to DDB2). This could be tested by mass spec analysis following pull-down to identify potential binding partners. Then a mutagenic screen to determine if knockout of any of these has an effect on UV sensitivity or DNA repair following UV irradiation.

**A1.6 DDB2 and Rad16 maybe functional analogs in lesion detection step.**

The direct function of Rad16 in lesion detection is not known, however one place to start could be the localization to silent HML locus. The data presented here suggests Rad16 plays a role in SWI/SNF recruitment to the HML locus post-UV irradiation. DDB2 has been shown to associate with Brg1 (a component of the human SWI/SNF complex), therefore could use ChIP analysis to determine if in the absence of Rad16 and presence of DDB2, Snf6 is localized to the HML locus following UV irradiation.

**A1.7 SWI/SNF binding damaged DNA in absence of Rad4**

The temporal difference in Snf6 recruitment to the HML locus post-UV in the presence and absence of Rad4 is an intriguing observation. Perhaps an in vitro binding assay to look at SWI/SNF binding to both damaged and non-damaged DNA would be informative. Additionally, these experiments should be conducted with and without Rad4-Rad23 heterodimer. Also, these in vitro binding assays should be done on both naked DNA and re-constituted mono-nucleosomes. To further delineate the specific role
of SWI/SNF in the lesion detection process, binding should be investigated when the orientation of the lesion is altered, i.e. if the lesion faces toward or away from the nucleosome core.

**A1.8 Role of histone acetylation in recruitment of SWI/SNF and Rad4-Rad23 to silent chromatin following UV irradiation**

In the initial model proposed by Gong et al. (Gong, Fahy et al. 2006), histone modifications such as acetylation are hypothesized to play a role in recruitment of Rad4-Rad23 and/or SWI/SNF. Our data presented here indicate that SWI/SNF does not require Rad4 for recruitment. However, it does not address the importance of histone acetylation in this process. To do this the ChIP experiments described here should be done in strains deficient in histone acetyltransferase activity. The histone acetyltransferasees Rtt109 and Gcn5 have been implicated in acetylation UV-dependent lysine acetylation. Knockout of each of these independently and in combination will provide some clues as to the role of acetylation post-translational modification on recruitment of DNA repair proteins.