Investigating the role of nucleotide excision repair (NER) proteins in the repair of oxidative DNA damage

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UV-DDB, consisting of subunits DDB1 and DDB2, recognizes UV-induced photoproducts during global genome nucleotide excision repair (GG-NER). We recently demonstrated a noncanonical role of UV-DDB in stimulating base excision repair (BER). This provocative study raised several questions about the timing of UV-DDB arrival at 8-oxoguanine (8-oxoG), and the dependency of UV-DDB on the recruitment of downstream BER and NER proteins. Using two different approaches to introduce 8-oxoG in cells, we show that DDB2 is recruited to 8-oxoG immediately after damage and colocalizes with 8-oxoG glycosylase (OGG1) at sites of repair. Interestingly, OGG1 recruitment to 8-oxoG is significantly reduced in the absence of DDB2. NER proteins, XPA and XPC, also accumulate at 8-oxoG. While XPC recruitment is dependent on DDB2, XPA recruitment is DDB2-independent and transcription-coupled. Finally, DDB2 accumulation at 8-oxoG induces local chromatin unfolding. We propose that DDB2-mediated chromatin decompaction facilitates the recruitment of downstream BER proteins to 8-oxoG lesions.

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Preface

As I reflect on my journey in graduate school, my heart is filled with gratitude towards everyone who has supported me these past few years. First, I would like to thank my advisor, Ben Van Houten for giving me the opportunity to work in his lab and for his expert guidance and unwavering support. Ben's mantra of 'if you don't have time to do it right, when will you have time to do it again' has pushed me and everyone in the lab to be critical thinkers. Next, I extend my gratitude towards my thesis committee members, Patricia Opresko, Roderick O'Sullivan, Jacob Stewart-Ornstein and Karen Arndt for their valuable suggestions and feedback which helped bring my thesis to fruition.

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1.0 Introduction

Every cell in our body suffers tens of thousands of DNA lesions per day [1, 2], which if left unrepaired, may lead to mutations, genome instability and cancer. DNA damage can occur from: 1) exogenous sources like ultraviolet (UV) light, ionizing radiation (IR), and chemical exposure from pollutants, or 2) endogenous processes such as replication errors, reactive oxygen species (ROS) or inflammation. Depending on the type of lesion formed in the DNA, six major repair pathways play a key role in maintaining genome stability, these include: direct reversal, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), recombination with two major sub-pathways [homologous recombination (HR) and non-homologous end joining (NHEJ)], and interstrand cross-link (ICL) repair, which combines features of several pathways including NER and recombination. There are also several dedicated translesion DNA polymerases that allow the replication machinery to bypass specific lesions, at the expense of lowered fidelity [3]. Furthermore, key DNA damage signaling pathways are controlled by transcription factors like tumor protein P53 (p53) and DNA kinases including ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related protein (ATR) and DNA-dependent protein kinase (DNA-PK). Although these pathways have been described to work independently, the past two decades have brought into focus the significant interplay between these pathways.

Specifically, ROS-induced oxidative DNA damage is primarily considered to be removed by the BER pathway. However, growing evidence indicates the involvement of NER proteins in oxidative DNA damage repair [4, 5], which is also the focus of this thesis.

1.1 Oxidative DNA damage

1.1.1 DNA lesions generated by oxidation

Reactive oxygen and nitrogen species (ROS/RNS), such as singlet oxygen, superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite, can be generated endogenously by normal cellular metabolism or inflammation, or by exogenous sources such as ultraviolet (UV) or ionizing radiation (IR) [2, 6, 7]. Oxidation can either directly or indirectly introduce a wide spectrum of base lesions in the DNA [8]. Due to the extensive DNA damage caused by oxidation, these lesions have been associated with a large number of human maladies including neurodegeneration, cancer and aging [9]. Some of the most widely studied DNA lesions resulting from oxidation are shown in Figure 1. One of the best characterized oxidative lesions is 8-oxoguanine (8-oxoG), the major product produced from the oxidation of guanine. Further oxidation of 8-oxoG results in the formation of spiroiminodihydantoin (Sp) and 5-guanidinohydantoin (Gh). Purine oxidation can also result in the formation of 5',8-cyclo-purine adducts. An important product of thymine oxidation is thymine glycol (TG). Cytosine is subject to methylation, resulting in the formation of 5-methylcytosine (5mC). Oxidative removal of 5-methylcytosine (5mC) occurs through an active enzymatic process in which 5mC is oxidized in three steps by a family of Ten-eleven translocation (TET) dioxygenases to form 5-hydroxymethyl-C (5hmC), 5-formylC (5fC), and 5-carboxylC (5caC).[1]

[1]Sections 1.0-1.3 were adapted from ref. 13. Please refer to Table 1 of Appendix E for frequencies of DNA lesions formed by oxidative DNA damage.

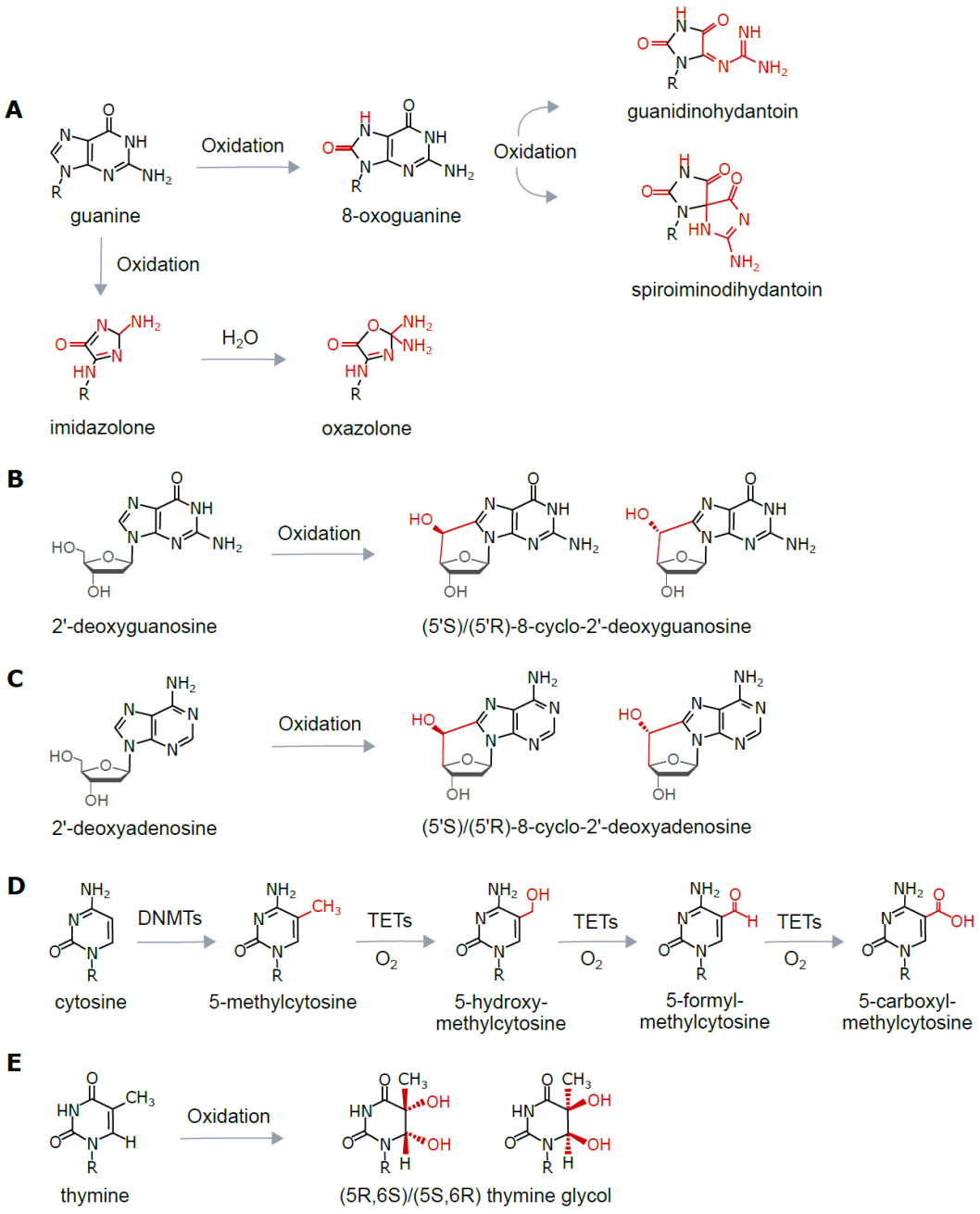

Figure 1: DNA Lesions generated by oxidation

A. Various oxidation products of guanine , B. Formation of cyclic guanosine by oxidation , C. Formation of cyclic adenosine by oxidation, D. Enzymatic oxidative demethylation of 5-methylcytosine, E. Oxidation of thymine to thymine glycol. ROS: Reactive oxygen species; DNMT: DNA methyltransferase; TET: Ten eleven translocation enzymes. From Ref. [10], with permission.

1.1.2 Base excision repair (BER)

Oxidative base lesions are commonly repaired via base excision repair (BER) pathway [11]. BER is initiated after a lesion-specific DNA glycosylase cleaves the glycosidic bond, which frees the lesion, and creates an abasic site [6] (Figure 2). Currently, there are 11 known mammalian DNA glycosylases that can be categorized as monofunctional or bifunctional. Monofunctional glycosylases only possess the ability to break the glycosidic bond between the damaged base and the sugar moiety, resulting in an abasic site, which is processed by AP endonuclease 1 (APE1) to form a 3'OH and a deoxyribo-5′-phosphate (dRP). This dRP is removed by the lyase activity of DNA polymerase β (pol β). Bifunctional glycosylases have an additional AP lyase activity which allows for cleavage of the phosphate backbone, creating a single strand break, leaving a free 5' phosphate and either a 3′-phospho-α, β-unsaturated aldehyde (3'-PUA) (β-elimination) or 3' phosphate (β,δ-elimination). APE1 acts on the β-elimination product, while polynucleotide kinase phosphate (PNKP) is required to process the 3'phosphate after β,δ-elimination. The resulting 3'OH is bound by Poly [ADP-ribose] polymerase 1 (PARP1) which recruits the BER complex consisting of DNA polymerase beta (pol β), X-ray cross complementing protein 1 (XRCC1), and DNA ligase. The one base gap is then filled by pol β and the nick in the DNA is sealed by DNA ligase [7]. The human 8-oxoG glycosylase (OGG1) is a bifunctional glycosylase responsible for the recognition and removal of 8-oxoG. Like several glycosylases, OGG1 is product inhibited, binding avidly to abasic sites, and turns over slowly in the absence of other proteins such as APE1 [7]. Sp and Gh are removed by the actions of the bifunctional glycosylases Endonuclease VIII-like glycosylase 1-3 (NEIL1-3), discussed in section 1.3.4. TG is removed by the bifunctional glycosylase Endonuclease III-like 1 (NTHL1). 5fC and 5caC are removed by the action of thymine DNA glycosylase (TDG), which is a monofunctional glycosylase.

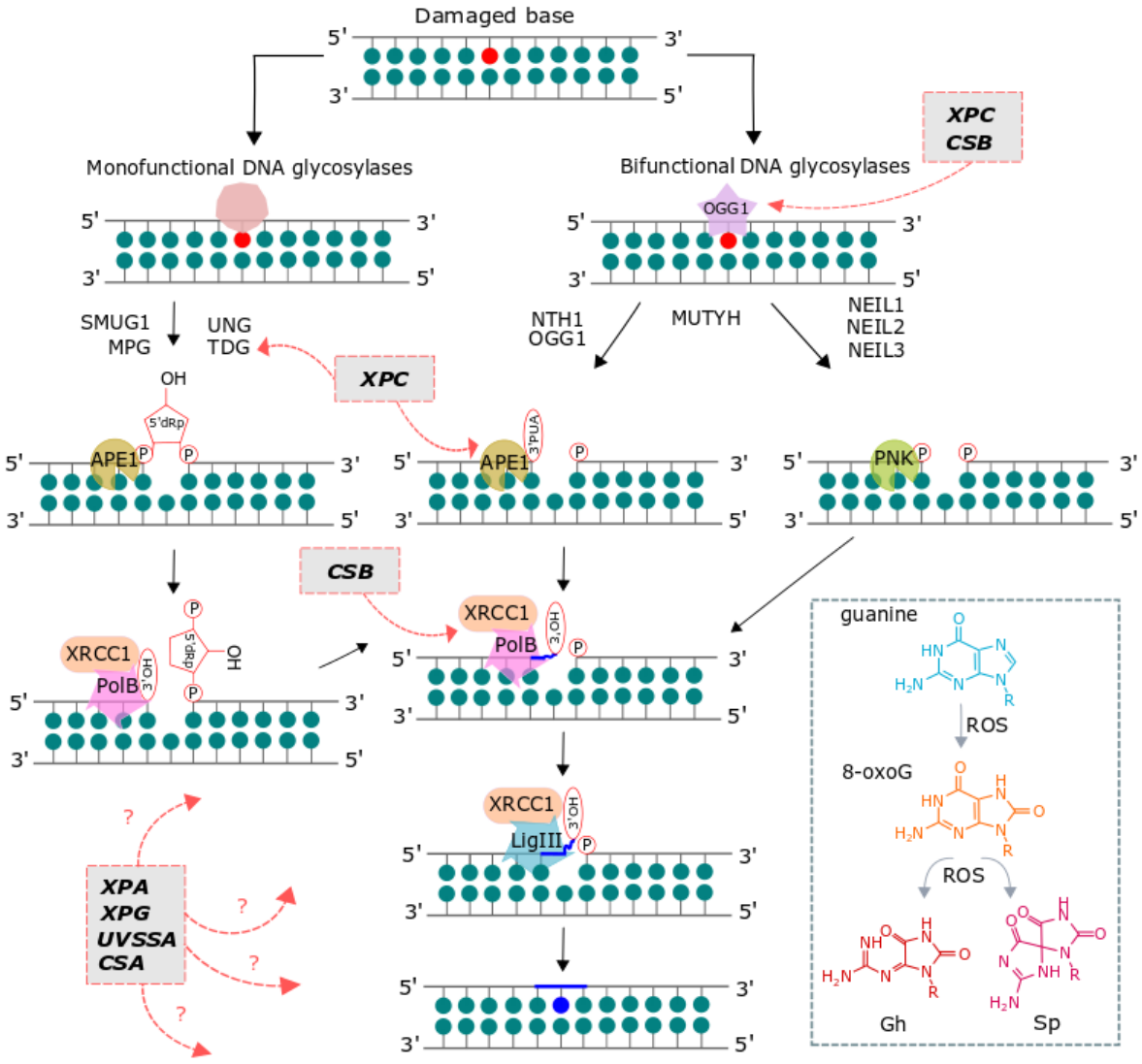

Figure 2: DNA glycosylase-initiated short-patch base excision repair (BER) in mammalian cells. The process consists of these main steps: Excision of the base lesion, incision by an AP endonuclease, end processing, gap filling and ligation. Insert shows the common oxidative lesions repaired by BER: 8-oxoguanine (8-oxoG), guanidinohydantoin (Gh), spiroiminodihydantoin (Sp). Grey boxes (red dashed outline) indicate the involvement of NER (XPA, XPC, XPG, CSA, CSB and UVSSA) proteins in BER. From Ref. [12], with permission.

1.1.3 BER in chromatin

As previously mentioned, several glycosylases are product-inhibited and need the activity of APE1 to turnover and work on other unrepaired lesions [13]. Another factor which can reduce the activity of BER proteins is the inaccessibility of oxidative lesions in the context of chromatin. DNA glycosylases have been shown to have impaired activity on damage when the lesion is contained within a nucleosome [14-22]. It is important to note, while certain glycosylases such as single-strand-selective monofunctional uracil-DNA Glycosylase 1 (SMUG1) are completely inhibited, others such as OGG1, 3-alkyladenine DNA glycosylase (AAG), or uracil-DNA glycosylase (UDG) can recognize outward facing lesions and can readily initiate BER [15, 17, 23, 24]. In addition, both NEIL1 and NTH1 have been shown to have reduced activity on TG substrates embedded in nucleosomes [23, 25, 26]. The issue of lesion accessibility in the context of chromatin is an important factor in other repair pathways including nucleotide excision repair (NER), which is discussed below [27, 28].

1.2 Nucleotide excision repair (NER)

1.2.1 Lesions repaired by NER

Nucleotide excision repair consists of a group of proteins that participate in the repair of lesions that cause significant helical distortion in the DNA structure, such as those induced by ultraviolet (UV) light, environmental mutagens like polycyclic aromatic hydrocarbons (PAHs) and certain chemotherapeutic agents like cisplatin [29, 30]. UVC (254 nm) produces mainly

cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP), while cisplatin forms intra- or interstrand Pt-adducts. Interestingly, longer wavelengths UVB (280-320 nm) and UVA (320-400 nm), which penetrate the earth's atmosphere, can produce a spectrum of lesions including photoproducts and oxidized bases, removed by NER and as well as BER.

1.2.2 Steps in NER

NER includes two sub-pathways: global genome NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER operates in the entire genome, including non-transcribed regions and silent chromatin, while TC-NER recognizes and repairs bulky lesions in the transcribed DNA strands of active genes. Damage recognition in GG-NER is initiated by two proteins, UV-DDB and XPC-RAD23B. In response to UV-induced DNA damage, UV-DDB in complex with CUL4 and RBX forms a ubiquitin E3 ligase complex and binds to the chromatin to ubiquitylate histones, making the lesion more accessible to downstream repair proteins in the NER pathway, including XPC-RAD23B. XPC-RAD23B binds with high affinity to the strand opposite to the distorted lesion, which begins the damage verification step of NER [31]. During the damage verification step of GG-NER, the transcription factor TFIIH is recruited by XPC-RAD23B protein [32-34]. TFIIH consists of 10 subunits, including the helicases XPB and XPD, that are responsible for opening up the DNA around the lesion [35]. XPD binding to the lesion facilitates the recruitment of the pre-incision complex (XPA, RPA, XPG) [36-38]. XPB is believed to act as a translocase to help reel the DNA into the pre-incision complex. XPA and RPA recruit the heterodimeric endonuclease, XPF-ERCC1. Recruitment of XPF-ERCC1 produces an endonucleolytic incision 5' to the lesion. DNA polymerases δ, ε or κ begin to fill in the repair patch, which stimulates the 3' endonucleolytic activity of XPG, leading to the release of an

oligonucleotide of 22-27 nucleotides containing both the lesion and TFIIH [39]. DNA polymerase (δ/ ε) and ligase I then fill in and ligate the gap [40]. TC-NER, on the other hand, is triggered by stalled RNA polymerase at a DNA lesion during transcription, causing the Cockayne syndrome proteins (CSB and CSA), and other lesion accessory proteins (UVSSA, XAB2, and HMGN1) to be recruited at the lesion site. XAB2 facilitates recruitment of XPA and subsequently TFIIH, at which step TC-NER converges with the GG-NER [41].

1.2.3 Diseases associated with NER

Defects in these NER proteins impair the ability to repair UV damage, causing autosomal recessive disorders including xeroderma pigmentosum (XP) (mutations in *XPA-G, XPV*) characterized by extreme sensitivity to sunlight and increased risk to skin cancer in exposed areas. About 20-30% of these patients also develop neurodegeneration. Also mutations in *CSA* and *CSB*, affecting only TC-NER, result in Cockayne syndrome (CS), with patients presenting developmental impairment and neurodegeneration, related to premature aging [42-44].

While both BER and NER pathways have been conventionally associated with specific substrates, growing evidence shows a significant cooperation between these two repair mechanisms, and has recently been reviewed in [4, 5, 45]. The relevance of this potential interaction includes the fact that NER deficient (XP and CS) patients may develop developmental and neurological symptoms, related to premature aging, that can be due to endogenous lesions, such as DNA damage induced by oxidation, which are normally considered substrates for BER. Thus, understanding BER and NER interplay may help us to better understand the causes for the symptoms of premature aging found in these patients and even during the normal aging process.

1.3 Involvement of NER in the repair of oxidative DNA damage

Oxidation of DNA can lead to a myriad of base lesions in the cell, including single-strand breaks and oxidized bases (Figure 1) [8]. Due to its low redox potential, guanine is the most readily oxidized base [46] leading to the formation of 8-oxoguanine (8-oxoG). This modification is one of the most abundant oxidative lesions in the genome, with an estimated steady-state level of about 1–2 lesions/10^6 guanines [1, 2, 47]. 8-oxoG is pre-mutagenic and if unrepaired, can cause G:C to T: A transversions [48-50]. Accumulation of mutations can lead to genomic instability, which is associated with various maladies such as ageing, cancer and neurodegeneration [9, 51].

Base lesions, such as 8-oxoG, do not significantly distort the nucleosome structure [52], and several biochemical studies, using purified OGG1 on reconstituted nucleosomes, have shown that OGG1 activity is severely inhibited when 8-oxoG is buried in the nucleosome [53]. Although, in some sequence contexts, lesions facing outward are more accessible for initiation of repair [21, 54]. Therefore, one major question in the field is how glycosylases act on occluded lesions hidden in a sea of undamaged bases that are organized into a highly compact chromatin structure [55, 56]. To this end, several chromatin remodelers and histone modifiers such as RSC (Remodeling the Structure of Chromatin), FACT (facilitates chromatin transcription) and ISWI (Imitation SWItch/SNF) have been suggested to help facilitate the repair of 8-oxoG, see reviews [14, 57-59]. Interestingly, several studies have suggested the involvement of nucleotide excision repair (NER) proteins in the repair of 8-oxoG, reviewed in [4, 5, 10, 12, 29, 45] and discussed further below.[2]

[2]Section 1.3 was adapted from ref. 15. Please refer to Appendix F for description of crosstalk between NER and BER pathways.

1.3.1 Cooperative interactions of NER proteins in processing 8-oxoG:

As described above, 8-oxoG is processed by OGG1 through BER, although recent studies show that other proteins and sub-pathways may partner in this process. One of the earliest experiments suggesting an involvement of NER proteins in the repair of oxidized lesions was an *in vitro* study from the Sancar laboratory. They showed that cell free extracts from human cell lines either lacking or containing mutated NER proteins (XP-A, XP-B, XP-C, XP-D, XP-F and XP-G) had markedly reduced ability to excise two major oxidized lesions, 8-oxoG and thymine glycol (TG) [60]. They went on to show that the complete NER system reconstituted with purified XPA, RPA, TFIIH (containing XPB and XPD), XPC-RAD23B, XPG, and ERCC1-XPF proteins, were necessary and sufficient to excise 8-oxoG or TG. While these studies indicated that NER proteins are capable of acting on two common oxidized bases, whether NER proteins had a direct role in BER by interacting with BER proteins or intermediates was uncertain. The authors suggested that perhaps NER is a relatively slow back-up system for BER.

D'Ericco, Dogliotti, and colleagues provided the first direct evidence that XPC plays a role in the protection against oxidative stress [61]. They demonstrated that keratinocytes and fibroblasts with mutations in XPC were extremely sensitive to potassium bromate ($KBrO_3$) and ionizing radiation (IR). Using LC/MS and HPLC-ED, they were able to show the accumulation of 8,5'-cyclopurine 2'-deoxynucleosides and slow removal of 8-oxoG and 8-oxoA in cells lacking XPC. Biochemical assays with purified proteins showed stimulation of DNA glycosylase OGG1 by XPC-RAD23B and far western blots showed that purified XPC-RAD23B interacted directly with OGG1. Unlike XPC, at the concentrations surveyed, XPA was not capable of stimulating OGG1. This study indicates that XPC-RAD23B facilitated recognition of 8-oxoG in an OGG1-dependent pathway. It is interesting to note that XP-C patients, in addition to high skin cancer rates, also have

a higher incidence of internal cancer development [62, 63]. Thus, reduced kinetics of oxidatively generated DNA damage might be a major contributor to these internal cancers. Moreover, oxidized damage are also associated with increased risk of neurodegenerative diseases [64, 65]. While XP-A, XP-B, XP-D and XP-G patients may show neurodegeneration symptoms, XP-C patients show no signs of neurological defects. Thus, it is possible that XPC might be acting as a cofactor in the repair process, therefore its loss alone does not display major effects.

In a separate study by Kassam and Rainbow (2007), methylene blue plus visible light (photoactivated MB, which generates singlet oxygen) was used to produce 8-oxoG in an adenovirus-encoded β-galactosidase (β-gal) reporter gene, and a host cell reactivation (HCR) assay was used to demonstrate that human cells deficient in XPC showed lower HCR as compared to WT cells, supporting a role for XPC in the processing of 8-oxoG [66]. Similarly, XP-A and XP-C NER deficient cells were found to be more sensitive to photoactivated MB, compared to NER proficient cells [67]. Problems dealing with the oxidized damage, in XP-A and XP-C cells, were confirmed with observations of cell cycle delay (increased G2/M arrest) and genotoxic stress (H2AX phosphorylation). These results confirm NER proteins participate in the processing of oxidized DNA damage, although which type of lesion (including 8-oxoG) is involved was not clear.

In order to better understand the potential roles of XPA and XPC in the removal of oxidized bases, Dogliotti and coworkers [68] measured the rates of 8-oxoG removal by HPLC–ED in mouse embryo fibroblasts (MEFs) derived from NER (*Csb*$^{m/m}$, *Csa*$^{-/-}$ *Xpa*$^{-/-}$ *Xpc* $^{-/-}$ and combinations of these) and/or *Ogg1*$^{-/-}$ deficient mouse. Following treatment with the oxidizing agent, potassium bromate ($KBrO_3$), *Ogg1*$^{-/-}$ deficient cells displayed a dramatic deficiency in the rate of 8-oxoG removal. NER deficient mutants (*Csb*$^{m/m}$, *Csa*$^{-/-}$ *Xpa*$^{-/-}$ *Xpc* $^{-/-}$) also displayed reduced rates of

removal as compared to WT MEFs. Furthermore, *Csb*$^{-/-}$ *Xpa*$^{-/-}$ and *Csb*$^{-/-}$ *Xpc*$^{-/-}$ double mutants were more deficient in repair as compared to the single mutants. On the other hand, *Xpc*$^{-/-}$ *Xpa*$^{-/-}$ double mutant did not show slower repair kinetics as compared to the single mutant MEFs, suggesting that XPC and XPA function through the same pathway, while CSB is OGG1-dependent, but XPA/XPC independent. These mouse experiments were confirmed in human XP-A primary fibroblasts that were more sensitive to $KBrO_3$ as compared to WT fibroblasts. Furthermore, SV40-transformed XP-A deficient cell line (XP12SV40) with OGG1 knocked down, showed slower 8-oxoG repair kinetics than either the XP-A cells alone or when XPC was knocked down. Whether this enhanced repair of 8-oxoG through the action of XPA, XPC, and CSB is mediated through canonical BER is unclear. Why XPA did not stimulate OGG1 activity in their previous study, but a deficiency in XPA showed a slower repair rate of 8-oxoG remains to be reconciled. Also, the involvement of these proteins could vary in the context of chromatin accessibility. Finally, it is interesting to note that the *Xpa*$^{-/-}$/*Xpc*$^{-/-}$ and *Csb*$^{m/m}$/*Ogg1*$^{-/-}$ double mutant mice are viable and do not show evidence for neurodegeneration [69, 70].

XP-G deficient cells were also found to be sensitive to the treatment with photoactivated MB, indicating that XPG protein, and thus NER, participate in the processing of oxidized DNA damage [71]. This was observed for cells from a severely affected patient, with neurological problems, carrying an *XPG* mutation that completely abrogates the protein. The increased sensitivity was also confirmed by HCR of plasmids treated with photoactivated MB. Interestingly, two different *XPG* missense alleles, from patients with no neurological symptoms (but with XP and increased frequency of skin tumors), showed sensitivity to UV-light induced DNA damage, but not to oxidized lesions induced by singlet oxygen. These results indicate that XPG protein might participate on the removal of UV-induced lesions by NER, with an independent function for

oxidized base damage, and defects on this latter function is in fact relevant for the induction of neurological symptoms in XP-G patients.

1.3.2 Cellular imaging of 8-oxoG processing involving CSB and XPC:

Menoni, Hoeijkmakers and Vermeulen used a novel imaging tool to study the role of XPC and CSB in the repair of oxidized lesions in living cells [72]. By using a photosensitizer (Ro 19-8022) and 405 nm laser light, they were able to generate localized oxidized lesions in specific regions of the nucleus. XPC-GFP and CSB-GFP both were seen to be recruited to the sites of damage. CSB appeared to be recruited faster than XPC, possibly due to different intrinsic mobility or chromatin binding properties. Indeed, they reported that CSB was prominently recruited in the nucleolus (regions with high transcription activity) and XPC accumulated more densely in the heterochromatic region, consistent with their roles in TC-NER and GG-NER of UV-induced photoproducts, respectively. Interestingly they reported, but did not show the data, that neither XPB nor XPA was recruited to the damage site even after 5-10 minutes of damage induction. These data suggesting that CSB and XPC recruitment was independent of subsequent steps in NER is in contrast to the work by Dogliotti and coworkers who showed that both XPA and XPC might facilitate 8-oxoG removal [68].

In a more recent study by Vermeulen's group, the role of CSB in 8-oxoG repair was further elaborated [73]. Using the live-cell imaging approach described above, it was shown that OGG1 recruitment to the damage site was independent of CSB, but the recruitment of the BER scaffolding protein XRCC1 was stimulated by CSB in a transcription-dependent manner. It is possible that as a chromatin remodeler, CSB helps XRCC1 loading under certain circumstances, perhaps in transcribed genes or at specific genomic regions that are not accessible to the downstream BER

proteins. Alternatively, since XRCC1 is recruited to nicks resulting from OGG1 and APE1's action on 8-oxoG, perhaps CSB-mediated repair is initiated downstream of OGG1 if the nick stalls the transcription machinery.

1.3.3 Comet-FISH assay reveals an involvement of XPA, CSB, and UVSSA in TCR of 8-oxoG:

As noted above, the role of XPA in the processing of 8-oxoG adducts has been controversial and contrasting studies have been published. Guo, Hanawalt and Spivak, in an elegant tour-de-force study, combined single-cell electrophoresis (Comet assay) with fluorescence *in situ* hybridization (FISH) and established the involvement of XPA and CSB preferentially in transcription-coupled 8-oxoG removal [74]. For these experiments, 5'- and 3'-ends of ATM gene were labelled with different fluorescent probes. The increase in the distance between the probes after damage was an indication of single strand breaks. The repair rates of transcribed and non-transcribed strands in CS-B and XP-A cells were similar, indicating that they played a role in transcription-coupled repair (TCR) of 8-oxoG. They also showed that elongating Pol II and UVSSA were necessary for this process consistent with TCR. The authors speculated that after initial recognition and incision by OGG1 and APE1, the single stranded break formed causes a block to transcription, recruiting TCR proteins to continue repair. This model is consistent with the work by Vermeulen cited above. XPC, since it is involved in GG-NER, was not investigated in this study.

1.3.4 Oxidized guanine lesions are excised more efficiently by competing BER than NER pathways:

The base damage, 8-oxoG is susceptible to further oxidation, leading to the formation of spiroiminodihydantoin (Sp) and 5-guanidinohydantoin (Gh), which are recognized by the DNA glycosylase NEIL1 [75-79].

Very recently, Shafirovich and colleagues examined the excision of these lesions in intact human cells and the relative contribution of BER and NER in the processing of these lesions [80]. In this study, an internally labelled hairpin substrate containing these lesions were transfected into HeLa cells. DNA was isolated at different time points and analyzed by Polyacrylamide gel electrophoresis (PAGE). The BER activity was determined by the presence of a 65nt incision product, while the presence of a 24-30nt excision product indicated NER activity. The hairpins with both Gh and Sp lesions exhibited BER, as well as NER activity, suggesting a competition between these two pathways in repair. Addition of unlabeled hairpin with a known BER substrate 5-OHU caused significant reduction in the BER product, but an increase in the NER product. This suggests that the participation of these two pathways depends on the local concentration of the recognition factors that recognize and bind to the same lesions in a competitive manner.

1.4 A new role of UV-DDB in the removal of 8-oxoG

1.4.1 UV-DDB structure

UV-damaged DNA binding protein (UV-DDB) is a heterodimeric protein consisting of DDB1 (127kDa) and DDB2 (48kDa). UV-DDB is part of a larger complex containing CUL4A/B and RBX1 that possess E3 ligase activity, and associates with chromatin in response to UV radiation (Figure 3A) [81, 82]**.** UV-DDB ubiquitylates histones to destabilize the nucleosome, thereby allowing downstream repair proteins, such as XPC-RAD23B, to access the lesion [36, 38].

UV-DDB can bind to a wide spectrum of lesions with high affinity [83, 84]. How UV-DDB can efficiently scan DNA for damage, while at the same time bind damaged DNA with the highest affinity of any damaged DNA-binding proteins is intriguing. One proposed mechanism is 'conformational proofreading', which involves both UV-DDB and the DNA undergoing different conformational changes to attain highly specific damage recognition [85]. Two co-crystals of UV-DDB have been resolved on DNA containing either a 6-4PP or an abasic site analog, tetrahydrofuran (THF) [86, 87]. These structures revealed important contacts between UV-DDB and damaged DNA, giving insights into the mechanism of damage recognition.

DDB1 is a large tri-β-propeller substrate adaptor protein with β-propeller domains denoted as BPA, BPB, and BPC, with a C-terminal helical domain referred to as CTD [88]. DDB2 is organized as a seven-bladed WD40 β-propeller (residues 103–421), preceded by an N-terminal domain (residues 1–102), with the hairpins of repeats 4-7 making extensive contacts around the damaged site (Figure 3B). The damaged base with the 3' adjacent base was flipped into a hydrophobic pocket in DDB2 and the 3' base was stabilized by a stacking interaction with Trp203 (W203). These flipped out bases leave a two-base gap in the DNA duplex, which is filled by

Phe334 (F), Gln335 (Q), and His336 (H) that form a beta-hairpin knuckle-like structure inserted through the minor groove. The structures also showed a set of salt-bridges between the Arg112 (R112), Lys132 (K132), and Lys244 (K244) on the damaged strand, and the Arg332 (R332) and Arg370 (R370) contacting the non-damaged strand.

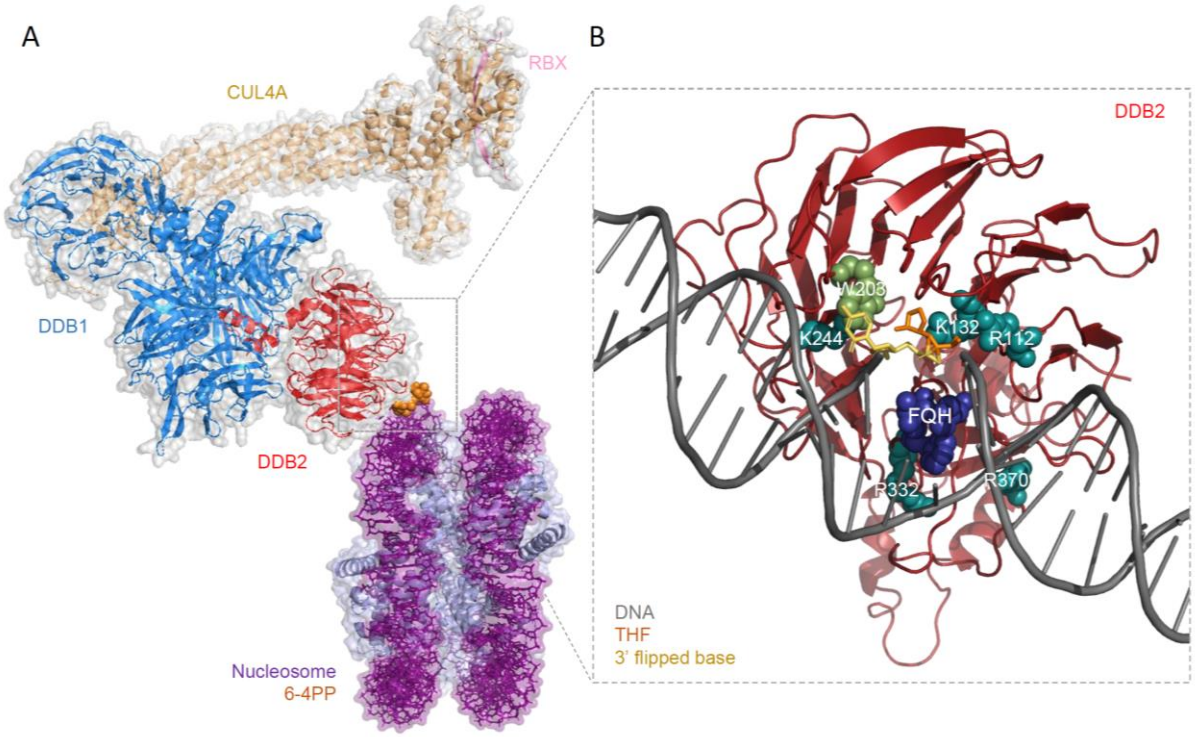

Figure 3: Structure of UV-DDB

A. Molecular model of UV-DDB-CUL4A-RBX complex bound to a nucleosome. DDB1 (blue); DDB2 (red); CUL4A (tan); RBX (pink); nucleosome (purple); 6-4 photoproduct (orange). Built from PDB codes: 4A0K and 6R8Y, [89, 90]. B. Damage recognition interface of DDB2 (red ribbon) with tetrahydrofuran, THF (orange)-containing DNA (gray) with adjacent 3' base flipped out (yellow). This flipped out base is stabilized by W203 (green) and an FQH knuckle (dark blue) fills the void made by the two flipped out bases. Important salt contacts with positively charged amino acids (teal) with phosphate backbone are shown. K244 when mutated to E causes XP-E. PDB codes: 4E54, see [86].

1.4.2 DDB2 and Cancer

Mutations in the *DDB2* gene cause XP complementation group E (XP-E) and can affect DDB2's function during DNA damage recognition and repair. So far, eight amino acid changes, including several frameshift/truncation mutants, altered splice mutants and deletions, have been discovered (Table 2, Appendix C). Specifically, a DDB2 Lys244 to Glu mutation was shown to cause loss in photolesion recognition and subsequent repair [91]. Furthermore, this mutant led to increased sliding on the DNA [85]. Variants that map to the DDB2-DNA interface (Arg239Ile, Asp307Tyr, Thr305Asn, Pro357Leu) would be expected to reduce or eliminate UV-DDB binding activity. Mutations localized within the DWD (DDB1-binding WD40 protein) box motif (Asn349Δ/Leu350Pro, Arg273His) impair the interaction with DDB1, inactivating UV-DDB's E3 ligase activity [92, 93].

In mice, apart from DDB2's role in protection against UV- induced DNA damage [94, 95], it was also shown that DDB2 can protect against spontaneous internal tumors [96]. DDB2 knockout mice were shown to have overall lower survival even in the absence of any external DNA damage and developed spontaneous tumors including adenocarcinoma of the lung and mammary gland as well as several forms of sarcoma [96]. Interestingly, The Cancer Genome Atlas (TCGA) data show that higher levels of *DDB2* gene expression is associated with higher survival in patients with endometrial, cervical and breast cancer (https://www.proteinatlas.org/ENSG00000134574-DDB2/pathology). Together, these data suggest that loss of DDB2 probably leads to accumulation of endogenously formed DNA lesions, perhaps mediated by ROS or other naturally occurring reactions in the cell. Therefore, it would be of interest to study the mutational signature of these tumors.

1.4.3 UV-DDB induced nucleosome destabilization

1.4.3.1 Interaction of UV-DDB with nucleosomes containing site-specific lesions

UV-DDB was found to bind to nucleosomes containing DNA photoproducts and was proposed to be an early responder in the repair of UV damage [97, 98]. A major advancement was made recently using cryo-EM to examine UV-DDB's interactions with a nucleosome containing defined damage sites at various positions along the nucleosome [90]. DNA damage recognition and repair is hindered by the presence of tightly bound histones wrapped around DNA. Specifically, lesions facing inward are more challenging to access by DNA repair proteins. However, UV-DDB was shown to directly bind damaged DNA on the 5S nucleosome (an *in vitro* nucleosome model system consisting of 5S RNA genes from Xenopus) and shift the DNA register up to three bases, altering the nucleosome architecture to allow access to occluded sites [90].

1.4.3.2 UV-DDB is an E3 ligase and ubiquitylates histone H2A

UV-DDB is an active ubiquitin E3 ligase when bound to cullin4A/B (CUL4A/B) and Roc1 (RBX). The DDB2-DDB1-CUL4A-RBX (CRL^{DDB2}) ubiquitin ligase auto-ubiquitylates itself and also ubiquitylates XPC [32]. While mono-ubiquitylated XPC is stabilized at sites of UV-induced DNA damage, the binding of UV-DDB is destabilized, due to poly-ubiquitylation of DDB2. This work led to the concept that UV-DDB needs to poly-ubiquitylate itself to allow proper hand off of UV-induced photoproducts to XPC during GG-NER, [82, 99, 100]. Furthermore, poly-ubiquitylated DDB2 is extracted from the chromatin by the p97 segregase VCP, a protein unfoldase, and subsequently degraded by the 26S proteosome [82, 101].

DDB1-$CUL4B^{DDB2}$ E3 ligase can specifically bind to mono-nucleosomes containing UV damage and mono-ubiquitylate histone H2A and H3 [97]. Mono-ubiquitylated H2A at Lys119 and

Lys120 helps facilitate the destabilization of nucleosome containing UV-induced photoproducts, as mutating these residues to Arg prevented the dissociation of poly-ubiquitylated DDB2 from the UV damage containing nucleosome. Both CUL4A and CUL4B have been shown to interact with UV-DDB and support ubiquitylation of histone H2A [102]. Finally, while UV-DDB can mono-ubiquitylate H3, H3 ubiquitylation is not necessary for UV-DDB-mediated destabilization of the nucleosome [97].

1.4.3.3 DDB2 can directly change the core histone density at UV-induced DNA lesions

A number of chromatin remodelers have been shown to play an important role in the regulation of nucleotide excision repair (reviewed in [103]). A novel and DDB1-CUL4A-RBX (CRL)-independent role was identified for DDB2 in unfolding of higher order chromatin structures at the sites of UVC damage [104]. Using a LacR-tagged DDB2 construct in various cell lines consisting of integrated LacO arrays, it was shown that tethering of LacR-DDB2 could significantly reduce density of GFP-tagged H1, H2A and H4. Furthermore, the decondensation of chromatin by DDB2 was independent of DDB1-CUL4A E3 ligase activity and dependent on ATP hydrolysis, indicating that ATP-dependent chromatin remodeling factors might be involved in the process. Also, this process required poly(ADP-ribose) polymerase (PARP1) activity, which has been linked to chromatin remodeling in the context of double-strand breaks [105, 106].

Using FRAP in cells stably expressing SNAP-tagged histone 3.3, it was demonstrated that DDB2 is necessary and sufficient for changing the histone density at locally induced UV damage sites, causing a redistribution of SNAP-H3.3 [107]. Knockdown of chromatin remodeling factors, ALC1 (amplified in liver cancer 1) and INO80 (inositol regulatory gene 80) did not affect the histone dynamics, suggesting that DDB2 binding is upstream of any chromatin remodeling activity. Furthermore, in contrast to the study discussed above [104], redistribution of histone H3.3

at sites of UV damage was PARP1-independent. How PARP is mechanistically linked to chromatin remodeling during NER is still unclear and requires further investigation.

1.4.4 Biochemical and single-molecule studies suggest UV-DDB as a damage sensor in BER

The earliest evidence of recognition of abasic (AP) sites by UV-DDB was shown using an electrophoresis DNA binding assay [84]. A 148 bp DNA probe was end-labeled with ^{32}P and damaged by UV to induce CPD and 6-4PP. Unlabeled DNA containing AP sites was used as a competitor. Partially purified UV-DDB from HeLa cell extracts was run on a gel along with the DNA. While the DNA containing AP sites was able to inhibit UV-DDB binding to UV damaged DNA, the affinity was 17-fold lower for the AP sites.

More direct evidence of recognition of AP sites by UV-DDB was established by using recombinant purified UV-DDB and DNA substrates with a site-specific CPD, 6-4PP and AP site [83, 108]. A 6-fold higher affinity for CPD, 83-fold higher affinity for 6-4PP and a 46-fold higher affinity for an AP site was observed as compared to undamaged DNA. A mismatch substrate was also tested with a 50-fold higher affinity as compared to undamaged DNA. This broad substrate specificity is probably because UV-DDB does not detect the damage site directly but recognizes helix-distorting lesions that can be flipped into the binding site of DDB2 (Figure 3B).

Recently, our lab established a novel role for UV-DDB in the repair of oxidative DNA damage [109]. Using a combination of biochemical, single-molecule and cellular studies, we demonstrated that UV-DDB can recognize and aid in the repair of 8-oxoG lesions. EMSA assays conducted in the presence of 5 mM Mg^{2+} showed that UV-DDB preferentially bound abasic sites, CPD and 8-oxoG, with equilibrium dissociation constants, K_d, of 3.9, 30, and 160 nM, respectively, with high specificity as compared to undamaged DNA (K_d = 1108 nM) [109, 110].

We further showed that when the EMSAs are done in the presence of Mg^{2+} in the binding buffer, gel, and running buffers, UV-DDB has enhanced specificity for both abasic sites and 8-oxoG. While Mg^{2+} greatly diminishes binding to a non-damaged 37 bp duplex by 26-fold from 42 nM to 1100 nM, Mg^{2+} only decreased binding to abasic site and 8-oxoG:C base pairs by 4-5-fold, greatly enhancing the specificity window of UV-DDB. We next showed that UV-DDB stimulated OGG1 and APE1 activity on 8-oxoG:C and abasic site-containing substrates by ~3-fold and ~9-fold, respectively. Using a single-molecule DNA tightrope assay where DNA containing an abasic site every 2 kb is suspended between 5μm poly-L-lysine coated beads and quantum-dot labeled purified proteins are observed in real time [111], UV-DDB was found to undergo limited linear diffusion in the presence of Mg^{2+} in the flow cell as compared to strict 3D searching on DNA with little or no linear diffusion in the absence of Mg^{2+}. By orthogonally labeling UV-DDB and OGG1 or APE1 with different colored Qdots, we also showed that UV-DDB could form transient complexes with OGG1 and APE1 on the damaged site [109]. Moreover, UV-DDB facilitated the dissociation of these proteins from the damaged site, suggesting that UV-DDB can help turnover OGG1 and APE1 from the abasic site. Using an *in vitro* BER reaction, the activity of pol β was measured through the incorporation of radiolabeled dCTP into the gap created by the dual action of APE1 incision and polβ dRpase activity. This assay revealed that addition of UV-DDB increased BER product formation by 30-fold. Additionally, we were able to show that the newly incorporated dCTP could be ligated into full length product, indicating that UV-DDB does not have an inhibitory effect on downstream steps in BER.[3]

[3]Section 1.4 was adapted from ref. 110. Please refer to Appendix G for further discussion on UV-DDB's role in maintaining genome stability.

1.5 Hypotheses and Scope

To test the hypothesis that UV-DDB plays a role in 8-oxoG repair in the context of cellular chromatin and to gain mechanistic insights into how the NER proteins, DDB2, XPC and XPA coordinate the processing of 8-oxoG in chromatin, we use two independent systems (fluorogen activation protein and photosensitizer Ro 19-8022 plus 405 nm, see section 1.6) to introduce 8-oxoG in cells. This present study specifically addresses the following questions:

1) *Is UV-DDB required for recruitment of OGG1 to 8-oxoG?* Previously, we have shown that purified UV-DDB can recognize 8-oxoG embedded in a 37-bp duplex DNA. Here, we introduce 8-oxoG specifically in different regions of the genome to directly visualize UV-DDB accumulation.

2) *Do NER proteins, XPC and XPA facilitate 8-oxoG processing in cells?* Cells deficient in XPC or XPA exhibit delayed repair of 8-oxoG, after treatment with potassium bromate ($KBrO_3$), an oxidant that predominantly forms 8-oxoG lesions [61, 68]. Using a system consisting of a photosensitizer (Ro 19-8022) plus 405 nm light to introduce predominantly 8-oxoG lesions [112], it was demonstrated that XPC accumulated to oxidative damage generated at heterochromatic regions [72, 73]. Interestingly, contrasting models have been presented for the potential role of XPA in the removal of 8-oxoG [72, 74]. While these studies suggest a role for NER proteins in facilitating 8-oxoG repair, a unified model of how these proteins work in synchrony is lacking.

3) Does the *CRL^{DDB2} complex participate in 8-oxoG recognition and mediate dissociation of DDB2 from 8-oxoG sites?* Previous studies have shown that the CRL^{DDB2} complex helps destabilize nucleosomes at UVC-induced DNA damage sites. Furthermore, the CRL complex auto-ubiquitylates DDB2 to allow for its extraction from chromatin and subsequent degradation

by the 26S proteosome. Here, we aimed to determine whether the CRL complex regulates DDB2 similarly at sites of 8-oxoG damage.

4) *How does binding of DDB2 to 8-oxoG impact the chromatin state at the damage site?* It has been demonstrated that DDB2 binding to UVC-induced damage is sufficient to cause local chromatin unfolding at the damage sites. We sought to demonstrate this at 8-oxoG sites by measuring chromatin changes in the presence and absence of DDB2.

Finally, we propose a new model for 8-oxoG processing that directly involves the NER proteins, DDB2, XPC and XPA, and two sub-pathways: a) a global genome pathway where DDB2 binds 8-oxoG lesions to change the local chromatin environment facilitating the recruitment of XPC and OGG1, and b) a transcription-coupled repair pathway that is initiated when BER intermediates stall RNA polymerase and act as a transcription block.

1.6 Approach

1.6.1 Fluorogen activating protein (FAP) system to introduce 8-oxoG in telomeric DNA

Studying oxidative DNA damage *in vivo* has been challenging due to the unavailability of tools that introduce specific base damage without the formation of other lesions such as single-strand or double-strand breaks [113]. To overcome this problem, we used a recently developed chemoptogenetic approach to target 8-oxoG specifically at telomeres (Figure 4) [109, 114]. This approach utilizes a fluorogen-activating protein (FAP) in combination with a photosensitizer dye, di-iodinated malachite green (MG-2I) [115]. Upon binding to FAP, the FAP plus MG-2I combination is excited by near-infrared wavelength (660 nm) to generate singlet oxygen. Here,

FAP is fused to a telomere binding protein, TTAGGG repeat binding factor 1 (TRF1), (FAP-TRF1) [109, 114]. Singlet oxygen is highly reactive and short-lived and can cause localized damage at telomeres [116]. Treatment with dye (MG-2I) and light generates singlet oxygen that oxidizes guanines at telomeric DNA to form ~ 1-2 8-oxoG lesions/telomere [114].

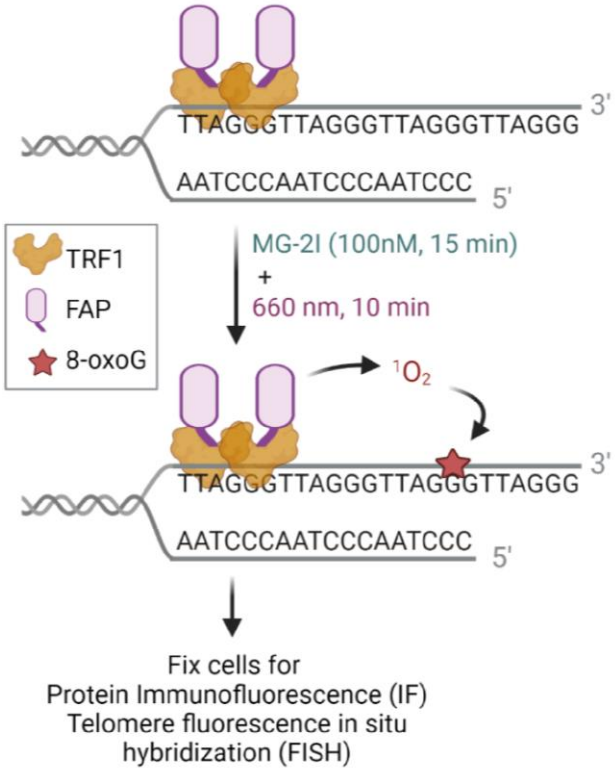

Figure 4: Schematic of FAP-TRF1 system

Cells stably expressing FAP-TRF1 were treated with dye (100 nM, 15 min) plus light (660 nm, 10 min) to introduce 8-oxoG lesions at telomeres.

1.6.2 Photosensitizer Ro 19-8022 plus 405 nm to generate 8-oxoguanine in genomic DNA

Ro19-8022 is a potent type II photosensitizer with an absorption maximum at 427 nm. Ro19-8022 has high polarity and can easily penetrate cellular membranes. It was shown that the combination of Ro 19-8022 and visible light resulted in DNA damage profile similar to that caused

by singlet oxygen in isolated DNA and AS52 Chinese hamster ovary cells, [112]. Furthermore, Sequence analysis revealed GC→TA and GC→CG transversions (see section 2.7.2.2 for method).

2.0 Materials and Methods

2.1 Mammalian cell culture

2.1.1 Cell lines

The U2OS-FAP-TRF1 and RPE-FAP-TRF1 stable lines were obtained by transfecting pLVX-FAP-mCer-TRF1 plasmid in U2OS, and RPE-hTERT cells respectively, and then selected in 500 μg/ml G418 (Gibco) [114]. Single-cell cloning was used to select for cells with expression of FAP-mCer-TRF1 construct at telomeres. U2OS-FAP-TRF1 cells were cultured at 5% oxygen in Dulbecco's Modified Eagle Medium (DMEM) containing 4 g/l glucose (Gibco). RPE-FAP-TRF1 cells were cultured at 5% oxygen in Dulbecco's Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12 1:1) containing 2.438 g/l sodium bicarbonate (Gibco). Cells were supplemented with 10% fetal bovine serum (Gibco), 1×penicillin/streptavidin (Life Technologies) and 500μg/ml G418.

SV40-immortalized MRC-5 cells stably expressing OGG1-GFP or XRCC1-YFP (Menoni H. et al., NAR, 2018), hTERT-immortalized human fibroblasts VH10 stably expressing GFP-DDB2 [117] and hTERT-immortalized fibroblasts GM01389 (DDB2-deficient) [118] stably expressing GFP-DDB2 were cultured at 37 °C in a humidified atmosphere with 5% CO2 in a 1:1 mixture of DMEM (Gibco, 41699-052) and Ham's F10 (Lonza, BE02-014F) supplemented with 10% fetal calf serum (FBS, FBS-12A) and 1% penicillin-streptomycin (Sigma, P0781).

2.1.2 Knockout (KO) and knockdown (KD) cell line generation

DDB2 knockout cells were generated in U2OS-FAP-TRF1 and RPE-FAP-TRF1 cells. HEK293T cells were co-transfected with a lentiviral construct expressing SpCas9 and a guide RNA targeting DDB2 exon 1 (Genscript DDB2 CRISPR Guide RNA1, CCGAGATTGTATTACGCCCC), along with the Sigma CRISPR & MISSION® Lentiviral Packaging Mix (Sigma, SHP002). Briefly, $2.5x10^5$ HEK293T cells (ATCC) were seeded in a 6-well plate. The next day 500 ng of the lentiviral vector, 4.6µl of the lentiviral packaging mix and 2.7µl was incubated in 30.3µl of OptiMEM. After incubation at room temperature for 15 minutes, the mix was added dropwise to each well containing 2 ml serum-free DMEM. The cells were incubated for 24 hours at 37°C and 5% oxygen. Next day, the media was replaced with 2 ml of fresh complete DMEM. Between 36-48 hours post-transfection, the supernatant was collected and filtered through a 0.2µm filter. Fresh 2 ml complete DMEM was added to the HEK293T cells for the second harvest. The first harvest was added with 2µl 10 mg/ml polybrene (Millipore #TR-1003-G) to the U2OS-FAP-TRF1 cells plated in a 6-well plate and incubated overnight at 37°C. The procedure was repeated for the second lentiviral harvest, between 60-72 hours post-transfection. The cells were then incubated overnight at 37°C and 5% oxygen. Next day, fresh media was added, and cells were allowed to recover for 6-8 hours. Cells were then selected with 1.5 µg/ml puromycin for 2 days in a 6 cm dish. Cells were then moved to a 75 cm^2 flask under selective pressure for an additional 2 days before harvesting for protein extraction and single cell cloning without puromycin. The clones were tested for DDB2 expression by western blot (abcam #ab181136) and immunofluorescence (abcam #ab51017). Clone 10 and Clone 37 were used for U2OS-FAP-TRF1 and RPE-FAP-TRF1 respectively.

2.2 DNA damage generation in cells

2.2.1 Dye plus Light treatment to generate 8-oxoG at telomeres

Cells were plated on coverslips in 35 mm dishes (100,000 cells/dish). 48 hours post transfection, cells were incubated with 100 nM MG-2I dye for 15 minutes at 37°C, 5% oxygen in phenol red-free DMEM. Cells were then exposed to 660 nm light (100 mW/cm^2) for 10 minutes (unless specified otherwise) to induce the production of singlet oxygen. Cells were pre-treated with transcription inhibitors for 90 minutes: α-amanitin (Sigma #A2263) and Cdk7 Inhibitor VIII, THZ1-Calbiochem (Sigma# 5323720001). Cells were fixed or harvested for further experiments.

2.2.2 Photosensitizer plus 405 nm to generate 8-oxoguanine in genomic DNA

Photosensitizer Ro 19-8022 was used to generate oxidative DNA damage (a kind gift from F. Hoffmann-La Roche, Ltd). The following inhibitors were used: NEDD8 neddylation activating enzyme inhibitor (NAE1 inhibitor, MLN4924, Boston Biochem) and CSN5-catalysed cullin de-NEDDylation inhibitor (CSN5 inhibitor, SB-58-SN29, kindly provided by Novartis) [119].

2.2.3 Local UV-C damage

Cells were washed with PBS. Using a 254 nm lamp, cells were exposed to 60 J/m^2 UV-C either globally or through a 2 μm polycarbonate filter (Millipore Sigma; #TTTP04700).

2.3 Plasmids

mNeon-DDB2 was made by Gene Universal Inc., by cloning the human DDB2 cDNA between BglII-XhoI sites of pmNeonGreen-C1 plasmid. DDB2(human)-mCherry was made by Gene Universal Inc., by removing the mNeonGreen sequence by digesting with AgeI and BglII and cloning the mCherry sequence between XhoI-HindIII sites of pmNeonGreen-DDB2 plasmid. DDB2(mouse)-mCherry, GFP-DDB1(mouse) and GFP-CUL4A were provided by Dr. Wim Vermeulen [117, 120]. OGG1-GFP was provided by Dr. A. Campalans [121]. DDB2-Flag was purchased from ORIGENE (RC200390). DDB2(K244E)-Flag mutant was made using the QuickChange II Mutagenesis kit (Agilent, #200523).

2.4 siRNA transfections

40 nM siRNA was transfected using Lipofectamine 2000 (Thermo Fisher Scientific, #11668027) in serum-free DMEM, according to the manufacturer's instructions. Fresh complete media was added 4-6 hours post transfection. Immunofluorescence and western blots were performed 48 hours post transfection, unless specified otherwise.

siRNAs used: Control siRNA: siGENOME non-Targeting siRNA Pool #2 (Dharmacon D-001206-14-05); OGG1: siGENOME Human OGG1 (Dharmacon M-005147-03-0005); DDB2: 5'-AACUAGGCUGCAAGACUU-3'; DDB1: 5'-AACGGCUGCGUGACCGGACAC -3'; CUL4A: 5'-GAAGAUUAACACGUGCUGGdTdT -3'; XPC: siGENOME Human XPC (SMARTpool, Dharmacon M-016040-01-0005); CSB: 5'- GUG UGC AUG UGU CUU ACG A -3'.

2.5 Western blotting

Cell pellets were resuspended in 1X lysis buffer (Cell signaling #9803) containing 1mM protease inhibitor (Millipore Sigma; #539134). Supernatants were obtained by centrifugation at 15,000 RPM for 15 minutes at 4˚C. Protein was quantified using Bio-Rad protein assay (Bio-Rad, #5000006). Equal amounts of protein were diluted in 2X sample buffer (Bio-Rad; #1610737) and loaded on 4-20% tris-glycine polyacrylamide gels (Invitrogen; XP04202BOX). Proteins were transferred onto a polyvinylidene difluoride membrane and blocked in 20% nonfat dry milk (diluted in PBST: phosphate-buffered saline containing 0.1% Tween-20) for 1 hour at room temperature. Membranes were incubated with primary antibodies for 2 hours at room temperature or overnight at 4˚C. Membranes were washed 3X 10 minutes in PSBT and incubated with peroxidase conjugated secondary antibodies for 1 hour at room temperature. Membranes were washed again before developing using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific; #34095). Primary antibodies used: DDB2 (1:1000; abcam #ab181136), OGG1(1:1000; abcam #124741), CUL4A (1:1000; CST #2699S), DDB1 (1:1000; Invitrogen #37-6200), β-actin (1:30,000; Sigma #A2228). Secondary antibodies used: anti-rabbit IgG (1:50,000 Sigma #A0545), or anti-mouse IgG (1:50,000 Sigma #A4416).

For figures 14 and 15: Cells were collected in 2x sample buffer (125 mM Tris-HCl pH 6.8, 20% Glycerol, 10% 2-β-Mercaptoethanol, 4% SDS, 0.01% Bromophenol Blue), homogenized passing through a syringe tip and boiled at 98°C for 5 min. Protein lysate was separated by SDS-PAGE and transferred to a PVDF membrane (0.45 µm, Merck Millipore). The membrane was blocked in 3% BSA and then incubated with primary and secondary antibodies for 2 h or overnight. Antibodies used were anti-DDB2 (ab181136, Abcam), anti-CUL4A (ab72548, Abcam), anti-CSA (ab137033, Abcam), anti-AQR (A302-547A, Bethyl Laboratories). Secondary antibodies were

conjugated with CF IR Dye 680 or 770 (Sigma) and visualized using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences).

2.6 Cell growth assays

2.6.1 Colony formation assay

U2OS-FAP-TRF1 (WT and DDB2 KO) cells were plated in 6-well plates 24 hours prior to treatment. The next day, cells were treated with KBrO3 (Sigma 309087), (0–20 mM) for 1 h at 37 °C. After treatment, cells were trypsinized and counted, and 800 cells were plated in 6 cm dishes in triplicate for each condition. Cells were then allowed to recover for 8 days. On day 8, cells were fixed with 4% formaldehyde in PBS for 15 minutes at room temperature and colonies were stained using a 0.1% crystal violet, 20% methanol solution for 30 minutes at room temperature. The plates were washed with water and dried overnight before counting.

2.7 Cellular imaging

2.7.1 Widefield imaging

2.7.1.1 8-oxoG immunofluorescence

100,000 cells were plated on coverslips in 35 mm dishes. siRNAs were transiently transfected for the experiments. 48 hours post transfection, cells were fixed for 8-oxoG staining using the Trevigen 8-oxo antibody (cat # 4354-MC-050). Briefly, cells were fixed with 1:1 MeOH,

acetone for 20 minutes on ice and coverslips were allowed to air dry. Fixed cells were next treated with 0.05N HCl for 5 minutes on ice. After washing cells 3 times with 1X PBS, coverslips were incubated with 100µg/ml RNAse in 150mM NaCl, 15mM sodium citrate for 1 hour at 37°C. Next, cells were washed sequentially in 1X PBS, 35%, 50% and 75% EtOH, for 3 minutes each. Cellular DNA was then denatured in situ with 0.15N NaOH in 70% EtOH for 4 minutes. After washing briefly 2x with 1X PBS, 0.2 µg/ml Hoechst 33342 (Thermo fisher scientific, cat #H3570) in 1X PBS was used to stain DNA for 10 minutes. Coverslips were washed sequentially in 70% EtOH containing 4% v/v formaldehyde, 50% and 35% EtOH, and 1X PBS for 2 minutes each. Finally, coverslips were incubated in 5µg/ml proteinase K in 20mM Tris, 1mM EDTA, pH 7.5 (TE) for 10 minutes at 37°C, washed several times with 1X PBS and blocked with 1%BSA, 10% normal goat serum in 1X PBS, 1hour at RT. Cells were washed 3x with 1X PBS, and incubated with anti-8-hydroxyguanine antibody (1:250) diluted in 1X PBS containing 1% BSA, 0.01% Tween 20 at 4°C O/N in a humidified chamber. Next day, cells were washed several times with 1X PBS containing 0.05% Tween 20 for 5 minutes each and incubated in fluorescent secondary antibody conjugate, Donkey anti-mouse Alexa488 (1:1000; 1Thermo Fisher Scientific #A21202) in 1X PBS containing 1% BSA, for 1hr in the dark, at room temperature. Finally, cells were washed several times with 1X PBS containing 0.05% Tween 20 and rinsed with de-ionized water before mounting with Prolong Diamond Anti-Fade (Catalog #P36970; Molecular Probes).

2.7.1.2 Immunofluorescence and fluorescence in situ hybridization (IF-FISH) to visualize recruitment of repair proteins at telomeres

100,000 cells were plated on coverslips in 35 mm dishes. Plasmids were transiently transfected for the experiments. 48 hours post transfection, cells were treated with dye plus light, and allowed to recover for indicated time periods. Cells were incubated with ice-cold CSK buffer

(100 mM NaCl, 3 mM MgCl2, 300 mM glucose, 10 mM Pipes pH 6.8, 0.5% Triton X-100) for 2 minutes before fixing with 4% paraformaldehyde for 10 minutes. Cells were washed thrice with PBS and permeabilized with 0.2% Triton X-100 for 10 minutes. After permeabilization, cells were blocked for 1 hour at room temperature (10% goat serum, 1% BSA in PBS). Primary antibodies were added to the cells and incubated overnight at 4 °C. Next day, cells were washed thrice with PBS, and incubated with secondary antibodies for 1 hour at room temperature. After three PBS washes, cells were fixed again with 4% paraformaldehyde for 10 minutes, washed in PBS, and dehydrated in 70%, 90% and 100% ethanol for 5 minutes each. The hybridization solution (70% Di Formamide, 1× Maleic acid, 10 mM Tris, pH 7.5, 1× $MgCl_2$, 0.1 μM PNA probe) was prepared and incubated at 85 °C for 3–5 min. PNA probes used: PNA Bio, F1004; (CCCTAA)3-Alexa488 or PNA Bio, F1013; (CCCTAA)3-Alexa647. After the coverslips dried, cells were hybridized for 10 minutes at 85 °C and incubated at room temperature for 2 hours in a humid chamber, in the dark. After 2 hours, coverslips were washed twice in hybridization wash buffer (70% formamide, 10 mM Tris-HCl pH 7.5) for 15 minutes each. Next, coverslips were washed thrice with PBS and incubated with DAPI (1:5000) for 10 minutes at room temperature. Finally, coverslips were washed once with PBS and dH_2O, and mounted on slides with Prolong Diamond Anti-Fade (Catalog #P36970; Molecular Probes).

Primary antibodies used: mCherry (1:250; Abcam #ab167453), GFP (1:100, Santa Cruz #B-2), Flag (1:500; CST #14793S), TRF1 (1:500; abcam #10579). Secondary antibodies used: Donkey anti-mouse Alexa488 (1:1000; 1Thermo Fisher Scientific #A21202), Goat anti-Rabbit Alexa-594 (1:1000; Thermo Fisher Scientific #A11012).

2.7.1.3 Quantification of protein colocalization at telomeres

Images were acquired on the Nikon Ti inverted microscope with a 60X objective (1.4 NA) using a z stack of 0.2 μm. The exposure time of each channel was kept consistent throughout samples. Images were deconvoluted and analyzed using NIS Elements 5.2 advance research software.

For the quantification of foci, the region of interest (ROI) tool was used to label the nuclei. Next, in the measurement tab, a separate binary layer was created for the repair protein foci and the telomere foci. The intersection tool was then used to identify the third binary layer, which corresponded to the colocalized foci. The intensity threshold for each channel was kept consistent throughout the samples. The foci counts were exported to Excel for analysis. The colocalized foci number was normalized to the telomere foci number of each nucleus to get the percent telomeres colocalized with the repair protein, which was reported.

2.7.1.4 Proximity ligation assay

10,000 cells were plated in each well of an 8-chambered tissue culture treated glass slide (Falcon, #354118). DDB2-mCherry and OGG1-GFP were transiently transfected for the experiments. 48 hours post transfection, cells were treated with dye plus light, and allowed to recover for indicated time periods.

Cells were incubated with ice-cold CSK buffer for 2 minutes before fixing, permeabilizing and blocking as mentioned above. Primary antibodies were added to the cells and incubated overnight at 4 °C. Next day, probe incubation, ligation and amplification were performed using the Sigma-Aldrich PLA kit (#DUO92101) according to the manufacturer's instructions.

Images were acquired on the Nikon Ti inverted microscope with a 60X objective (1.4 NA) using a z stack of 0.2 µm. Images were deconvoluted and analyzed using NIS Elements 5.2 advance research software. PLA foci per nucleus was reported.

2.7.2 Confocal imaging

2.7.2.1 Telomere volume measurements

U2OS-FAP-TRF1 cells were imaged on a Sweptfield confocal system with a 1.2 pinhole at 100x magnification and a 1.5x coupler using a z-stack of 0.13µm. RPE-FAP-TRF1 cells were imaged on the Nikon A1 confocal system using a 60x magnification, a pinhole of 1.2 and a z-stack of 0.1µm. All imaging conditions were kept consistent throughout samples. Images were deconvoluted using the Richardson Lucy method and the number and volumes of telomeres was analyzed using NIS Elements advance research GA3 software using a custom GA3 script.

2.7.2.2 Local DNA damage induction using photosensitizer Ro 19-8022

Cells were examined in normal culture medium and maintained at 37°C and 5% CO2 within a large chamber included in the Leica TCS SP5 confocal microscope. Local DNA damage was induced in a sub-nuclear area with a diameter of 1.5µm as described before [73]. For the induction of direct single strand breaks (SSBs) a 405 nm laser-pulse was used. For the induction of oxidative DNA damage, cells were first incubated for 10 min with 50µM photosensitizer Ro 19-8022 and micro-irradiated as described above. Resulting accumulation curves were corrected for background values and normalized to the relative fluorescence signal before local irradiation. Data are presented as mean ± SEM from at least three independent, pooled experiments.

2.8 Statistical analysis

Statistical analysis was performed as indicated in figure legends. Means of two groups were compared using Student's t test with a 95% confidence interval. Multiple comparisons were performed by one-way ANOVA or Two-way ANOVA with Sidak post-test. All the analyses were performed on GraphPad Prism (V8.2) software.

3.0 Results

3.1 Loss of DDB2 leads to accumulation of endogenous 8-oxoG

To determine if DDB2 is involved in 8-oxoG repair, we sought to investigate if there were unrepaired 8-oxoG lesions in the cell when DDB2 was knocked down. DDB2 and OGG1 were knocked down in U2OS-FAP-TRF1 cells and 8-oxoG levels were measured by immunofluorescence using an antibody that recognizes 8-oxoG in DNA, 48 hours post transfection with siRNAs (Figure 5A and B). There was a significant increase in endogenous 8-oxoG levels in the absence of DDB2 and OGG1, suggesting that DDB2 is involved in 8-oxoG processing in cells. It is to be noted that these cells are maintained at 5% O_2, to minimize background levels of oxidative stress.

To determine whether loss of DDB2 had long term effects on cell growth after oxidative damage we performed cell survival assays. We treated WT and DDB2 knockout (KO) cells (Figure 5C) with $KBrO_3$ before performing a colony formation assay. We found that cells deficient in DDB2 were more sensitive to oxidative DNA damage induced by $KBrO_3$ (Figure 5D). Taken together, these results indicate that DDB2 plays a critical role in 8-oxoG processing within cellular DNA.

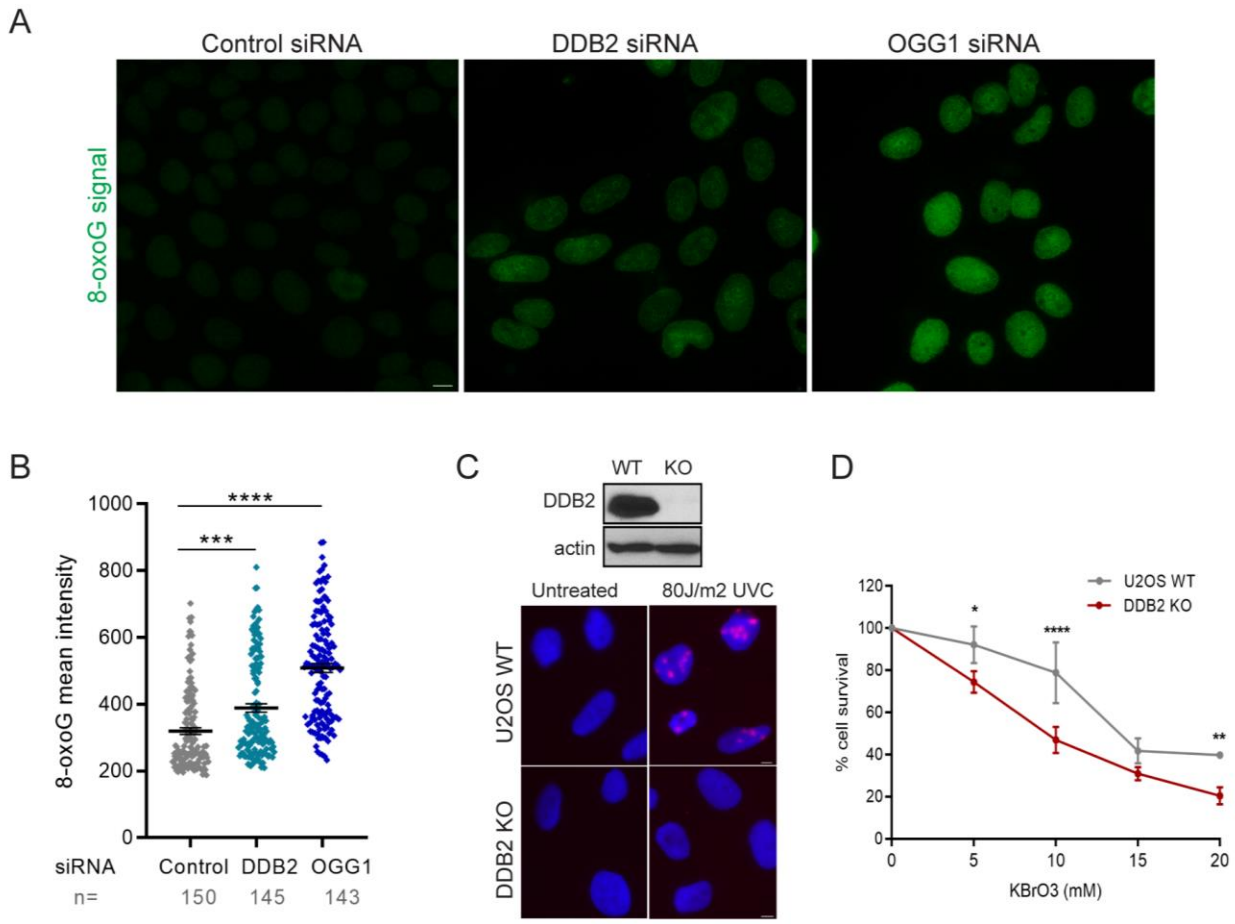

Figure 5: Loss of DDB2 leads to accumulation of 8-oxoG.

A. U2OS-FAP-TRF1 cells were transfected with control, DDB2 or OGG1 siRNA and 8-oxoG signal was measured by immunofluorescence, 48 hours post transfection. B. Mean nuclear intensity of A. Data represents three experiments, mean ± SEM. C. Western blot and Immunofluorescence (after local UVC exposure through 2 µm PC membrane) for DDB2 in U2OS-FAP-TRF1 wildtype (WT) and DDB2 knockout (KO) cells. D. Clonogenic cell survival curves in U2OS WT and DDB2 knockout (KO) cells treated with a range of concentrations of KBrO3. Data (D) shows one representative experiment (performed in triplicate), mean ± SD. One-way ANOVA (B) and two-way ANOVA (D) were performed for statistical analysis: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale: 5 µm.

3.2 Robust recruitment of DDB2 to telomeric 8-oxoG lesions

Using the FAP chemoptogenetic system we previously showed that mCherry-tagged mouse DDB2 was recruited to 8-oxoG in human cells immediately after damage [109]. The recruitment of DDB2 preceded that of OGG1 suggesting that DDB2 may be the first responder in 8-oxoG recognition. Here, we confirmed and extended these results using a human DDB2-mCherry expressed in U2OS cells stably expressing FAP-TRF1 (U2OS-FAP-TRF1) and show that DDB2 is recruited to 8-oxoG after dye plus light treatment (Figure 6A, 6B). As a parallel approach, we visualized the fluorescence of mNeon-DDB2 without using antibodies or DDB2-Flag in U2OS-FAP-TRF1 and RPE-hTERT cells stably expressing FAP-TRF1 (RPE-FAP-TRF1) and observed robust recruitment of DDB2 after dye plus light treatment (Figure 6C, 6D). These results directly demonstrate that DDB2 recruitment to telomeric 8-oxoG is not cell type dependent. Moreover, N-terminal (mNeon-DDB2) or C-terminal tags (DDB2-mCherry, DDB2-Flag) result in similar recruitment frequencies (Figure 6C, 6D). In order to further validate that DDB2 is associated with telomeres after 8-oxoG damage, we utilized a proximity ligation assay (PLA) (Figure 6E, 6F, 6G). Antibodies against mCherry-tagged DDB2 and TRF1 were used and the PLA signal in untreated and dye plus light treated cells was examined. We observed a significant increase in PLA signal after dye plus light treatment (Figure 6H), indicating that DDB2 is recruited to telomeres after 8-oxoG damage.

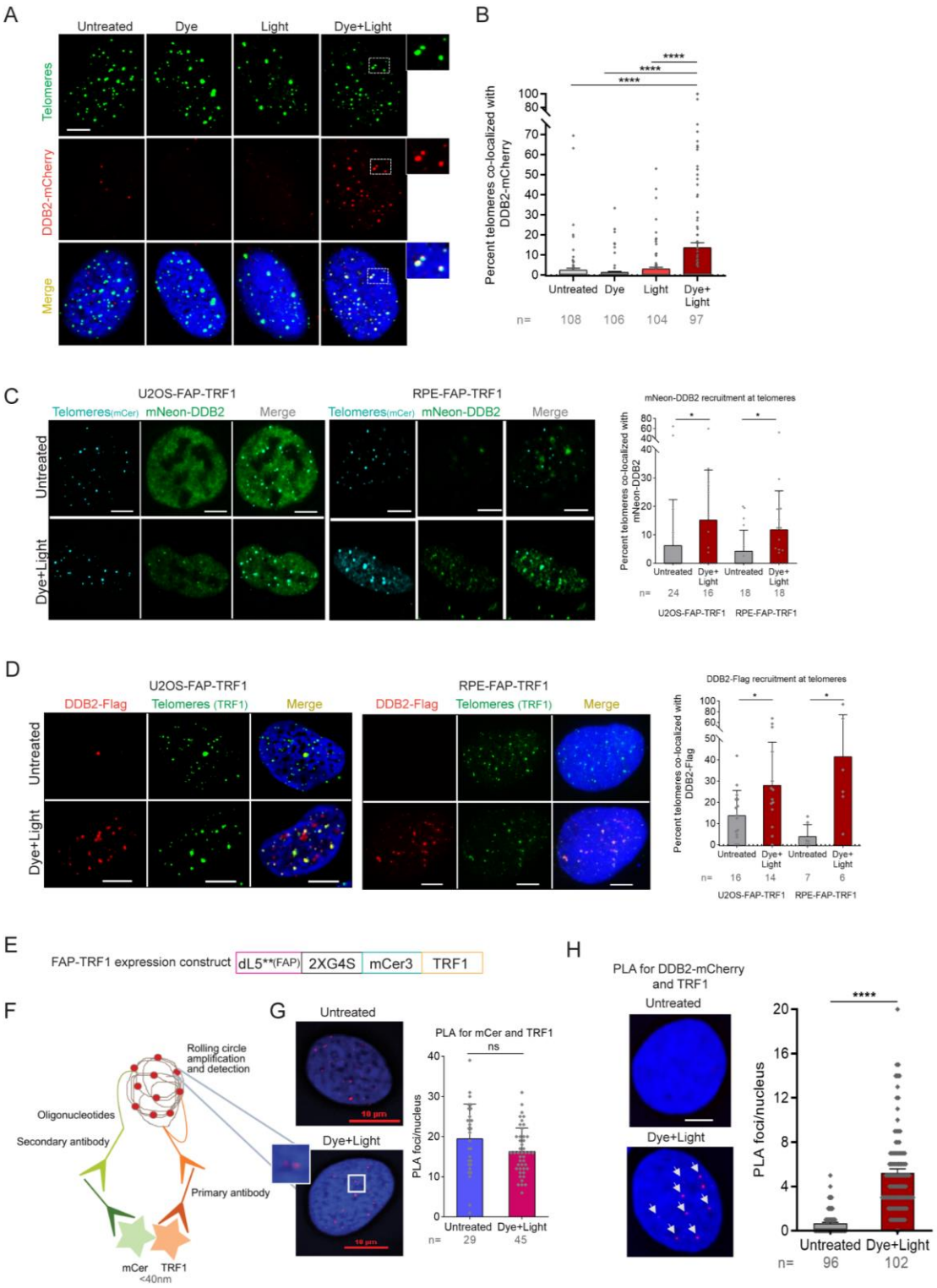

Figure 6: : Robust recruitment of DDB2 to telomeric 8-oxoG.

A. Recruitment of DDB2-mCherry to 8-oxoG sites at telomeres in untreated, dye alone, light alone and dye plus light treated cells. B. Percentage telomeres colocalized with DDB2-mCherry in A. C.

Recruitment of mNeon-DDB2 at telomeres after dye (100 nM, 15 min) plus light (660 nm, 10 min) treatment in U2OS and RPE-hTERT cells. mCerulean and mNeon fluorescence was directly observed under the microscope. D. Recruitment of DDB2-Flag at telomeres after dye (100 nM, 15 min) plus light (660 nm, 10 min) treatment in U2OS and RPE-hTERT cells. E. Schematic of the FAP-TRF1 overexpression construct stably expressed in U2OS and RPE-hTERT cells. F. Schematic representation of proximity ligation assay (PLA). G. Antibodies against mCerulean (mCer3) and TRF1 were used as a positive control to validate the PLA conditions. H. Proximity ligation assay (PLA) for mCherry and TRF1 in untreated cells and cells treated with dye (100 nM, 15min) and light (660 nm, 10 min). Data represent mean ± SEM (B) or mean ± SD (C, D) from two independent experiments. 'n' represents the number of cells scored for each condition. One-way ANOVA (B) and Student's two-tailed t-test (C, D, G, H) were performed for statistical analysis: *p<0.05, **p<0.01, ****p<0.0001, ns = not significant. Scale: 5 μm.

3.3 XP-E K244E variant does not recognize 8-oxoG lesions and UV photoproducts.

Mutations in DDB2 can cause xeroderma pigmentosum E (XP-E), a rare skin disorder characterized by extreme light sensitivity and increased risk of skin cancer [122]. We examined whether an XP-E variant K244E (Lys 244 to Glu) (Figure 7A) that is unable to bind specifically to UV-induced damage sites [85, 91] (Figure 7B) can recognize 8-oxoG lesions in cells. We visualized the accumulation of WT or K244E DDB2-Flag at telomeric 8-oxoG in U2OS-FAP-TRF1 cells. Compared to WT, we observed a 2-fold reduction in DDB2 (K244E) binding to damaged telomeres (Figure 7C, 7D), suggesting that DDB2 uses a similar damage recognition mechanism for UV-induced photoproducts and 8-oxoG. Taken together, these results indicate that DDB2 plays a critical role in 8-oxoG recognition within cellular telomeric DNA.

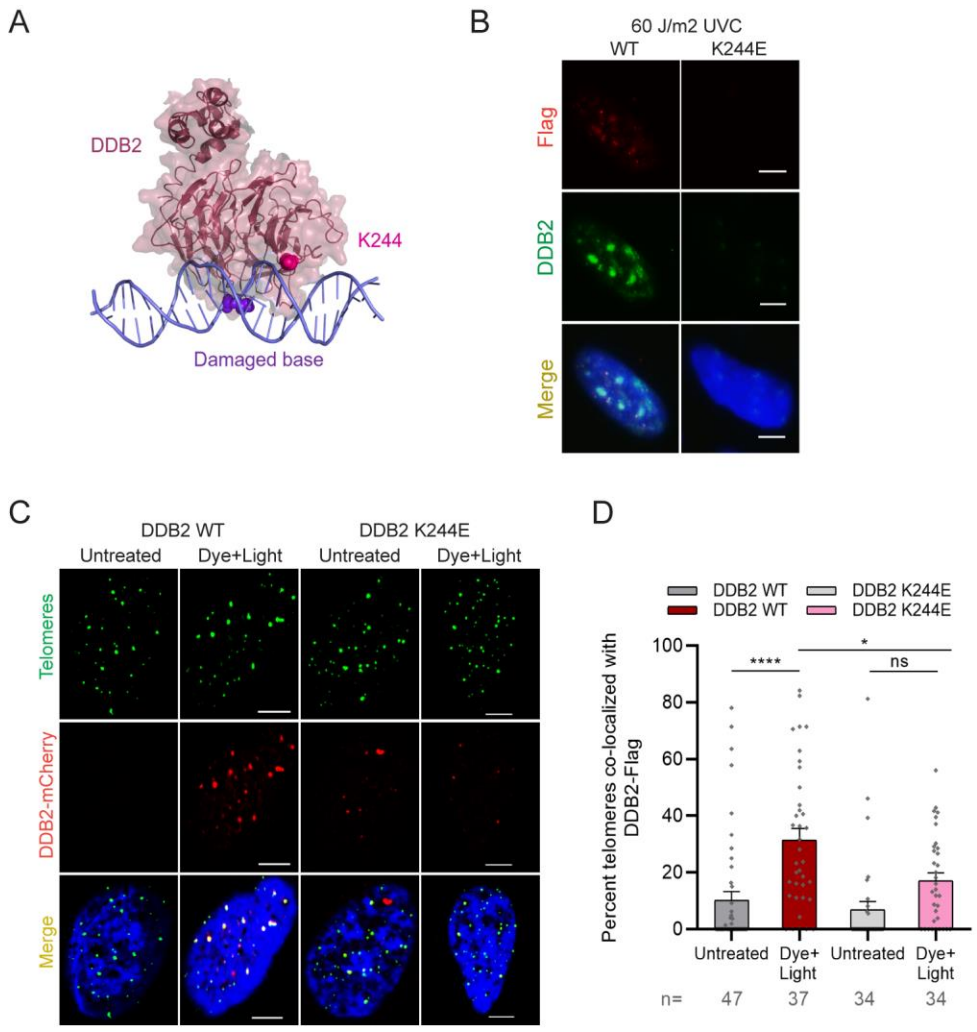

Figure 7: Robust recruitment of WT DDB2, but not XP-E variant K244E, to UVC-induced DNA damage and telomeric 8-oxoG.

A. Structure of DDB2 (dark red) bound to damaged (Purple) duplex DNA. Lysine 244 (pink) was mutated to glutamic acid (K244E). B. Immunofluorescence for DDB2-Flag (after local UVC exposure through 2µm PC membrane) in U2OS-FAP-TRF1 cells expressing WT or K244E variant of DDB2-Flag. C. Recruitment of DDB2-WT and DDB2-K244E to 8-oxoG sites at telomeres after dye plus light treatment. D. Percentage telomeres colocalized with DDB2-Flag in C. Data represents mean ± SEM. 'n' represents the number of cells scored for each condition. One-way ANOVA (D) was performed for statistical analysis: *p<0.05, ****p<0.0001. Scale: 5µm.

3.4 DDB2 is required for efficient OGG1 recruitment to 8-oxoG.

To evaluate the spatial and temporal association of DDB2 with OGG1 at sites of 8-oxoG damage, we employed PLA over a period of three hours after dye and light treatment. We observed a robust PLA signal from DDB2 and OGG1 immediately after dye and light treatment that decreased to background levels by three hours (Figure 8A, 8B). These results strongly support the concept that DDB2 and OGG1 transiently associate during processing of 8-oxoG. Biochemical and single-molecule results from our group have previously shown that UV-DDB stimulates the turnover of OGG1, and the two proteins transiently interact at abasic sites [109]. Strikingly, using IF we observed a higher accumulation of DDB2 at sites of damage in the absence of OGG1 at 30 minutes post dye and light treatment (Figure 8C, 8D). By fitting these kinetic data to an exponential decay, we calculated an approximate 3-fold longer half-life ($t_{1/2}$) of DDB2 in the absence of OGG1 (Control siRNA = 30.65 min, OGG1 siRNA = 89.91 min). These data suggest that DDB2 continues to re-bind unrepaired lesions in the absence of OGG1.

While the above-mentioned data suggest that DDB2 recruitment precedes OGG1, we wanted to examine whether DDB2 is absolutely required for OGG1 recruitment to 8-oxoG sites. To that end, we monitored the accumulation of OGG1 at damaged sites in the presence or absence of DDB2 (Figure 9B). Remarkably, when DDB2 was knocked down using siRNA, we observed a 3-fold reduction in OGG1 accumulation at both 30 minutes and an hour after dye and light treatment (Figure 9A, 9C). Consistent with these results, complete knockout of DDB2 resulted in a significant reduction of OGG1 accumulation at early times and longer retention at later times (Figure 9D, 9E). Together, these results establish that DDB2 is required for rapid and efficient recruitment and turnover of OGG1 at 8-oxoG sites.

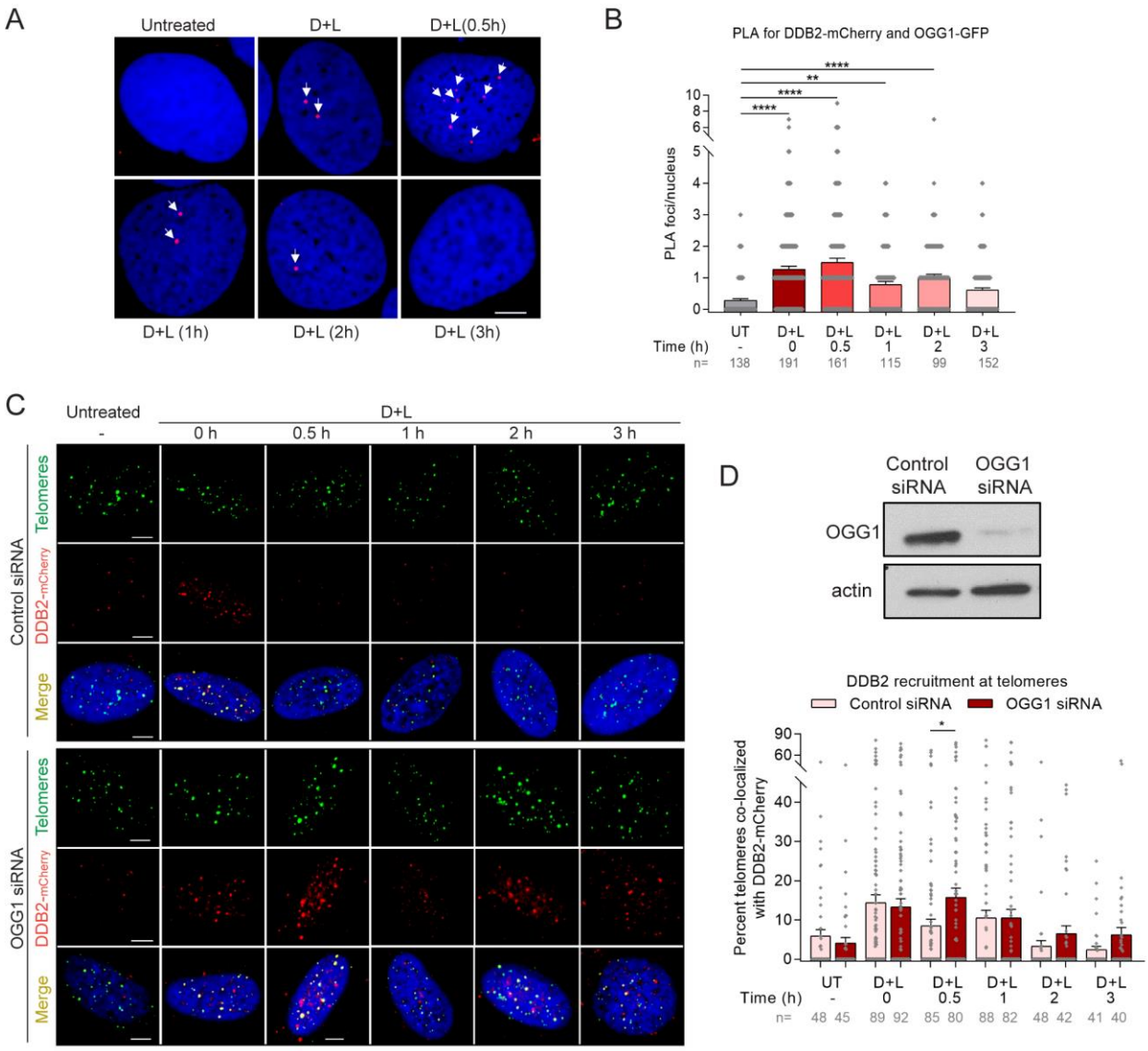

Figure 8: DDB2 is recruited to 8-oxoG in the absence of OGG1.

A. DDB2-mCherry and OGG1-GFP associate at 8-oxoG sites as shown by PLA after dye (100 nM, 15 min) plus light (600 nm, 10 min) treatment, over a period of three hours. Antibodies against mCherry and GFP were used. B. Quantification of PLA. C. Accumulation of DDB2-mCherry at telomeric 8-oxoG post dye plus light treatment in U2OS-FAP-TRF1 cells transfected with control or OGG1 siRNA. D. Western blot for OGG1 expression in U2OS-FAP-TRF1 cells transfected with control or OGG1 siRNA, 48 hours post siRNA transfection. E. Percent telomeres colocalized with DDB2-mCherry as shown in C. Data represents mean ± SEM from two independent experiments. 'n' represents the number of cells scored for each condition. One-way ANOVA: *p<0.05, **p<0.01, ****p<0.0001. Scale: 5 µm.

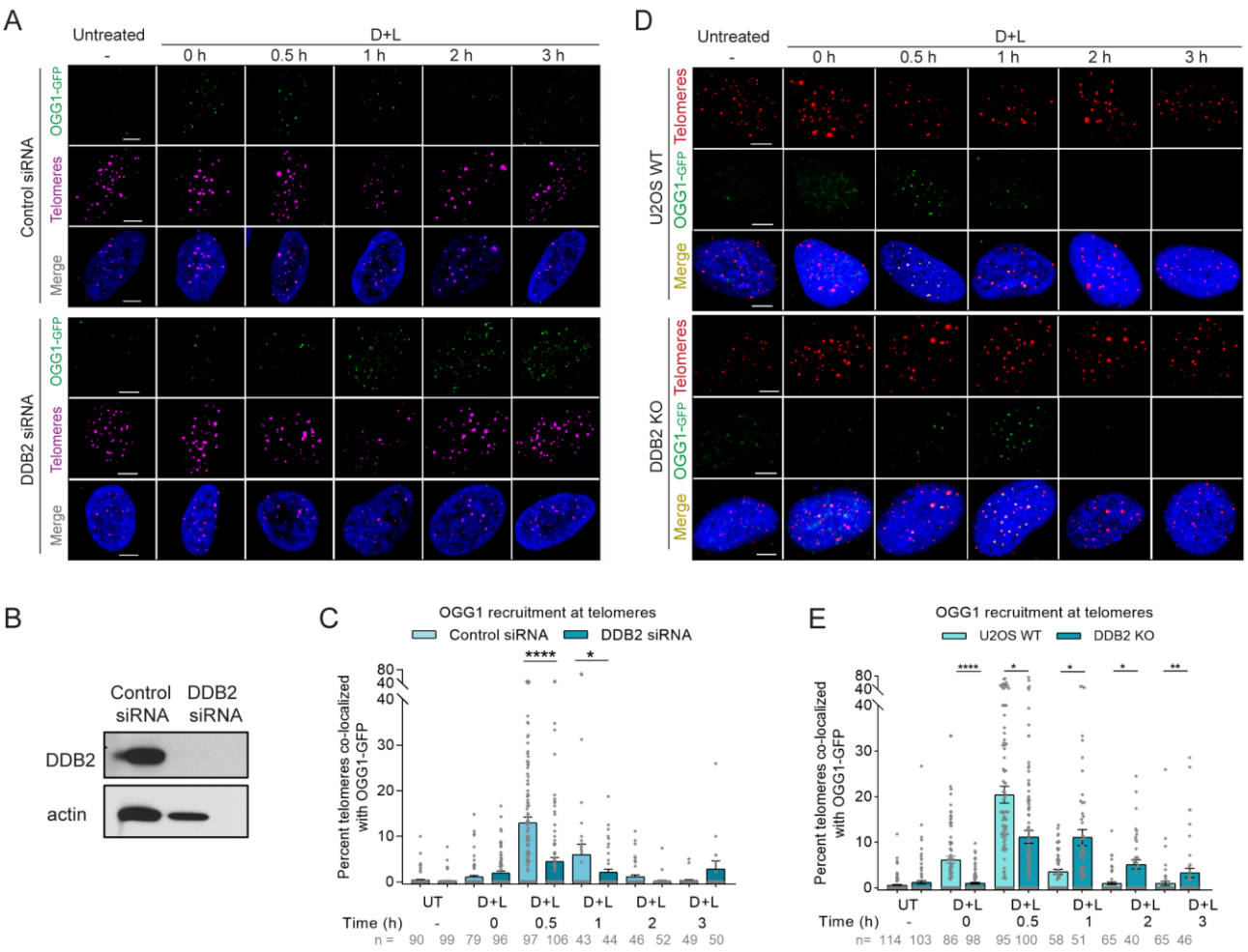

Figure 9:DDB2 is required for efficient OGG1 recruitment to 8-oxoG.

A. Recruitment of OGG1-GFP at damaged telomeres in cells transfected with control or DDB2 siRNA. B. Western blot for DDB2 expression in U2OS-FAP-TRF1 cells transfected with control or DDB2 siRNA, 48 hours post siRNA transfection. C. Percent telomeres colocalized with OGG1-GFP as shown in A. D. Recruitment of OGG1-GFP to damaged telomeres after dye plus light treatment in U2OS WT and DDB2 KO cells. E. Quantification of D. Data represents mean ± SEM from two independent experiments. 'n' represents the number of cells scored for each condition. One-way ANOVA: *p<0.05, **p<0.01, ****p<0.0001. Scale: 5 µm.

3.5 DDB2 recruits XPC to telomeric 8-oxoG, while XPA recruitment is transcription-coupled and independent of DDB2.

In GG-NER, UV-DDB facilitates the recruitment of XPC [32], which binds to the non-damaged strand and helps flip out the lesion on the opposite strand, facilitating the recruitment of the transcription factor TFIIH. Current models suggest that XPA is recruited simultaneously with TFIIH and is involved in both GG-NER and TC-NER [44]. To determine whether DDB2 mediates the recruitment of XPC and XPA to 8-oxoG, we examined the accumulation of GFP-tagged XPC or XPA over a period of three hours after dye plus light treatment. In WT cells, we observed both XPC and XPA are recruited to 8-oxoG within 30 minutes post dye plus light treatment (Figure 10A-D, Appendix D.1, Figure 19A, 19B), confirming the involvement of these proteins in 8-oxoG repair [61, 68, 72, 74]. Interestingly, knocking out DDB2 decreased XPC accumulation 3-fold (Figure 10A, 10B). However, the recruitment of XPA to 8-oxoG damage was not affected even in the complete absence of DDB2 (Figure 10C, 10D). These data suggest that XPC is recruited downstream of DDB2. Contrary to XPC, XPA appears to be recruited in a DDB2-independent repair pathway.

Spivak and colleagues have shown that cells lacking XPA were deficient in 8-oxoG repair in the transcribed strand [74]. To examine whether XPA is being recruited to sites of 8-oxoG damage through transcription-coupled repair (TCR) process, we pretreated cells with transcription inhibitors, α-amanitin and THZ1, and analyzed the accumulation of XPC or XPA 30 minutes after treating with dye plus light. As expected, we saw no difference in XPC recruitment at 8-oxoG sites (Figure 10E, 10F). Strikingly, we saw a 2-3-fold reduction in XPA accumulation in the presence of transcription inhibitors (Figure 10G, 10H) or when cells were transfected with CSB siRNA (Figure 11B, 11D), indicating that XPA participates in TCR of 8-oxoG. The presence of TCR at

8-oxoG sites is noteworthy because 8-oxoG itself lacks transcription-blocking capacity [123]. However, it has been shown that BER intermediates (abasic sites and/or single-strand breaks) can efficiently block transcription [123, 124]. Transcription of the C-rich strand 'CCCTAA' at telomeres by Pol II gives rise to a class of long noncoding RNAs containing telomeric repeats (TERRA) [125]. While studies suggest TERRA plays a role in regulating telomere function and homeostasis, its mechanism of action is largely unknown [126]. The FAP-TRF1 system damages the G-rich strand of telomeres containing the 'TTAGGG' repeat, which is the non-transcribed strand. Therefore, TCR of telomeric 8-oxoG is counterintuitive. We believe this could be due to two possible reasons: (1) It has been shown that single-strand nicks in the non-transcribed strand favors the formation of R-loops, which involves the transcribed strand and efficiently blocks transcription, needing the TCR machinery to be recruited [127-129]; (2) U2OS cells maintain their telomeres through the recombination-mediated alternative lengthening of telomeres (ALT) pathway. ALT cells contain a 'TCAGGG' variant repeat throughout the telomeres [130], therefore guanines are present in the complementary C-rich transcribed strand. The repair of this oxidized guanine might require TCR. To test these two possibilities, recruitment of TCR proteins should be measured in ALT-negative cell lines.

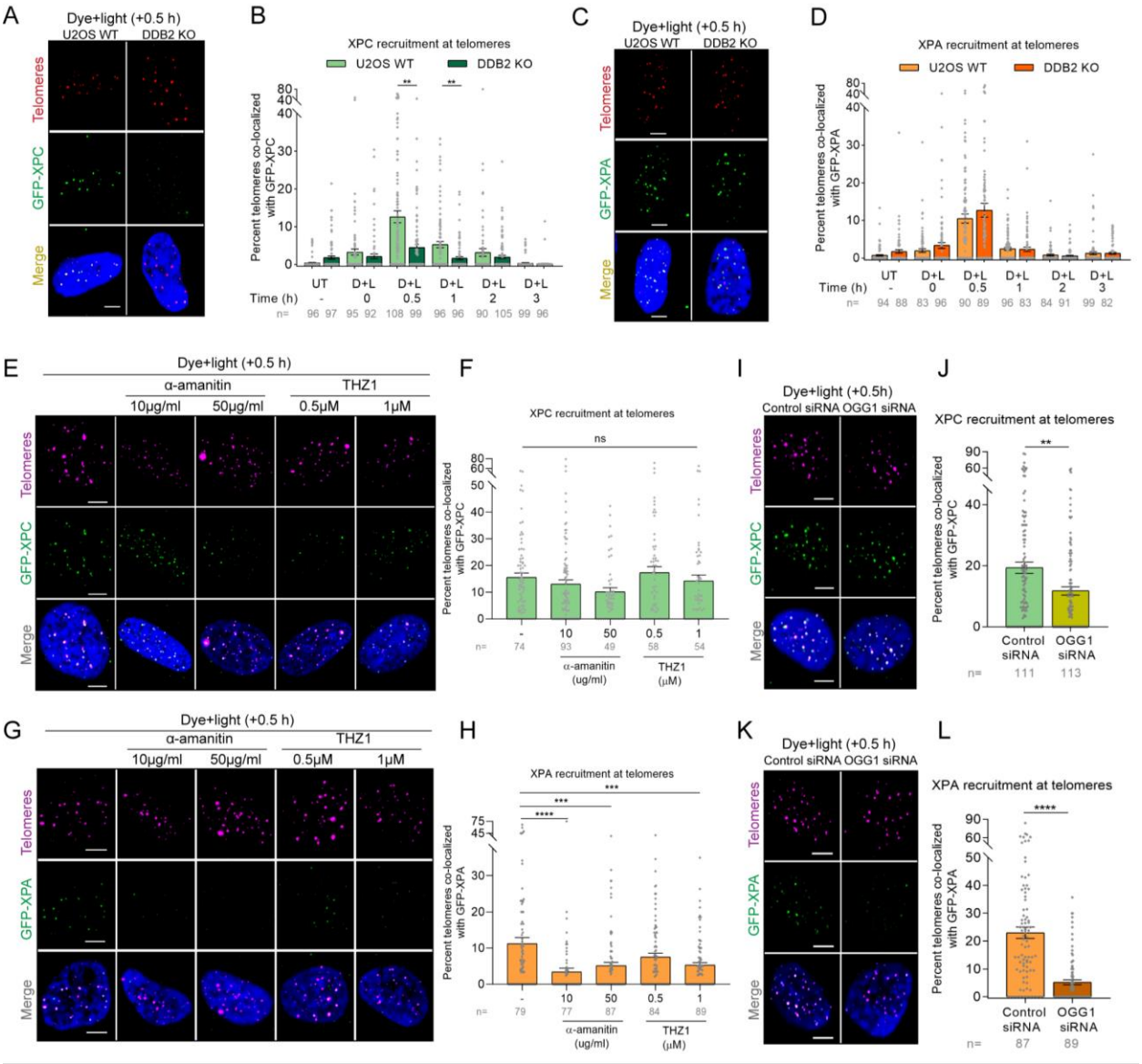

Figure 10: DDB2 and OGG1 recruit XPC to telomeric 8-oxoG, while XPA recruitment is transcription-coupled and independent of DDB2.

A and C. Representative images showing recruitment of GFP-XPC (A) or GFP-XPA (C) to 8-oxoG at telomeres after dye (100 nM, 15 min) and light (600 nm, 10 min) treatment in U2OS WT and DDB2 KO cells, 30 minutes post treatment. B and D. Percentage telomeres colocalized with GFP-XPC (B) or GFP-XPA (D) after treatment, over a period of three hours. E and G. Representative images of GFP-XPC (E) or GFP-XPA (G) accumulation at damaged telomeres 30 minutes after dye plus treatment in cells pre-treated with transcription inhibitors α-amanitin and THZ1. F and H. Quantification of E (F) and G (H). I and J.

Colocalization of GFP-XPC with telomeres after dye plus light treatment in cells transfected with control or OGG1 siRNA. K and L. Colocalization of GFP-XPA with telomeres after dye plus light treatment in U2OS-FAP-TRF1 cells transfected with control or OGG1 siRNA. Data represents mean ± SEM from two independent experiments. 'n' represents the number of cells scored for each condition. One-way ANOVA (B, D, F, J) and Student's two-tailed t-test (J, L): **p<0.01; ***p<0.001; ****p<0.0001. Scale: 5 μm.

As mentioned earlier, Pol II stalls at BER intermediates, and formation of BER intermediates requires the action of OGG1 and/or APE1. To test whether XPA recruitment depends on OGG1-mediated processing of 8-oxoG, we knocked down OGG1 using siRNA (Figure 9A). We found that recruitment of XPA was decreased ~5-fold in the absence of OGG1 (Figure 10K, 10L). However, in OGG1 KD cells, XPC was still recruited to sites of 8-oxoG, although there was a slight 25% reduction (Figure 10I, 10J). Furthermore, OGG1 recruitment to telomeres was not dependent on XPC (Figure 11A, 11C). It is possible that XPC stabilization at 8-oxoG requires timely dissociation of DDB2 and subsequent recruitment of OGG1, similarly to our recent observation that timely dissociation of DDB2 and recruitment of the downstream GG-NER factor TFIIH stabilizes XPC binding to UV damage [101]. Moreover, it has been shown that XPC can stimulate OGG1 activity on 8-oxoG-containing duplex oligonucleotide by 3-fold [61]. In summary, our results indicate that 8-oxoG is processed at telomeres through two separate and distinct pathways: (1) a global repair pathway, involving GG-NER proteins, where DDB2 and XPC work together to enable OGG1 recruitment to 8-oxoG, and (2) a transcription-coupled repair pathway involving XPA, that is initiated when repair intermediates interfere with transcription.

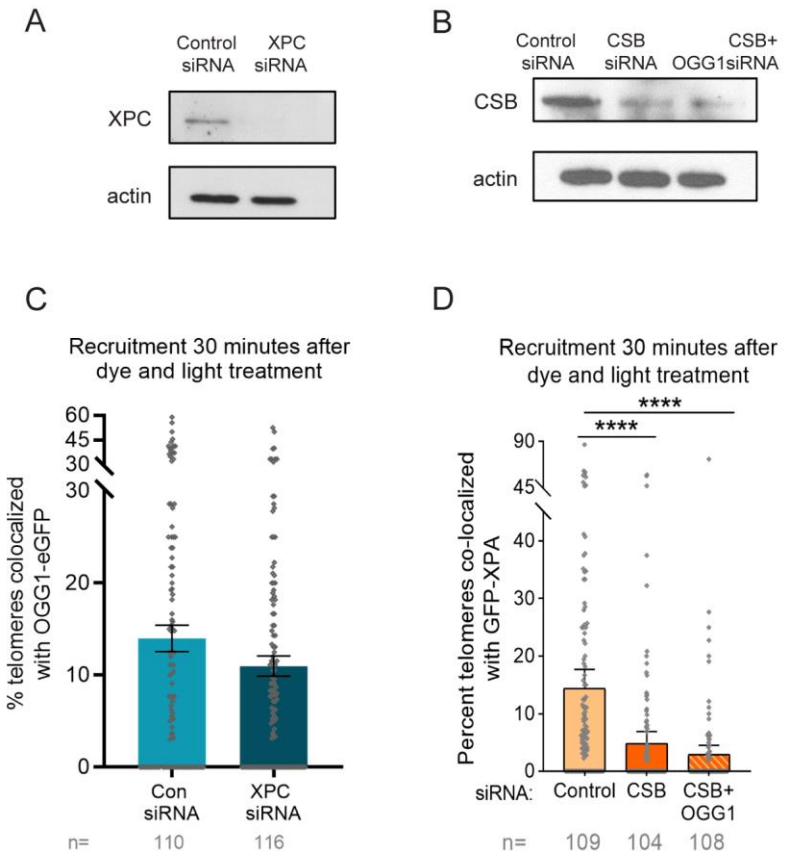

Figure 11: OGG1 is required for XPC and XPA accumulation during processing of 8-oxoG.

A. Western blot showing XPC levels in cells treated with control or XPC siRNA, 48 hours post transfection. B. Western blot showing CSB levels in cells treated with control or CSB siRNA, 48 hours post transfection. C. Colocalization of OGG1-GFP with telomeres after dye plus light treatment in U2OS-FAP-TRF1 cells transfected with control or XPC siRNA. D. Colocalization of GFP-XPA with telomeres after dye plus light treatment in cells transfected with control, CSB or CSB and OGG1 siRNA. Data represents mean ± SEM from two independent experiments. 'n' represents the number of cells scored for each condition. Student's two-tailed t-test (C) and One-way ANOVA (D): **p<0.01; ***p<0.001; ****p<0.0001. Scale: 5 µm.

3.6 DDB2 binds sparse telomeric 8-oxoG lesions independently of the DDB1-CUL4A-RBX1 E3 ligase.

DDB2 was discovered as part of a heterodimeric complex, UV-DDB, consisting of DDB2 itself and the larger subunit DDB1 [131]. UV-DDB forms a larger complex with the CUL4A-RBX1 ubiquitin E3 ligase (CRL^{DDB2}) and binds to UV damage to ubiquitylate histones and allow for chromatin relaxation and subsequent accessibility to downstream repair proteins, including XPC [110]. Interestingly, longer retention of UV-DDB at the damage site, either due to high affinity to the lesion or high lesion density, can obstruct downstream repair [101]. Therefore, timely removal of DDB2 is necessary for efficient repair. The CRL^{DDB2} E3 ligase complex can auto-polyubiquitylate DDB2 to allow for its extraction from chromatin by the Valosin-containing Protein (VCP)/p97 Segregase and subsequent degradation by the 26S proteosome [101]. Other studies have shown that DDB2, in the absence of other factors, can cause chromatin decompaction and together with ATP-dependent chromatin remodelers, alter the nucleosome structure around photoproducts after UV damage [104, 107, 117].

To evaluate the role of the CRL^{DDB2} complex in 8-oxoG repair, we measured the accumulation of DDB2 after siRNA-mediated depletion of DDB1 or CUL4A (Figure 13A, 13B). We found that DDB2 binds to relatively sparse 8-oxoG sites (1-2 per telomere [114]) even in the absence of CRL (Figure 12A, 12B). To assess whether DDB2 dissociation from the damage site required the ubiquitylation action of DDB1 or CUL4A, we quantified the colocalization of DDB2-mCherry with GFP tagged DDB1 or CUL4A at 8-oxoG sites (Figure 12C-F). We observed that DDB2 rapidly accumulated at sites of damage and dissociated by 30 minutes. On the other hand, we saw a significant accumulation of both DDB1 and CUL4A by 30 minutes. However, very little colocalization (< 3%) was observed between DDB2 and DDB1 or CUL4A. Moreover, recruitment

of DDB1 and CUL4A at 30 minutes was independent of DDB2 (Figure 13C and 13D). It is possible that the significant recruitment of DDB1 or CUL4A at 30 minutes post damage is due to TCR at repair sites, since DDB1 and CUL4A also associate with CSA (CRL^{CSA}) during TC-NER to ubiquitylate CSB [132]. Ubiquitylation and degradation of CSB has been shown to be indispensable for post TC-NER recovery of RNA synthesis [132, 133].

The absence of colocalization between DDB2 and DDB1 or CUL4A could be due to low 8-oxoG density or differences in the repair of lesions in telomeres versus the bulk genome, since targeted damage to telomeric DNA only represents 0.02% of the genome. Therefore, much lower damage is being introduced after dye and light treatment as compared to studies that have used high doses (10-60 J/m^2) of UVC to damage the entire genome. Additionally, the binding affinity of UV-DDB to 8-oxoG is ~5-fold lower than to CPDs [109], decreasing its retention time on the lesion, thus potentially eliminating the necessity for CRL mediated ubiquitylation and degradation. To that end, we looked at the total cellular DDB2 amounts in cells after dye plus light treatment. As expected, we saw no significant degradation of DDB2 after dye and light treatment ($100mW/cm^2$, 10 or 20 minutes) or $KBrO_3$ treatment (40mM, 1hour), but we saw as much as a 4-fold decrease in DDB2 levels 4 hours after global UV damage ($60J/m^2$) (Figure 12G).

We recently demonstrated that DDB2 dissociation from UV damage is stimulated by recruitment of the downstream protein complex TFIIH, and longer retention on the damage site leads to CRL^{DDB2} mediated DDB2 polyubiquitylation and degradation [101]. We therefore examined whether DDB2 and CUL4A colocalize at 8-oxoG sites in the absence of the downstream protein OGG1, 30 minutes post dye plus light treatment. Compared to WT cells, we observed a 2.5-fold increase in DDB2 and CUL4A colocalization at damaged telomeres when OGG1 was knocked down (Figure 12H, 12I). As shown earlier (Figure 10K, 10L), XPA is not recruited to

telomeric 8-oxoG in the absence of OGG1, suggesting that 8-oxoG processing by OGG1 is required to form transcription blocking intermediates. To confirm that the CUL4A recruitment seen in OGG1 KD cells is not due to TCR, we treated OGG1 KD cells with α-amanitin and observed no effect on CUL4A recruitment (Figure 13E). Moreover, while CUL4A accumulation at damaged telomeres is significantly reduced in the absence of CSB, when both CSB and OGG1 is knocked down, an increase in CUL4A colocalization with telomeres is observed (Figure 13F), suggesting that in the absence of OGG1, CUL4A is required for DDB2 dissociation from telomeric 8-oxoG. In total, these results indicate that at lower lesion densities and when OGG1 is present, DDB2 dissociation from 8-oxoG may not require CRL^{DDB2} activity.

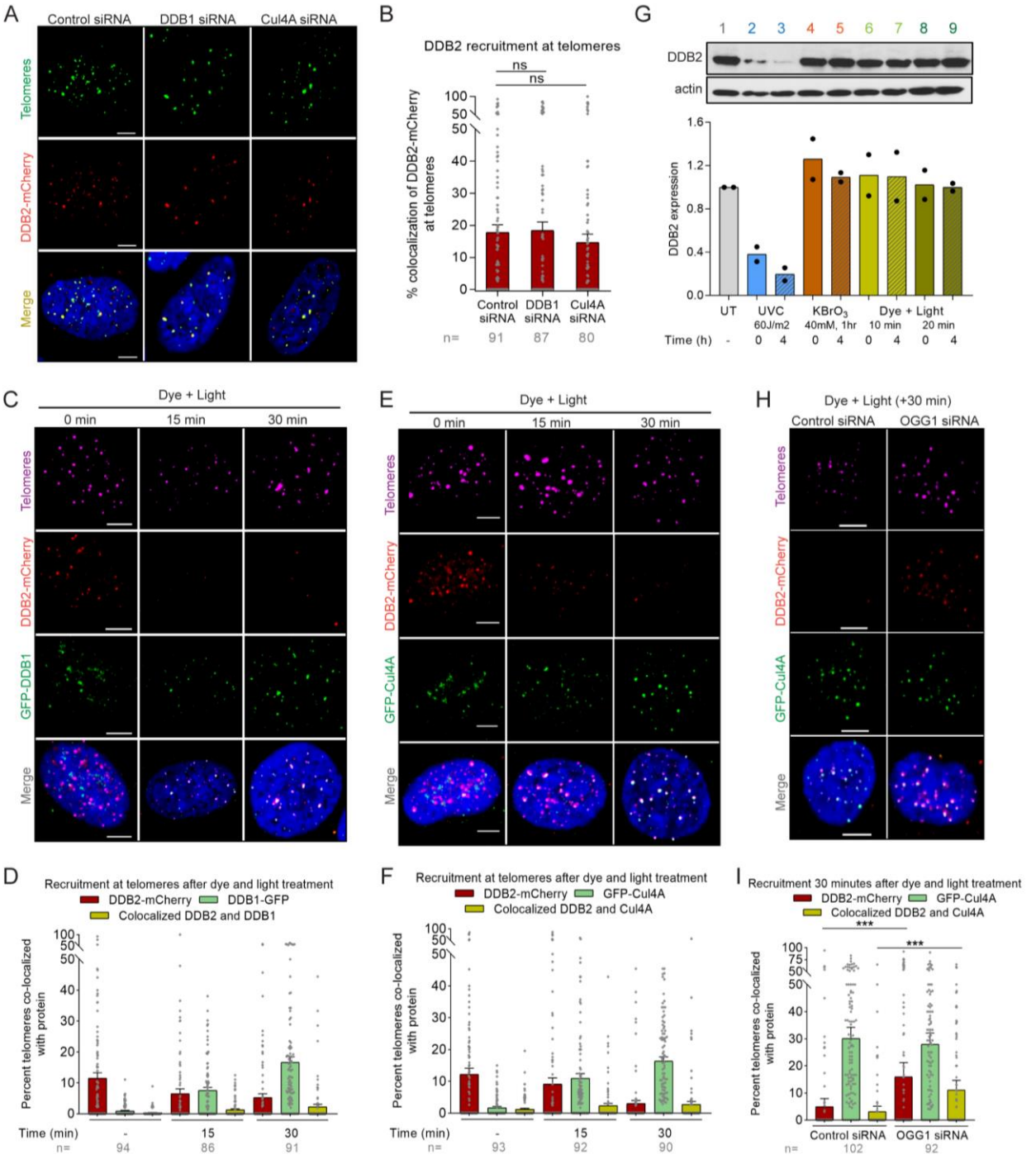

Figure 12: DDB2 binds sparse telomeric 8-oxoG independently of the DDB1-CUL4A-RBX1 E3 ligase.

A. Representative images showing recruitment of DDB2-mCherry to telomeric 8-oxoG sites in cells transfected with control, DDB1 or CUL4A siRNA. B. Quantification of A. C and E. DDB2-mCherry and GFP-DDB1 (C) or DDB2-mCherry and GFP-CUL4A (E) accumulation at 8-oxoG sites after dye

(100 nM, 15 min) plus light (600 nm, 10 min) treatment. D and F. Quantification of C and E respectively. G. Western blot for DDB2 in U2OS-FAP-TRF1 cells treated with UVC, potassium bromate (KBrO3) or dye plus light at indicated doses. Independent experiments are represented by black circles. H. Colocalization of DDB2-mCherry and GFP-CUL4A at damaged telomeres in U2OS-FAP-TRF1 cells transfected with control or OGG1 siRNA. I. Quantification of H. Data represents mean ± SEM from two independent experiments. 'n' represents the number of cells scored for each condition. One-way ANOVA (B), Student's two-tailed t-test (I) was performed for statistical analysis: *p<0.05, ***p<0.001, ns = not significant. Scale: 5 µm.

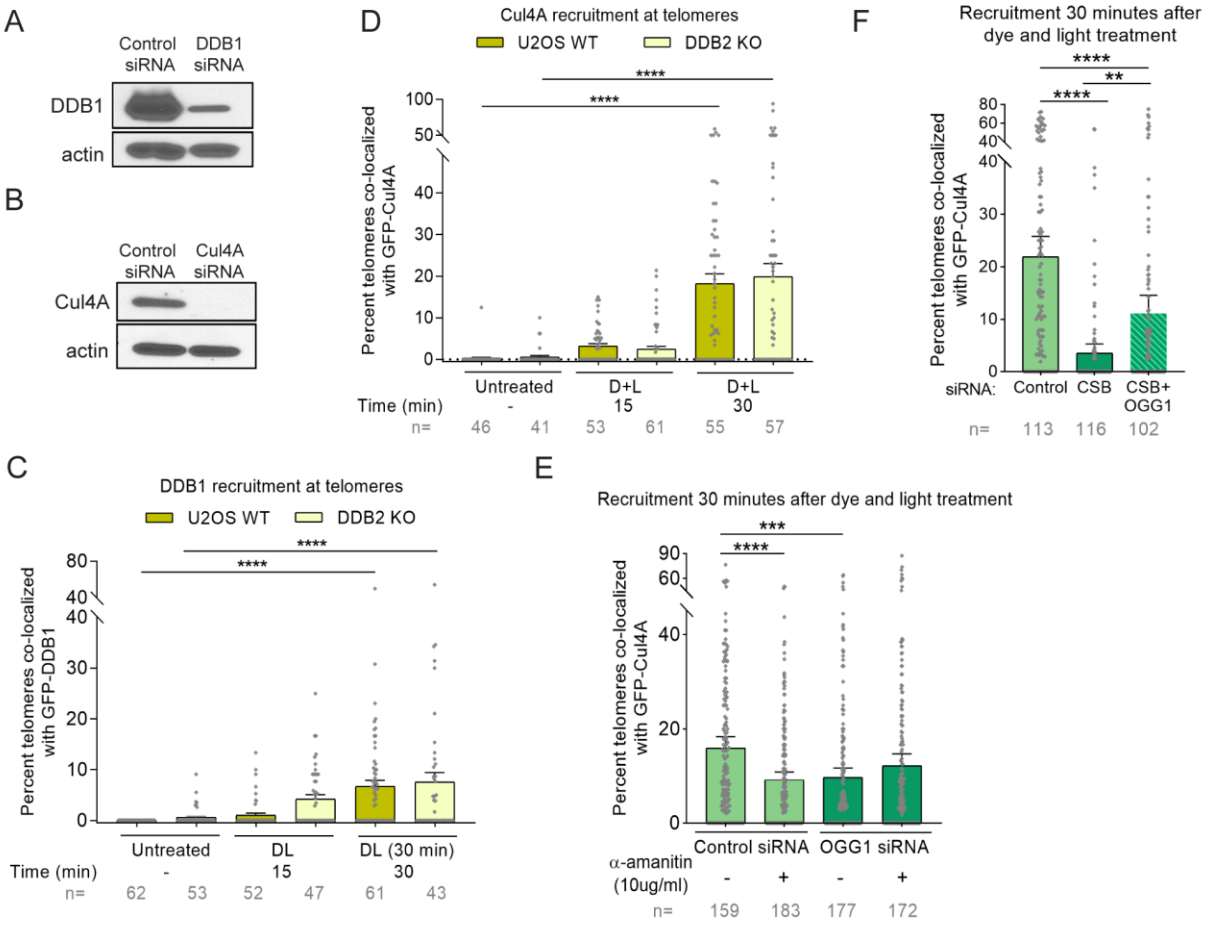

Figure 13: The DDB1-CUL4A-RBX complex is required for transcription-coupled repair of 8-oxoG.

A. Western blot for DDB1 and D. CUL4A in cells treated with DDB1 or CUL4A siRNA. C. GFP-DDB1 and D. GFP-CUL4A recruitment to 8-oxoG sites after dye (100 nM, 15 min) plus light (600 nm, 10 min) treatment in WT and DDB2 KO cells. E. Accumulation of GFP-CUL4A at damaged telomeres in U2OS-FAP-TRF1 cells transfected with control or OGG1 siRNA and pre-treated with transcription inhibitors α-amanitin. F. Percent telomeres colocalized with GFP-CUL4A in cells transfected with control, CSB or CSB and OGG1 siRNA. Data represents mean ± SEM from one (C, D) or three (E, F) independent experiments. 'n' represents the number of cells scored for each condition. One-way ANOVA: **p<0.01, ***p<0.001, ****p<0.0001.

3.7 DDB2 stimulates OGG1 recruitment to densely clustered 8-oxoG sites

As shown in Figure 12H and 12I, persistent binding of DDB2 to unrepaired 8-oxoG lesions results in the recruitment of the CRL complex. To validate DDB2's role in 8-oxoG recognition at higher lesion densities at non-telomeric sequences, we employed an independent approach using a photosensitizer (Ro 19-8022) in combination with 405 nm laser pulse [72, 73] to locally induce 8-oxoG lesions at high density in specific sub-nuclear regions. We employed real-time live-cell imaging in three different cell lines stably expressing GFP-DDB2 or OGG1-GFP and observed rapid recruitment (within a minute) of both DDB2 and OGG1 at 8-oxoG sites (Figure 14A, 14B). The recruitment of DDB2 or OGG1 was not observed when only single-strand breaks were introduced (Figure 15A, 15B).

In undamaged cells, the CRL^{DDB2} complex is bound by the COP9 signalosome [134], which renders it inactive. Following UV damage, neddylation of CUL4A by NEDD8 makes CRL^{DDB2} an active ubiquitin ligase. We used two different inhibitors to study this process: 1) NAEi, which inhibits neddylation keeping CRL^{DDB2} inactive, and 2) CSN5i, which prevents deneddylation and keeps CRL^{DDB2} hyperactive causing continual ubiquitylation and subsequent degradation of DDB2, even in the absence of UV damage (Figure 14C and 14F). These inhibitors seem to be specific to CRL^{DDB2} as CSA levels were unaffected. Using the NEDD8 inhibitor (NAEi) and keeping UV-DDB inactive significantly reduced OGG1 accumulation (Figure 14D and 14E). Furthermore, when DDB2 is greatly depleted by the action of CSN5i, we observed a significant reduction of OGG1 recruitment to 8-oxoG sites (Figure 14D and 14E). Taken together these data suggest that DDB2 helps facilitate OGG1 recruitment to 8-oxoG sites irrespective of the genomic location. Moreover, either blocking CRL^{DDB2} or ubiquitylating and degrading DDB2

reduces OGG1 recruitment to 8-oxoG, indicating the involvement of CRL^{DDB2} during 8-oxoG repair when these lesions are at high densities in genomic DNA.

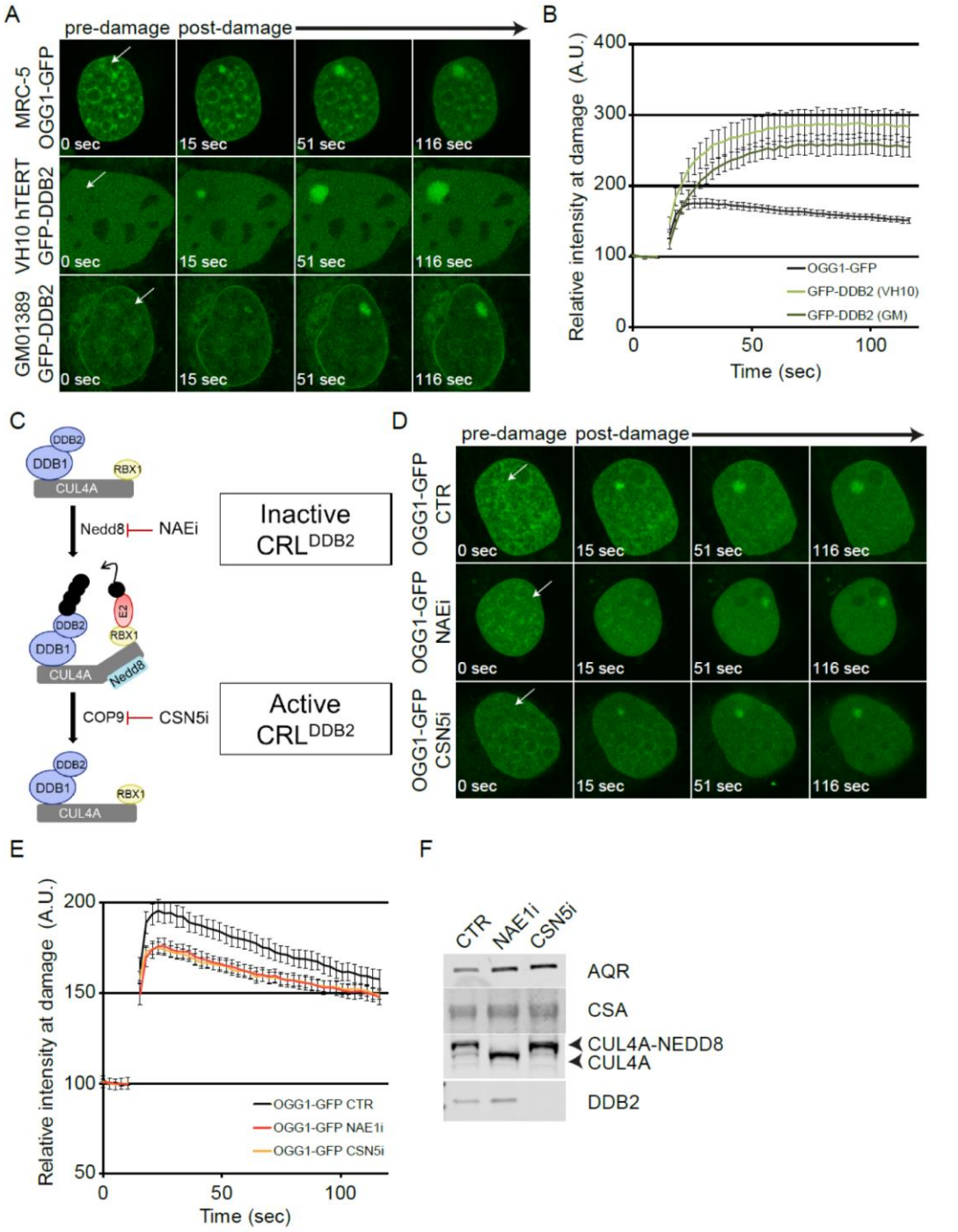

Figure 14: DDB2 stimulates OGG1 recruitment to densely clustered 8-oxoG sites.

A. Representative time-lapse pictures of OGG1-GFP and GFP-DDB2 accumulation at micro-irradiated (405 nm laser) sub-nuclear area, indicated by arrows, in the presence of 50μM Ro 19-8022 photosensitizer. B. Quantification of accumulation kinetics of OGG1-GFP and GFP-DDB2 (as shown in A). C. Schematic overview of the molecular interactions of DDB2 within the CUL4A-DDB1-RBX1 E3 ubiquitin ligase complex (CRL), which is required for the successive molecular interactions by ubiquitylation and subsequent DNA repair. The activation of CRL is mediated by covalent attachment of the ubiquitin-like activator NEDD8 on CUL4A and its proteolytic removal leads to the deactivation of ubiquitin ligase function. These crucial events can be fine-tuned by specific inhibitors MLN4924 (NAE1i) and SB-58-SN29 (CSN5i), acting on NEDD8-activating enzyme NAE1 and CSN5 respectively. D. Representative time-lapse pictures of OGG1-GFP accumulation at micro-irradiated (405 nm laser) sub-nuclear area, indicated by arrows, in the presence of 10μM Ro 19-8022 photosensitizer. Cells were pre-treated with DMSO (CTR), NEDDylation inhibitor (NAE1i) or de-NEDDylation inhibitor (CSN5i) for 1.5 hours. E. Quantification of accumulation kinetics of OGG1-GFP (as shown in D). F. Immunoblot analysis for DDB2, CUL4A, CSA and AQR (loading control) in MRC-5 expressing OGG1-GFP. Cells were treated with inhibitors as indicated in D. ***These data were generated by Arjan F. Theil from Department of Molecular Genetics, Oncode Institute, Erasmus MC, University Medical Center Rotterdam, Dr. Molewaterplein 40, 3015 GD, Rotterdam, The Netherlands***

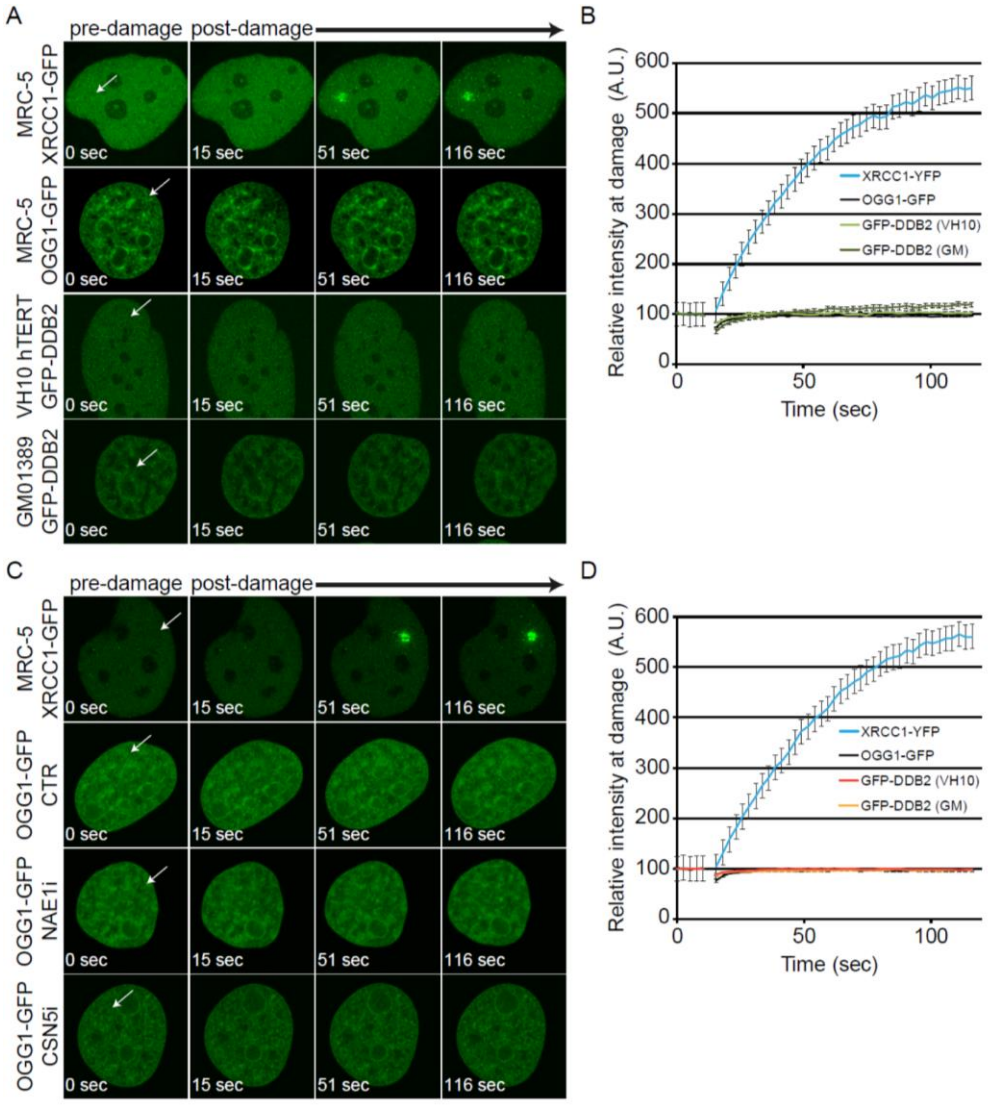

Figure 15: OGG1-GFP and GFP-DDB2 do not accumulate at SSBs.

A. Representative time-lapse pictures of XRCC1-YFP, OGG1-GFP and GFP-DDB2 accumulation at micro-irradiated (405 nm laser) sub-nuclear area, indicated by arrows. B. Quantification of accumulation kinetics of XRCC1-YFP, OGG1-GFP and GFP-DDB2 (as shown in A). C. Representative time-lapse pictures of XRCC1-YFP and OGG1-GFP accumulation at micro-irradiated (405 nm laser) sub-nuclear area, indicated by arrows. Cells were pre-treated with DMSO (CTR), neddylation inhibitor (NEDDi) or de-neddylation inhibitor (deNEDDi) for 1.5 hours. D. Quantification of accumulation kinetics of XRCC1-YFP and OGG1-GFP (as shown in C*). These data were generated by Arjan F. Theil from Department of Molecular Genetics, Oncode Institute, Erasmus MC, University Medical Center Rotterdam, Dr. Molewaterplein 40, 3015 GD, Rotterdam, The Netherlands*

3.8 DDB2 mediates chromatin decompaction at sites of telomeric 8-oxoG.

Intriguingly, we observed a gradual expansion of GFP-DDB2 and OGG1-GFP repair proteins at local 8-oxoG damaged sites after treatment with Ro 19-8022 and 405 nm light (Figure 14A). As mentioned earlier, previous studies have shown a role for DDB2 in chromatin decompaction [104, 107]. Moreover, in these studies, DDB2 was tethered to a Lac repressor (LacR) and expressed in cells containing Lac operator (LacO) sites. Binding of DDB2-LacR to the LacO led to an expansion of the LacO area, suggesting that binding of DDB2 is necessary and sufficient for decompaction of chromatin. Based on these previous findings and our data, we asked whether binding of DDB2 to 8-oxoG lesions at telomeres impacted the local chromatin structure.

To address whether DDB2 binding to telomeric DNA causes telomere expansion, 8-oxoG was induced at telomeres and telomere 3D volumes in WT cells were measured using confocal imaging (Figure 16A, Figure 20B, 20D). These results indicated that the telomeric chromatin relaxes after 8-oxoG damage. Interestingly, this increase in telomere volumes was not observed when DDB2 was knocked out (Figure 16B, Figure 20B, 20D), indicating that DDB2 plays a critical role in local chromatin unfolding at the sites of 8-oxoG damage. U2OS cells maintain their telomeres through the alternative lengthening of telomeres (ALT) pathway, which is characterized by a heterogenous telomere length and telomere clustering after double-stranded breaks [135]. To verify that the apparent telomere expansion we observed was not a result of ALT-associated telomere clustering, we measured the telomere volumes in a telomerase positive cell line, RPE-FAP-TRF1. We observed a significant increase in telomere volume in WT cells, but not in DDB2 KO cells (Figure 16C, 16D, Figure 20A, 20C, 20E). These data clearly demonstrate that DDB2 binds to 8-oxoG sites in the chromatin and mediates a local chromatin restructuring to allow downstream proteins to access the lesion. As DDB2 has no known chromatin remodeling activity,

whether this decompaction is a direct result of DDB2 binding or through the recruitment of other chromatin remodelers remains to be investigated.

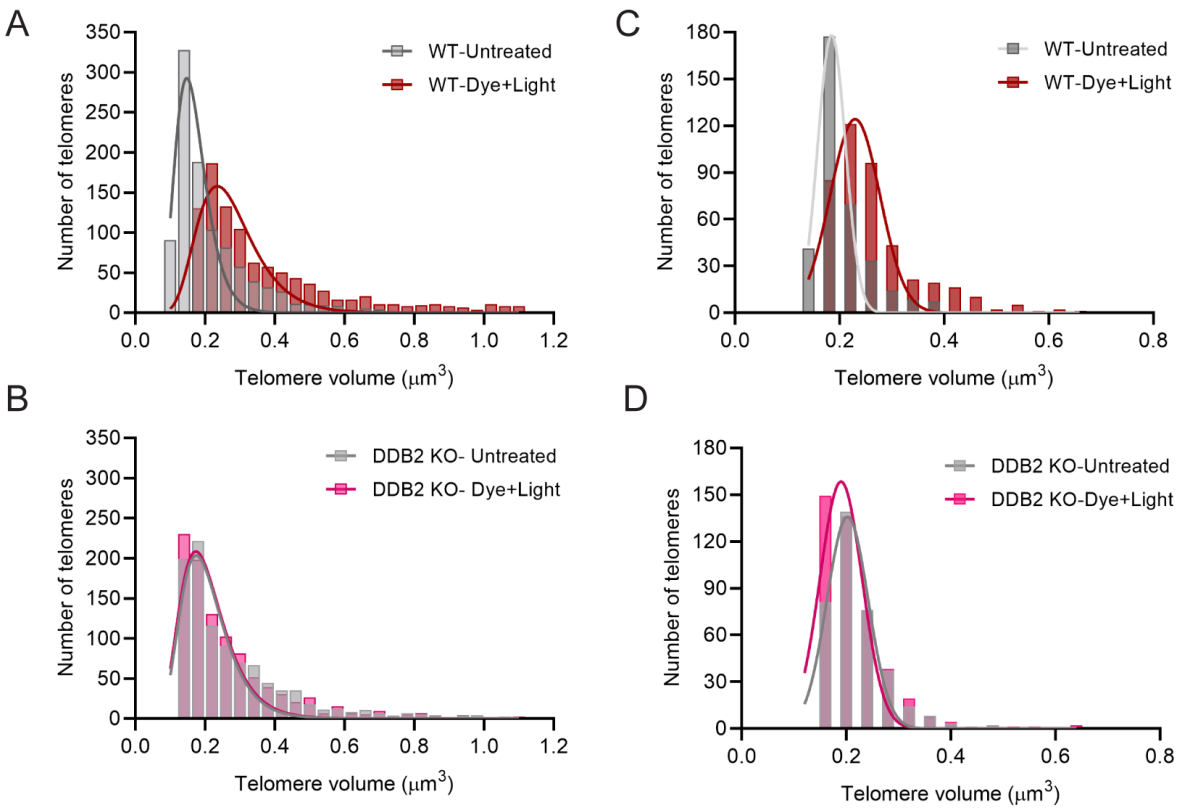

Figure 16: DDB2 mediates chromatin decompaction at sites of telomeric 8-oxoG.

A and B. Distribution of the largest 20% telomeres in untreated and dye plus light treated U2OS-FAP-TRF1 WT and DDB2 KO cells. Cells were fixed 30 minutes post treatment. C. and D. Distribution of the largest 20% telomeres in untreated and dye plus light treated RPE-FAP-TRF1 WT and DDB2 KO cells. Cells were fixed 30 minutes post treatment.

3.9 Acknowledgments

The authors wish to thank Dr. Elise Fouquerel for the U2OS-FAP-TRF1 cell line. We thank Dr. Marcel P. Bruchez for providing the MG-2I and 660 nm light source. We thank Dr. Jacob Stuart-Ornstein, Dr. Roderick O'Sullivan, and Dr. Karen Arndt for helpful discussions. We also thank our lab members, Dr. Matt Schaich, Sripriya Raja, Dr. Wei Qian, Dr. Zhou Zhong and Brittani Schnable for careful reading of the manuscript. This work was supported by NIH grants, R01ES019566, R01ES028686, and R35ES031638 (B.V.H.), R35ES030396 (P.L.O.), F32AG067710 (R.P.B.), the gravitation program CancerGenomiCs.nl (H.L., A.P. and W.V.) and TOP-CW grant (714.017.003) (A.P. and W.V.) from the Netherlands Organization for Scientific Research. Oncode Institute is partly financed by the Dutch Cancer Society.

4.0 Discussion

In this study we used two complementary tools to introduce 8-oxoG sites at telomeric or local sub-nuclear regions and provided direct evidence for the involvement of several NER proteins in 8-oxoG processing. We show that the GG-NER protein DDB2 initiates 8-oxoG processing in chromatin immediately after damage is introduced. Furthermore, we observe that recruitment of XPC to 8-oxoG is facilitated by DDB2, suggesting that both UV-DDB and XPC act as early recognition factors in the repair of 8-oxoG. Strikingly, DDB2 knockdown by siRNA showed almost a complete inhibition of OGG1 recruitment at telomeres (Figure 8F). Similarly, at locally induced 8-oxoG damage sites, a strong reduction of DDB2 by the COP9 signalosome deneddylation inhibitor, CSNi, which keeps the E3 ligase CRL^{DDB2} in a hyperactive state, led to a decrease in OGG1 recruitment (Figure 14E). XPA was also found to be recruited to sites of 8-oxoG damage at telomeres, and this recruitment is dependent upon OGG1 and transcription. We also found that in the absence of OGG1 or at high lesion density, UV-DDB was associated with CUL4A. Finally, we observed evidence for chromatin decompaction at 8-oxoG sites which was dependent upon DBB2.

4.1 Longer retention of DDB2 at unrepaired 8-oxoG lesions requires CRL^{DDB2} mediated DDB2 dissociation.

UV-DDB, as part of the CRL^{DDB2} complex, helps modify chromatin at sites of UV damage by ubiquitylating histones H2A, H3 and H4, and aids downstream NER [44]. Moreover, if UV-

DDB remains bound to the lesion, CRL^{DDB2} auto-polyubiquitylates DDB2 to allow for its dissociation and degradation. When 8-oxoG was produced at low density (1-2 per telomere) we found that recruitment and dissociation of DDB2 to 8-oxoG sites is independent of the CRL^{DDB2} complex. We speculate that at low 8-oxoG density in telomeric DNA, DDB2 binding is transient, so DDB2 can dissociate without degradation. Indeed, when we introduced a higher lesion density using the photosensitizer (Ro 19-8022) plus 405 nm laser illumination, we observed that OGG1 is not recruited effectively when CRL^{DDB2} is inhibited. Recruitment of downstream NER proteins, such as TFIIH, can facilitate the dissociation of DDB2 from UV lesions [101]. Consistent with these findings, we saw a significant increase in DDB2 and CUL4A accumulation at telomeric 8-oxoG sites in the absence of OGG1, indicating that lesion density and location dictate whether DDB2 alone or in complex with DDB1-CUL4A-RBX are necessary for efficient OGG1 recruitment. Future experiments will focus on determining whether DDB2 dissociation in OGG1 KD cells is facilitated by CRL^{DDB2} mediated DDB2 polyubiquitylation, subsequent action of VCP to extract ubiquitylated DDB2 from chromatin, and finally degradation by the 26S proteosome.

4.2 XPC and XPA participate in 8-oxoG processing through two independent sub-pathways.

Surprisingly, we observed both XPC and XPA recruitment to 8-oxoG. XPC recruitment was dependent upon DDB2. Why might XPC be recruited to sites of 8-oxoG processing, as these lesions are not expected to be processed by GG-NER? Biochemical experiments with purified XPC and OGG1 revealed that XPC can help turnover OGG1 at product inhibited abasic sites [61]. We have also shown with biochemical and single-molecule approaches that UV-DDB plays a

similar role [109]. Furthermore, it was recently shown that repair of oxidative DNA damage was slower in XP-C cells compared to normal fibroblasts [136]. Future studies will be necessary to show that these XPC and UV-DDB can work together to improve OGG1 access to damage and help turnover OGG1 during 8-oxoG processing, thereby stimulating the processing of 8-oxoG. Our present study also clearly demonstrates that XPA recruitment is mediated through transcription-coupled repair. Previous studies have shown contrasting evidence for XPA's role in 8-oxoG repair [61, 72, 74], which could have been due to differences in experimental techniques and conditions. Here, we show that XPA is recruited to telomeric 8-oxoG as part of a transcription-coupled pathway when processing of 8-oxoG by OGG1 leads to transcription-blocking intermediates. Furthermore, we also observed TCR-linked recruitment of DDB1 and CUL4A suggesting an involvement of the CRL^{CSA} complex in TCR of 8-oxoG. Future work will be necessary to determine if TC-NER recognition proteins, CSA and CSB, are recruited to actively transcribed regions at telomeric 8-oxoG to further define the interplay between GG-NER and TCR with BER.

4.3 Chromatin structure defines the critical players required for 8-oxoG processing.

Chromatin structure can drastically affect the amount of oxidative DNA damage and repair in cells [137, 138]. Specifically, it has been shown that heterochromatic regions are more susceptible to 8-oxoG damage [137], although this could be due to inefficient accumulation of BER proteins at heterochromatin compared to euchromatic regions [138]. Therefore, repair at heterochromatin may require additional factors including NER proteins. In this study, we observed a higher degree of DDB2 dependency on the recruitment of OGG1 when damage was introduced

at telomeric chromatin versus at sub-nuclear genomic regions (Figure 8F, 14E), suggesting that chromatin structure and lesion density play a key role in repair kinetics. Recent studies have established a chromatin decompaction role for DDB2 at sites of UV damage [90, 104, 107]. We demonstrate that when bound to 8-oxoG lesions, DDB2 facilitates chromatin expansion at sites of damage, as measured by increase in telomere volume. While DDB2 has been shown to lead to chromatin decompaction [107], it does not have any known chromatin remodeling properties. We therefore propose that DDB2 may mediate the change in chromatin state by recruiting other factors like chromatin remodelers. Chromatin remodelers and histone chaperones, such as RSC and FACT, have been shown to be involved in 8-oxoG repair [52]. Our DDB2 KO studies suggest that continued cellular absence of DDB2 activates compensatory pathways that facilitate less efficient recognition of 8-oxoG by OGG1. Future experiments are required to identify these additional factors. Furthermore, it is possible that DDB2 is required for 8-oxoG recognition in regions that are challenging for OGG1 to access. To that end, Thoma and colleagues have shown that UV-DDB can bind a lesion embedded in the nucleosome and even change the register of an occluded region by as much as 3 base pairs [90], suggesting a "pioneering repair factor" function for UV-DDB.

In NER, DDB2 is regulated by several post-translational modifications, including ubiquitylation, PARylation and SUMOylation [110]. For example, it has been suggested that PARP1 mediated poly-ADP-ribosylation (PARylation) of DDB2 and subsequent recruitment of SWI/SNF chromatin remodeler, ALC1, facilitates repair of UV damage [117]. PARP1 also plays an important role downstream in BER by accumulating at BER intermediates (abasic sites/ single-strand breaks) and recruiting repair factors, XRCC1 and Pol β. More recently, ALC1 has also been

shown to be required for BER [139, 140]. To that end, it would be of importance to study the crosstalk between DDB2 and PARP1 at 8-oxoG sites undergoing repair.

4.4 Working model

In summary, our data support a fundamentally new model for how 8-oxoG lesions are processed at telomeres and other genomic regions, which consists of DDB2-dependent and -independent pathways (Figure 18). We propose that DDB2, alone at telomeres and as a CRL^{DDB2} E3 ligase in other genomic regions, binds 8-oxoG damage and facilitates local chromatin decompaction, stimulating damage recognition by XPC and OGG1. In contrast, if damage occurs in actively transcribed regions, where the chromatin structure is more relaxed, OGG1 may recognize damage independent of DDB2. Binding of OGG1 or processing of 8-oxoG by OGG1 and/or APE1 can stall Pol II, blocking transcription and requiring the recruitment of TC-NER proteins, including XPA. When the lesion density is high, re-binding of DDB2 to unrepaired lesions can inhibit downstream repair, requiring CRL^{DDB2} mediated ubiquitylation and degradation of DDB2. Our study establishes a mechanistic role for NER proteins DDB2, XPC and XPA in 8-oxoG processing. It remains to be investigated whether involvement of DDB2 and XPC in 8-oxoG repair is specific to heterochromatic and more condensed genomic regions that are tightly bound by nucleosomes, and thus in the absence of these GG-NER proteins would not efficiently be recognized by OGG1. We propose that repair of 8-oxoG in heterochromatic DNA requires additional factors as these regions are inaccessible to BER. Furthermore, persistent damage at heterochromatin can alter the chromatin structure and make cells for susceptible to genomic instability [141].

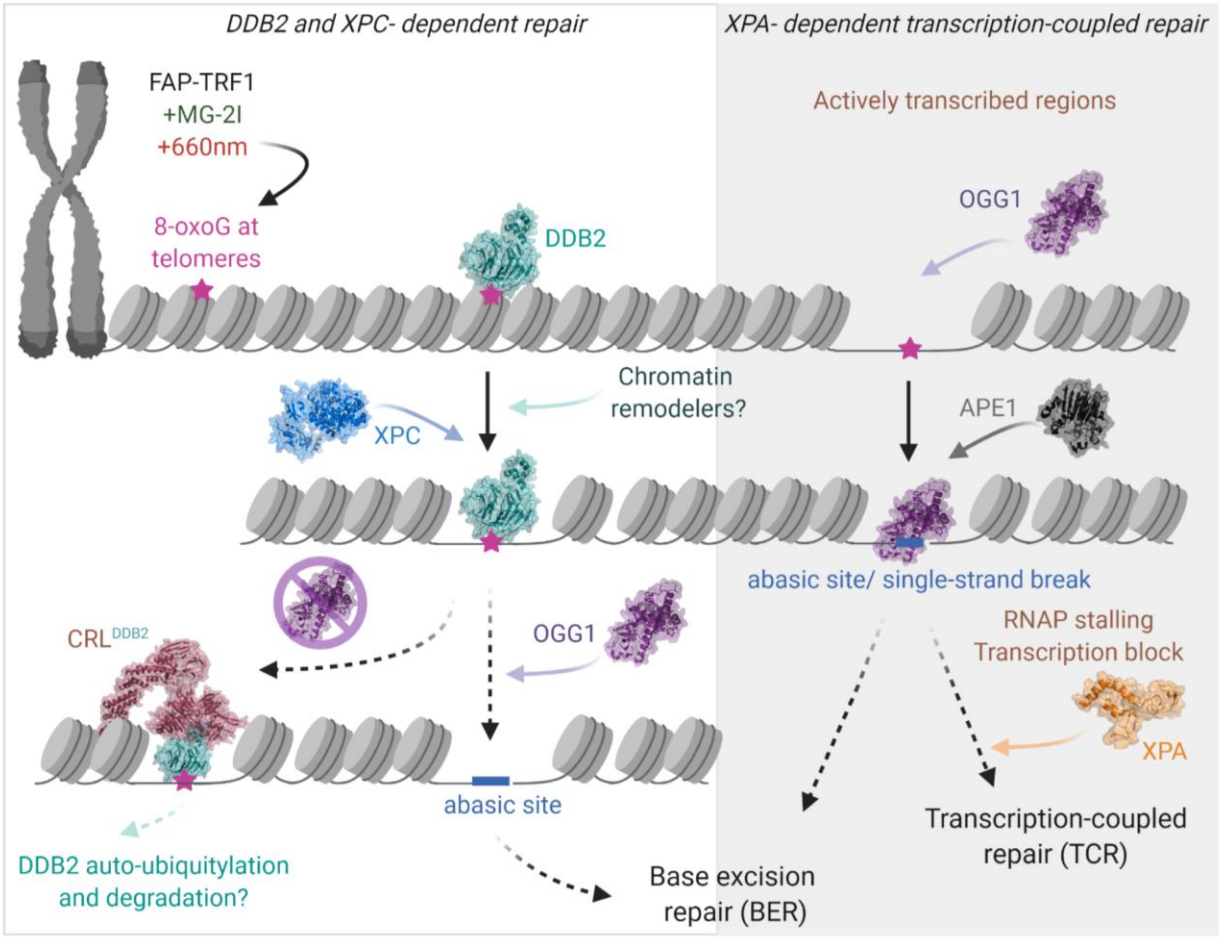

Figure 17: Unified working model: role of NER proteins in 8-oxoguanine repair.

Treatment of cells expressing FAP-TRF1 with dye (100 nM, 15 min) plus light (660 nm, 10 min) introduces 8-oxoG lesions at telomeres. In the DDB2-dependent repair pathway, DDB2 recognizes 8-oxoG lesions and facilitates chromatin relaxation through chromatin decompaction allowing the recruitment of XPC and OGG1 to the damage site. OGG1 recruitment facilitates the dissociation of DDB2. In the absence of downstream repair, DDB2 is retained longer at 8-oxoG sites requiring DDB1-CUL4A-RBX1 (CRL) mediated DDB2 dissociation. At actively transcribed strands, OGG1 can access the lesion independent of DDB2. 8-oxoG processing can lead to toxic BER intermediates that can act as a transcription block. Transcription-coupled repair (TCR) proteins, including XPA, participate in the repair of these BER intermediates.

4.5 Limitations of the study

One of the challenges in this study was the low lesion density introduced by the FAP system at telomeres (~1-2 8-oxoG/ telomere). To introduce a higher lesion density, we utilized an independent approach using a photosensitizer (Ro 19-8022) in combination with 405 nm laser pulse, which predominantly introduces 8-oxoG [72, 73]. An alternate approach using the FAP system would be to fuse the FAP protein to a more abundant DNA binding protein, for example histones. We have now established a cell line (U2OS-H2B-FAP), where the FAP is fused to histone H2B. This system can be employed for targeted generation of 8-oxoG at higher densities and can be used for live-cell imaging to study protein dynamics on oxidative DNA damage.

The second limitation is the transient overexpression of repair proteins, which could have changed the protein association and dissociation kinetics in the cell. To overcome this, future studies could be performed in cell lines selected for stable and low expression of tagged repair proteins or cell lines with endogenously tagged repair proteins.

4.5.1 Measuring 8-oxoG lesion density

Lesion density can be defined as the number of 8-oxoG lesion per million bases and can be measured using Comet assay, LC/MS or HPLC (see Appendix E, Table 1) [10].

Another method for measuring lesion density is to treat cellular DNA with 8-oxoG processing enzymes such as Formamidopyrimidine DNA Glycosylase (Fpg) or OGG1 and APE1. Briefly, cells can be treated with increasing amounts of dye and light to introduce different lesion densities. DNA extracted from these cells can be subjected to treatment with Fpg. Fpg is a bifunctional DNA glycosylase with DNA N-glycosylase and AP lyase activities. The glycosylase

activity will help remove the damaged base leaving an abasic site. The AP lyase activity cleaves the AP site, via β and δ-elimination, creating a 1 nucleotide DNA gap. Further treatment with S1 nuclease will convert the single nucleotide gaps to double stranded breaks in the DNA which can be visualized by gel electrophoresis. Damaged DNA will run faster than undamaged DNA and the distance run on the gel can be used to calculate the lesion frequency [142, 143]:

$$Weighted\ mean\ DNA\ length\ (MDL) = \Sigma\ (MWi\ x\ Ii)\ /\ \Sigma(Ii)$$

MWi: length of the DNA at each row (kb); Ii: integrated volume at each row

$$Lesion\ frequency\ =\ (MDL\ untreated\ /MDL\ treated)\ -1$$

$$\text{Fold change} = \text{Frequency in experimental sample/ Frequency in control sample}$$

5.0 Outlook

This thesis establishes a mechanistic role of three NER proteins, UV-DDB, XPC, and XPA in the processing of 8-oxoG through two distinct pathways involving global genomic and transcription-coupled mechanisms. While these data answer several questions in the field, they also provide many new future directions that would provide more insights into the synergistic role DNA repair pathways play in 8-oxoG repair.

5.1 UV-DDB mediated regulation of chromatin structure at 8-oxoG:

5.1.1 How does UV-DDB help in chromatin decompaction at 8-oxoG sites?

As discussed in section 3.8, DDB2 binding to telomeric 8-oxoG leads to local chromatin decompaction as measured by the increase in telomere volume. Previous studies have shown that DDB2 binding to UV damage is sufficient to cause histone rearrangements [104, 107]. Furthermore, absence of DDB2 abrogated the chromatin unfolding at these damage sites. Therefore, it would be interesting to understand how the histone dynamics change in the presence of 8-oxoG. This can be done by tagging histones with a fluorescent tag (Halo- or SNAP-) and using live-cell imaging to monitor changes in fluorescence after damage. Halo- and SNAP- tags are self-labelling proteins (SLP), that can be fused to a protein of interest (POI) [144]. Most often, SLPs are engineered version of enzymes that react covalently with a fluorescent substrate. Depending on the concentration of substrate added, a stable bond between the SLP and substrate

will be formed, and the POI is labelled as desired. Using cells stably expressing halo- or SNAP-tagged H3.3, localized 8-oxoG can be generated in the H2B-FAP cell line using a 660nm laser and H3.3 fluorescence can be measured. A decrease in fluorescence will suggest mobility of histones and chromatin opening. If UV-DDB is involved in regulating the chromatin dynamics at 8-oxoG sites, there would be no reduction in H3.3 fluorescence observed at the damage site in the absence of the protein.

Since UV-DDB does not have an ATPase or histone binding domain, it is more likely that it works with other factors that directly affect the chromatin dynamics, such as chromatin remodelers and histone chaperones. Previous studies show the involvement of ATP-dependent chromatin remodelers (ACRs) in NER as well as BER (Table 1) [57, 117, 145-153], although no direct evidence has been shown that ACRs are recruited to the sites of 8-oxoG lesions. Most studies so far have used RNAi knockdown of ACR complexes to examine the effect on BER [154], but interpretation of these data can be complicated as defects in ACRs can affect other global cellular processes like transcription, which, in turn, can impact BER activity. Additionally, the study of BER in cells is hampered due to the inability to induce purely 8-oxoG lesions in the cells without other DNA lesions [113]. The FAP system presents advantageous and can be used to identify chromatin remodelers recruited to 8-oxoG.

First, to explore chromatin remodelers and histone chaperones being recruited after damage, an unbiased proteomics approach that can be used is the proximity-based biotin ligase (BirA), that biotinylates nearby proteins within ~10nm [155]. The biotinylated proteins can then be pulled down by streptavidin beads and subjected to mass spectrometry and western blot analysis. Fusing BirA to DDB2 would help determine the remodelers being recruited by DDB2 to 8-oxoG.

Second, the chromatin remodelers identified by the proteomics approach could be validated by western blots and immunofluorescence after dye plus light treatment in DDB2 proficient and deficient cell lines. Finally, an effect on oxidative DNA damage repair could be determined by knocking down the chromatin remodelers and measuring the repair of 8-oxoG as well the sensitivity of cells to oxidizing agents.

5.1.2 Is UV-DDB regulated by post-translational modifications (PTMs) in the presence of 8-oxoG?

UV-DDB is modified on several residues by ubiquitylation, PARylation and SUMOylation (Figure 18).

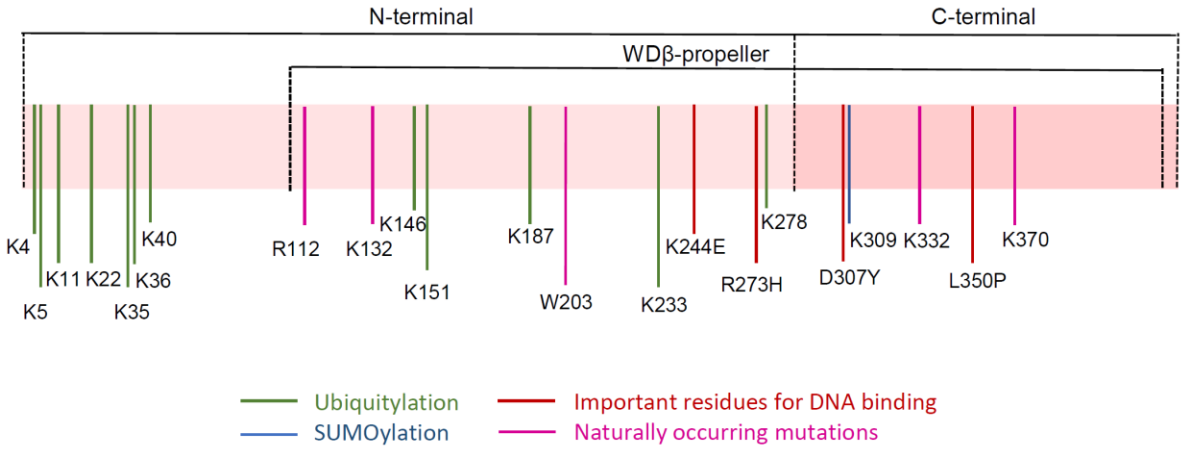

Figure 18: Schematic of DDB2 highlighting important residues.

5.1.2.1 Ubiquitylation

UV-DDB is regulated by ubiquitylation during GG-NER. UV-DDB is a ubiquitin E3 ligase, consisting of cullin4A (CUL4A) and RBX that auto-ubiquitylates itself and also ubiquitylates XPC [32]. While mono-ubiquitylated XPC is stabilized at sites of UV-induced DNA

damage, the binding of UV-DDB is destabilized, due to poly-ubiquitylation of DDB2 and subsequent degradation by the 26S proteosome. It has been reported that the N-terminal tail of DDB2 contains several lysines that are targeted for ubiquitylation by the CRL4 complex and are required for degradation of DDB2 after UV-induced damage [89]. In addition, structural studies have identified five potential ubiquitylation lysines outside the N-terminal domain (K146, 151, 187, 233, and 278).

These studies were performed in the context of UV damage and whether ubiquitylation of DDB2 is necessary after 8-oxoG damage still needs to be pursued. As discussed in section 3.6 and 3.7, the CRL^{DDB2} complex mediates DDB2 dissociation from 8-oxoG when the lesion density is high or in the absence of downstream repair. However, it is unclear how DDB2 is regulated by ubiquitylation after oxidative damage.

Is DDB2 ubiquitylated and degraded after oxidative damage? The H2B-FAP cell line stably expressing a tagged DDB2 can be used for these experiments. First, after high density of 8-oxoG is introduced, DDB2 levels can be measured by western blots to visualize protein degradation. Furthermore, no degradation should be observed in the presence of MG132, a proteosome inhibitor. If DDB2 degradation is observed, DDB2 pulldowns can be performed using the tag and probed for ubiquitylation using the FK2 antibody (to detect mono- and poly-ubiquitylated conjugates). Ubiquitylated DDB2 is actively extracted from the chromatin by the Valosin-containing Protein (VCP)/p97 Segregase [101]. Therefore, DDB2 degradation after 8-oxoG damage could also be measured in the absence of VCP.

Another experiment would be to mutate the lysines (to Arg) on DDB2 that were shown to be essential ubiquitylation sites after UV damage and measure DDB2 ubiquitylation and degradation by western blots. To ascertain if timely ubiquitylation and dissociation of DDB2 is

required for unperturbed 8-oxoG repair, accumulation of downstream proteins should be measured in the absence of VCP, or when the important lysine residues are mutated.

Does CRL^{DDB2} mediated ubiquitylation play a role in chromatin decompaction at the damage site? UV-DDB along with the CUL4A E3 ligase can specifically bind to mono-nucleosomes containing UV damage and mono-ubiquitylate histone H2A and H3 [97]. Mono-ubiquitylated H2A at Lys119 and Lys120 helps facilitate the destabilization of nucleosome containing UV-induced photoproducts, as mutating these residues to Arg prevents the dissociation of poly-ubiquitylated DDB2 from the UV damage containing nucleosome. Furthermore, it has been reported that DDB2 might have CRL-independent roles in chromatin unfolding at UV damage sites [107].

We have shown that DDB2 can recognize telomeric 8-oxoG even in the absence of DDB1 or CUL4A (see section 3.6). While we also observed a DDB2-dependent telomeric chromatin decompaction after 8-oxoG damage, whether CRL mediated DDB2 ubiquitylation was involved needs to be investigated.

First, ubiquitylation of histone H2A needs to be measured in H2B-FAP cells after 8-oxoG damage, in CRL proficient and deficient cells. This would indicate whether CRL^{DDB2} is directly involved in histone modification. Next, it would be important to determine whether the chromatin decompaction observed at 8-oxoG sites is a direct result of DDB2 binding or due to ubiquitylation and subsequent destabilization of histones by the CRL^{DDB2}. To test this, cells expressing SNAP-tagged H3.3 can be used, and the histone fluorescence can be measured around the damage site in a) the absence of DDB1 or CUL4A, b) by mutating H2A Lys 119 and 120 to Arg. If a change in histone fluorescence is still observed, it suggests that DDB2 unfolds chromatin in a CRL-independent manner.

If H2A ubiquitylation is still observed but in a CRL^{DDB2} independent manner, it is possible that other E3 ligases might be involved, including the polycomb repressive complexes 1 (PRC1) [156]. Therefore, an unbiased approach (perhaps an H2A-Bir, refer to section 5.1.1) would be required to identify the E3 ligases involved in histone ubiquitylation after oxidative DNA damage.

Alternatively, knocking down DDB1 and CUL4A can also affect transcription-coupled repair of 8-oxoG as CRL^{CSA} mediated ubiquitylation of CSB and Pol II is crucial for TCR [89, 132, 133]. Therefore, to assess the E3 ligase independent role of DDB2 in chromatin unfolding at the damage site without affecting TCR, DDB2 mutants could be expressed that can recognize and bind to the damage but are unable to interact with DDB1 (D307Y, L350P) [104].

5.1.2.2 PARylation

Poly(ADP-ribosyl)ation (PARylation) is posttranslational modification of proteins by linear or branched chains of ADP-ribose units, originating from NAD+. The main enzyme for PAR generation in cells during DNA damage is poly(ADP-ribose) polymerase 1 (PARP1).

DDB2 was shown to be poly-ADP-ribosylated (PARylated) by PARP1 in response to UV damage, subsequently leading to the recruitment of SWI/SNF chromatin remodeler, ALC1 to the damaged site [117]. Furthermore, using FRAP, it was observed that DDB2 had a prolonged retention time on the DNA in the absence of poly(ADP-ribose) glycohydrolase (PARG), suggesting that PARylation might stabilize the protein at the lesion site. Moreover, inhibition of PARP activity resulted in suppressed PARylation but increased ubiquitylation of DDB2, indicating that PARylation may prevent DDB2 auto-ubiquitylation and subsequent degradation [157].

In base excision repair (BER), PARP1 is known to play a role downstream by binding single-strand breaks (SSBs) formed by the action of APE1 and recruiting XRCC1 and Pol β for the completion of repair. While PARP1's role as an SSB repair protein is well established, little is

known about its chromatin remodeling function in BER. A recent study reported that PARP1 recruits ALC1 and mediates nucleosome remodeling at abasic sites, upstream of APE1 but downstream of the glycosylase [158].

Based on these studies, several questions arise that can be addressed using the H2B-FAP system. First, to assess if DDB2 is PARylated after 8-oxoG damage, PAR enriched proteins can be pulled down after dye plus light treatment by affinity capture with the PAR-binding macrodomain, AF1521, and validated by western blots [159]. Second, if PARP1 is recruited to PARylate DDB2, then recruitment of PARP1 to 8-oxoG and its colocalization with DDB2 could be visualized using immunofluorescence. Furthermore, as mentioned above, if PARP1-mediated PARylation of DDB2 stabilizes the protein and prevents its degradation, loss of PARP1 should lead to hyper-ubiquitylation of DDB2 by CRL^{DDB2} and degradation by the 26S proteosome. Finally, to address whether DDB2 mediated chromatin decompaction at 8-oxoG is PARP1-dependent, loss of tagged-histone fluorescence can be examined at the damage site in the absence of PARP1 or after its chemical inhibition. The crosstalk between ubiquitylation and PARylation needs to be further investigated to understand efficient base damage recognition in chromatin.

5.1.2.3 SUMOylation

Small ubiquitin-related modifiers (SUMOs) can regulate a variety of cellular processes including transcriptional regulation, signal transduction and maintenance of genome integrity by causing rapid changes in protein-protein interactions. SUMO is covalently attached to proteins through a cascade similar to that of ubiquitylation. DDB2 was shown to be SUMOylated post-UV damage and PIASy (protein inhibitor of activated STST proteins) was the major SUMO E3 ligase involved [160]. Three DDB2 lysine residues (Lys5, Lys77 and Lys309) were identified to be SUMOylated [161]. Lys309Arg completely abolished the DDB2 modification upon UV damage.

Moreover, when expressed in cells, this mutant was deficient in the removal of CPDs as well as in the recruitment of XPC as compared to the WT, indicating that the SUMOylation at Lys309 is functionally significant.

Although DDB2 SUMOylation was shown to be important for repair, how this modification regulates DDB2 has not been explored. Furthermore, whether DDB2 is SUMOylated after oxidative base damage is still unknown. Overall, studying all three PTMs and their crosstalk after 8-oxoG damage will provide insights on the mechanism of damage recognition by UV-DDB.

5.2 Coordination of DDB2, XPC and OGG1 at 8-oxoG

5.2.1 Is XPC modulated by CRL^{DDB2}?

As discussed in section 3.4, XPC is recruited to 8-oxoG in a DDB2-dependent manner. We hypothesized that DDB2 works with XPC to facilitate OGG1 accumulation at 8-oxoG. CRL^{DDB2} is known to ubiquitylate XPC after UV damage, helping with its stabilization on DNA [32]. Furthermore, subsequent SUMOylation and RNF111-mediated ubiquitylation leads to dissociation of XPC from UV damage [161]. To determine if XPC is ubiquitylated after 8-oxoG damage, pulldowns for XPC can be performed and probed for ubiquitin in the presence or absence of CRL^{DDB2}. Furthermore, it can be tested if the absence of CRL^{DDB2} affects the recruitment or dissociation of XPC. Finally, to test whether XPC ubiquitylation affects downstream repair, the ubiquitylation site (K48, K63) on XPC can be mutated to confirm whether recruitment of downstream repair proteins is delayed.

5.2.2 How is XPC regulated at 8-oxoG?

Surprisingly, loss of XPC did not affect OGG1 accumulation at 8-oxoG (section 3.5). In contrary, there was a small but significant decrease in XPC recruitment in the absence of OGG1. Two hypotheses might be tested to understand these data:

First, XPC plays a role downstream of OGG1. Consistent with this idea, it was previously shown that XPC helps OGG1 turnover from abasic site containing duplex DNA [61]. A way to test this would be to use an OGG1 mutant (OGG1-K249Q) that is able to recognize and bind to 8-oxoG but lacks both N-glycosylase and AP-lyase activities [162]. If XPC recruitment depends on OGG1 processing of the 8-oxoG lesion, then XPC may not accumulate when the OGG1-K249Q mutant is expressed.

Second, it is possible that XPC is not stabilized at 8-oxoG in the absence of OGG1. In GG-NER, stabilization of XPC at UV damage requires timely dissociation of DDB2 and recruitment of the downstream GG-NER factor TFIIH [101]. XPC binds the non-damaged strand opposite the lesion and has a higher preference for helix-distorting regions. It is possible both XPC and OGG1 bind at the site of 8-oxoG, but OGG1 binding to the damaged site helps stabilize XPC. One way would be to look at colocalization between OGG1 and XPC by Immunofluorescence or proximity ligation assay. More importantly, if the second hypothesis is true and OGG1 helps stabilize XPC at 8-oxoG, then XPC should still be recruited in OGG1 deficient cells expressing the OGG1-K249Q mutant.

To assess the interaction between XPC and OGG1 at a single-molecule level, the Lumicks optical trap microscope (C-Trap®) could be employed to visualize tagged XPC and OGG1 on duplex DNA containing 8-oxoG attached between two optically trapped beads.

5.2.3 Does XPC recognize 8-oxoG or abasic sites?

Cellular experiments regarding XPC's involvement in 8-oxoG repair have been contradictory and complicated. Therefore, biochemical and single-molecule approaches can be used to understand substrate recognition.

Electrophoretic mobility shift assays (EMSAs) could be performed with purified XPC on 8-oxoG and downstream BER substrates (abasic sites, single stranded nicks) to determine which substrate is recognized better by XPC. As mentioned earlier, XPC prefers helix-distorting regions, therefore, one would predict that it has a higher affinity to abasic sites. Additionally, XPC's ability to turnover OGG1 and/or APE1 must be systematically measured using excision assays. A duplex DNA containing 8-oxoG or an abasic site analog THF (tetrahydrofuran) could be incubated with limiting amounts of OGG1/APE1. The excision activity of these substrates can then be measured in the presence or absence of XPC. We have previously shown that UV-DDB can stimulate OGG1 and APE1 by 3- and 8-fold respectively [109].

To directly visualize whether XPC and OGG1 can bind DNA together, atomic force microscopy (AFM) could be used. AFM is a powerful tool to study protein-DNA interactions at a single-molecule level [111, 163]. A probe tip at the end of an oscillating cantilever scans the sample and allows for three-dimensional imaging. Some examples of measurements that can be obtained are: 1) protein specificity and affinity to DNA, 2) DNA bending and protein-induced DNA bending, and 3) stoichiometry of proteins binding to DNA. 8-oxoG or THF could be introduced on duplex DNA and imaged in the presence of purified XPC-RAD23B. 1) Protein specificity will help us determine if XPC has more affinity to 8-oxoG or THF. 2) Bending introduced by 8-oxoG and THF can be measured to determine XPC's preference for bent DNA. 3) Both purified OGG1 and XPC could be incubated with the DNA substrates to determine the

volumes of bound proteins. Volumes directly correlate with stoichiometry and will help determine if XPC and OGG1 bind the damage together or separately.

5.3 Transcription-coupled repair (TCR) of 8-oxoG

5.3.1 How is TCR of 8-oxoG initiated?

5.3.1.1 Does RNA polymerase stall at telomeric 8-oxoG?

In TCR, elimination of damage is initiated by a stalled polymerase at the lesion [38]. Transcription-blocking capacity of 8-oxoG has been debated for over a decade, with different studies suggesting no blocking to weak blocking [123, 164, 165]. As discussed in section 3.4, NER protein XPA was observed to be recruited to 8-oxoG in a transcription-coupled manner. Pretreatment of cells with transcription inhibitors dramatically reduced XPA accumulation. Furthermore, XPA recruitment is diminished in the absence of OGG1, suggesting that OGG1-mediated processing of 8-oxoG is required for TCR. We hypothesize that abasic sites and single stranded nicks formed by the action of OGG1 act as a transcription block allowing for TCR to be initiated.

It is known that stalling of Pol II at a DNA lesions leads to the ubiquitylation of its largest subunit RPB1 at K1268 [166, 167], mediated by the $CRL4^{CSA}$ E3 ligase complex. In agreement with this, we do observe DDB2-independent recruitment of DDB1 and CUL4A to telomeric 8-oxoG which was abrogated in the presence of transcription inhibitors. One potential future experiment would be to measure the ubiquitylation and degradation of RPB1 after 8-oxoG damage. Furthermore, mutating K1268 will help determine if the ubiquitylation is functionally significant.

5.3.1.2 Why is TCR initiated when 8-oxoG is introduced at the non-transcribed strand?

Interestingly, the FAP-TRF1 system introduces 8-oxoG at the G-rich sequence of the telomere, which is not transcribed, therefore why TCR is initiated is an unresolved question. One explanation could be that BER intermediates such as single-strand nicks in the non-transcribed strand can favor formation of R-loops, which involved the transcribed strand and efficiently block transcription [127]. To determine whether generation of 8-oxoG in cells, especially at telomeres introduces R-loops, two antibodies can be used after dye plus light treatment: a) S9.6 monoclonal antibody, that specifically recognizes RNA: DNA hybrids; or b) an RNase H mutant (D210N), which recognizes DNA/RNA hybrids but cannot digest its RNA strand. If a significant accumulation of R-loops is observed after 8-oxoG damage, cells could be treated with RNase H (an enzyme that degrades RNA within RNA: DNA hybrids) to reduce R-loops, which should lead to a significant abrogation in recruitment of TCR proteins.

As discussed in section 3.5, U2OS cells maintain their telomeres through the ALT pathway. ALT cells contain a 'TCAGGG' variant repeat throughout the telomeres [130], therefore guanines are present in the complementary C-rich transcribed strand which could be oxidized after the dye plus light treatment. To examine whether TCR is initiated due to the oxidation of the guanine on the C-rich strand, recruitment of TCR proteins can be visualized in cell lines that contain little to no 'TCAGGG' repeats, such as HeLa [168].

5.3.2 Is TCR of 8-oxoG processed specifically by NER proteins?

We also measured XPA recruitment to telomeric 8-oxoG in CSB deficient cells and found that XPA accumulation was CSB dependent suggesting that TC-NER recognition protein CSB is involved in initiation of transcription-coupled 8-oxoG repair (section 3.5). Future experiments

should focus on directly visualizing recognition proteins CSB and CSA at telomeric 8-oxoG. Previous studies have reported that CSB recruitment to oxidative DNA damage is transcription dependent [72, 73].

To ascertain whether repair of 8-oxoG proceeds through the traditional TC-NER pathway, accumulation of downstream TC-NER proteins (XPB, XPD, XPG, XPF) need to be measured and their dependency on OGG1 needs to be determined.

5.4 Involvement of UV-DDB in 8-oxoG repair in the context of chromatin structure

5.4.1 Can UV-DDB stimulate OGG1 on 8-oxoG embedded in reconstituted nucleosomes?

Chromatin acts as a natural barrier during several cellular processes including access to DNA lesion during DNA damage repair. Using reconstituted 601 nucleosomes and positioning 8-oxoG at three different rotational orientations (In, Mid, Out) at the dyad axis, it was shown that OGG1 activity was completely inhibited on 8-oxoG embedded in a nucleosome [53]. Glycosylase activity was measured as product formation when OGG1 was incubated with the lesion containing nucleosome. Interestingly, when the lesion was moved off the dyad axis, OGG1 showed reasonable activity suggesting that lesion positioning relative to the nucleosome can affect accessibility [54]. Surprisingly, lesion accessibility did not correlate with solution accessibility: OGG1 had most activity on the ‘Mid’ positioned 8-oxoG but lower activity on ‘In’ and ‘Out’ facing lesion. It is possible that the histone tails limit the access to the outward facing lesion. Consistent with this idea, it was observed that OGG1 activity increased when histone tails were acetylated, modeling a more relaxed chromatin structure [54].

More recently, a cryo-EM study demonstrated that UV-DDB can bind occluded lesions on reconstituted nucleosome containing the abasic site analog, tetrahydrofuran (THF) [90]. UV-DDB was able to bind the outward facing lesion without disturbing the nucleosome architecture. In the case of inward facing THF, UV-DDB shifted the translational register of the 5S nucleosome and bound the lesion in an exposed position. Interestingly, this activity of UV-DDB was independent of ATP-dependent chromatin remodelers *in vitro.*

We have previously shown that UV-DDB can stimulate OGG1 activity on 8-oxoG containing duplex DNA by 3-fold [109]. It is possible that UV-DDB assists OGG1 in damage recognition by shifting the nucleosome register and exposing inward facing lesions. First, it needs to be determined if UV-DDB can recognize 8-oxoG embedded in a nucleosome at different positions. Once verified, purified OGG1 can be tested with 8-oxoG containing nucleosome substrates in the presence or absence of UV-DDB to examine whether UV-DDB can stimulate OGG1 activity on the nucleosome.

5.4.2 Is DDB2 required for 8-oxoG removal in more heterochromatin?

Genome-wide sequencing revealed that there is a higher accumulation of 8-oxoG in lamina associated domains (LADs) [137]. This could possibly be because these domains are at the periphery of the nucleus and therefore, more susceptible to oxidative stress. Additionally, LADs are heterochromatic and it has been reported that BER is less active in heterochromatic regions as compared to euchromatin [138].

Keeping in mind UV-DDB's role in chromatin decompaction and its ability to shift the nucleosome register, one hypothesis would be that UV-DDB facilitates 8-oxoG repair in specific

genomic regions (transcriptionally silent heterochromatic regions), when the lesion is difficult to access by OGG1. This hypothesis can be tested using imaging and genomic approaches:

5.4.2.1 Cellular model to image recruitment of DDB2 to heterochromatic regions:

In order to track repair in heterochromatin by imaging, a cellular model is required where heterochromatic regions are easily distinguishable. For this purpose, a cell line like the NIH/3T3 mouse embryonic fibroblast could be used where the pericentric heterochromatin domains form clusters that can be easily visualized by any DNA stain. Using this cell line, it was recently demonstrated that DDB2 regulates heterochromatin compaction after UV damage by stimulating displacement of the linker histone H1 [169]. By stably expressing H2B-FAP in NIH/3T3, recruitment of repair proteins including DDB2 and OGG1 can be monitored at 8-oxoG specifically at heterochromatin or euchromatin. Using this system, following questions can be addressed by live-cell imaging or immunofluorescence:

a) Does DDB2 regulate chromatin decompaction specifically at heterochromatin? Histone rearrangements can be examined at the damaged site in heterochromatin and euchromatin in DDB2 proficient and deficient cells.

b) Is repair in heterochromatin initiated by global-genome repair proteins, DDB2? The time taken for DDB2 to accumulate at 8-oxoG at heterochromatin versus euchromatin could be analyzed. Furthermore, whether OGG1 recruitment to heterochromatic regions is dependent on DDB2 can be determined.

c) Does OGG1 preferentially accumulate at euchromatic 8-oxoG? This would suggest that OGG1 activity is inhibited in condensed chromatin.

d) Do TC-NER proteins preferentially accumulate at 8-oxoG in euchromatin? Euchromatin includes actively transcribed regions. Accumulation of TC-NER proteins specifically at

euchromatin would suggest that TC-NER takes over 8-oxoG repair when 8-oxoG or downstream BER intermediates stall transcription.

5.4.2.2 CUT&RUN approach to determine preferential binding of DDB2 after 8-oxoG damage:

CUT&RUN (cleavage under targets and release using nuclease)-sequencing is a method used to analyze protein interactions with DNA by fusing a DNA-binding protein to a micrococcal nuclease (MNase). The MNase then cleaves the DNA around the DNA-binding protein and the bound DNA can then be sequenced to determine the binding site.

In cells expressing H2B-FAP and a tagged-DDB2 or OGG1, CUT&RUN can be employed using an antibody to determine the global distribution of 8-oxoG. As a positive control, OGG1-K249Q could be used that would bind but not remove 8-oxoG. Furthermore, enrichment at specific genomic regions can be assessed to evaluate whether DDB2 preferentially binds to more condensed regions such as CpG islands or satellite DNA. Additionally, the bound sequence can be analyzed for mutations such as G:C to T:A transversions in DDB2 and/or OGG1 deficient cells, validating their role in 8-oxoG repair. Chromatin immunoprecipitation and slot blots for CPDs was used to show that loss of DDB2 impeded repair of CPDs in H3K9me3-enriched (heterochromatin) compared to H3K9ac-enriched chromatin (euchromatin) [170]. Similarly, it would be instrumental to determine if there are more G:C to T:A transversions in H3K9me3-enriched regions when DDB2 is knocked down.

5.5 Concluding remarks

In this dissertation, I systematically elucidated the role of three NER proteins, UV-DDB, XPC and XPA in processing 8-oxoguanine base damage. I presented a model and proposed that repair of 8-oxoG involves two sub-pathways: a global genome repair that is initiated by GG-NER protein DDB2 and XPC, and a DDB2-independent pathway that can be initiated by OGG1. If 8-oxoG is introduced at actively transcribed regions and BER intermediates act as a transcription block, then repair of 8-oxoG is processed by TC-NER proteins including CSB and XPA.

Appendix A Abbreviations

5caC – 5-carboxylcytosine

5fC – 5-fluorocytosine

5hmC – 5-Hydroxymethylcytosine

5mC – 5-Methylcytosine

6-4 PP – 6–4 pyrimidine–pyrimidinone

8-oxoG – 8-oxoguanine

AAG – Alkyladenine DNA glycosylase

ACR – ATP-dependent chromatin remodelers

ALC1 – Amplified in liver cancer 1

ALT – Alternative lengthening of telomeres

AP – Apurinic/apyrimidinic site

APE1 – Apurinic/apyrimidinic Endonuclease 1

ATM – Ataxia-telangiectasia mutated

ATR – Ataxia telangiectasia and Rad3 related protein

BER – Base excision repair

CPD – Cyclobutane pyrimidine dimer

CS – Cockayne syndrome

CSA – Cockayne syndrome A protein

CSB – Cockayne syndrome B protein

CTD – C-terminal domain

CUL4A – Cullin 4A

CUT&RUN – Cleavage under targets and release using nuclease

DNA-PK – DNA-dependent protein kinase

DWD – DDB1-binding WD40 protein

EM – Electron microscopy

EMSA – Electrophoretic mobility shift assay

ERCC1 – ERCC excision repair 1, endonuclease non-catalytic subunit

FACT – Facilitates chromatin transcription

FAP – Fluorogen activating protein

Fpg – Formamidopyrimidine DNA Glycosylase

GG-NER – Global genome nucleotide excision repair

Gh – 5-guanidinohydantoin

HCR – Host cell reactivation

HMGN1 – High mobility group nucleosome binding domain 1

HR – Homologous recombination

ICL – Interstrand DNA crosslink

INO80 – Inositol regulatory gene 80

IR – Ionizing radiation

ISWI – Imitation SWItch/SNF

MG-2I – Di-iodinated malachite green

MMR – Mismatch repair

NEIL – Endonuclease VIII-like glycosylase

NER – Nucleotide excision repair

NHEJ – Non-homologous end joining

NTHL1 – Endonuclease III-like 1

OGG1 – 8-oxo-G glycosylase

P53 – Tumor protein P53

PAGE – Polyacrylamide gel electrophoresis

PAH – Polycyclic aromatic hydrocarbons

PARP1 – Poly [ADP-ribose] polymerase 1

PLA – Proximity ligation assay

PNKP – Polynucleotide kinase phosphate

POI – Protein of interest

Pol II – RNA polymerase II

Pol β – DNA polymerase β

PRC1 – Polycomb repressive complexes 1

PUA – 3´-phospho-α, β-unsaturated aldehyde

RAD23B – Rad23 homolog B

RBX1 – Ring-box protein 1

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

RPA – Replication protein A

RSC – Remodels structure of chromatin

SLP – Self labeling protein

SMUG1 – Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase 1

Sp – Spiroiminodihydantoin

SUMO – Small ubiquitin-related modifiers

TC-NER – Transcription-coupled nucleotide excision repair

TCR – Transcription-coupled repair

TDG – Thymine DNA glycosylase

TERRA – Telomere-repeat-containing RNA

TET – Ten-eleven translocation

TFIIH – Transcription factor II

TG – Thymine glycol

THF – Tetrahydrofuran

TRF1 – TTAGGG repeat binding factor 1

UDG – Uracil DNA glycosylase

UV – Ultraviolet

UV-DDB – UV-damaged DNA binding protein

UVSSA – UV-stimulated scaffold protein A

VCP – Valosin-containing protein

XAB2 – XPA binding protein 2

XP – Xeroderma pigmentosum

XPA – Xeroderma pigmentosum group A

XPB – Xeroderma pigmentosum group B

XPC – Xeroderma pigmentosum group C

XPD – Xeroderma pigmentosum group D

XPF – Xeroderma pigmentosum group F

XPG – Xeroderma pigmentosum group G

XRCC1 – X-ray cross complementing protein 1

Appendix B Table of chromatin remodelers

Table 1: ATP-dependent chromatin remodelers and histone modifiers shown to be involved in NER and BER.

Family	Complex	ATPase	Role in NER	Role in BER	Association with UV-DDB
			Chromatin remodelers		
SWI/SNF	RSC	Sth1	x	Stimulates BER *in vitro* [15, 171] and in yeast [60]	x
	BAF	BRG1/BRM	Enhances NER [147]	x	Association with DDB2 [147]
INO80	INO80	INO80	Promotes NER [148, 149]	x	Association with DDB1 [149]
CHD	CHD1		Implicated in UV-induced damage [150]	x	
Uncategorized	CHDL1/ALC1		Involved in repair of UV-induced damage [117]	Recruitment by PARP1 [139, 140]	Recruitment mediated by PARP1 and DDB2 [117]
			Histone modifiers		
CBP/p300	Lysine acetyltransferase		UV-induced DNA damage response [151, 152]	x	Association with DDB2 [152]
ASHL1	Histone methyltransferase		Promotes CPD excision [153]	x	DDB2 recruits ASHL1 [153]

Appendix C XP-E causing mutations

Table 2: Characterization of published XP-E causing mutations

Patient / Cell line	Allele 1 Amino Acid	Allele 2 Amino Acid♦	Cancer	UDS	HCR	Biochemistry	Cellular Imaging
XP1GO	Thr305Asn		BCC, SCC, M [172]	NR	Reduced[172]	NR	NR
XP37BE[&]	Arg273His		BCC, SCC [172]	NR	Reduced[172]	*****DDB1 and CUL4A not detected by co-IP [173, 174]	No binding to local UV damage, *****Part of the DDB-CUL4A-RBX complex [120]
XP66BE[&]	Arg273His		M [172]	NR	Reduced[172]	*****DDB1 and CUL4A not detected by co-IP [173, 174]	No binding to local UV damage, *****Part of the DDB-CUL4A-RBX complex [120]
XP408BE/ GM01389/ GM01646	Leu350Pro	Asn349del	BCC, SCC, M [172]	50% [91, 175]	NR	EMSA, no binding activity **(E)** [91, 175]; DDB1and CUL4A not detected by co-IP [33, 91]; no mono-Ub-H2A on chromatin after UVC [33]	No binding to local UV damage, Fails to recruit DDB1 and Cul4A [104]
XP2RO/ GM02415[$]	Arg273His		BCC [172, 176]	40-60% [91, 175, 177]	NR	EMSA, no binding activity (E) [178] *DDB1 and CUL4A not detected by co-IP [173, 174]	No binding to local UV damage, *Part of the DDB-CUL4A-ROC complex [120]
XP3RO/ GM02450[$]	Arg273His		BCC [172, 176]	40-60% [91, 177]	NR	*DDB1 and CUL4A not detected by co-IP [173, 174]	No binding to local UV damage, *Part of the DDB-CUL4A-ROC complex [120];

Table 2 continued

Patient / Cell line	Allele 1 Amino Acid	Allele 2 Amino Acid♦	Cancer	UDS	HCR	Biochemistry	Cellular Imaging
XP82TO	Lys244Glu		None (as of 2011) [172, 176]	44% [91, 177]	NR	EMSA, no binding activity (E) [175, 178, 179] ; Partial binding activity detectable (P) [173]; no histone ubiquitination in nucleosome [173]	No binding to local UV damage [104, 173]; Slides on DNA in the absence of Mg^{2+} [85]
XP23PV	Leu235_Lys341del		BCC [91, 172]	65% [91, 175]	NR	EMSA, no binding activity **(E)** [91, 175]	No UV-induced chromatin decondensation [104]
XP25PV	Asp307Tyr No change		BCC, SCC [91, 172]	50% [91, 175]	NR	EMSA, no binding activity **(E)** [91, 175]; DDB1 not detected by co-IP [91]	No binding to local UV damage, No recruitment of DDB1 and CUL4A [104]
XP27PV	Lys244X Trp236Valfs10 Leu235_ ys341del		BCC, SCC, M [91, 172]	48% [91]	NR	EMSA, no binding activity **(E)** [91, 175];	NR
Ops1	Arg313X		BCC, SCC, M [172, 176, 179]	99-138% [179]	NR	EMSA, no binding activity **(E)** [179] DDB1 and CUL4A not detected by co-IP [173]	NR
XP115BR	Met383fs		None (as of 2016) [180]	~50% [180]	NR	NR	NR
XP105BR	Pro357Leu	Arg239Ile	BCC, SCC, M [180]	~50% [180]	NR	NR	NR
XP98BR	Trp54X		BCC, SCC, M [180]	~50% [180]	NR	NR	NR
XP100BR	Splice		BCC [180]	~50% [180]	NR	NR	NR

Abbreviations: BCC, Basal cell carcinoma; SCC, Squamous cell carcinoma; M, Melanoma, UDS, Unscheduled DNA synthesis assay; HCR, Host cell reactivation; Biochemistry includes electrophoretic mobility shift assays, western blotting and Co-IP; E: Cell extracts; P: Purified protein; Cellular Imaging examines both recruitment to local damage and FRAP experiments. NR: not reported

&: Siblings; $: Second cousins; ♦empty boxes for no allele 2 amino acid indicate homozygous mutation; * contrasting results from biochemistry and cellular studies. From Ref. [110], with permission.

Appendix D Supplemental figures

Appendix D.1 Related to figure 10

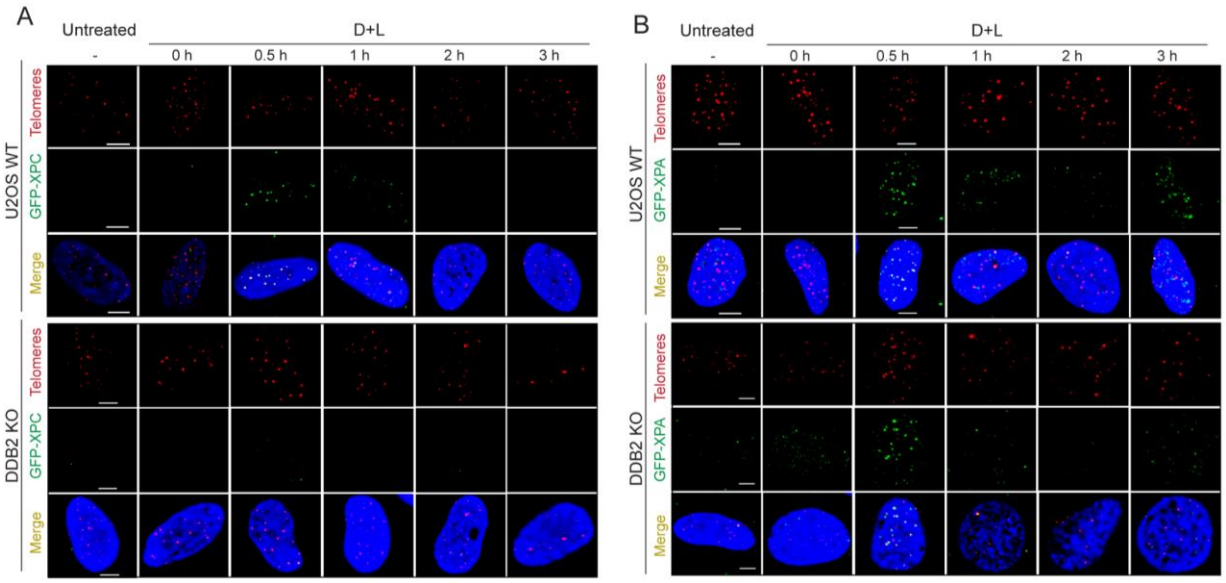

Figure 19: DDB2 recruits XPC to telomeric 8-oxoG, while XPA recruitment is transcription-coupled and independent of DDB2.

A and B. Representative images showing recruitment of GFP-XPC (A) or GFP-XPA (B) to 8-oxoG sites at telomeres after dye (100 nM, 15 min) and light (600 nm, 10 min) treatment in U2OS WT and DDB2 KO cells, over 3 hours. Scale: 5µm.

Appendix D.2 Related to figure 16

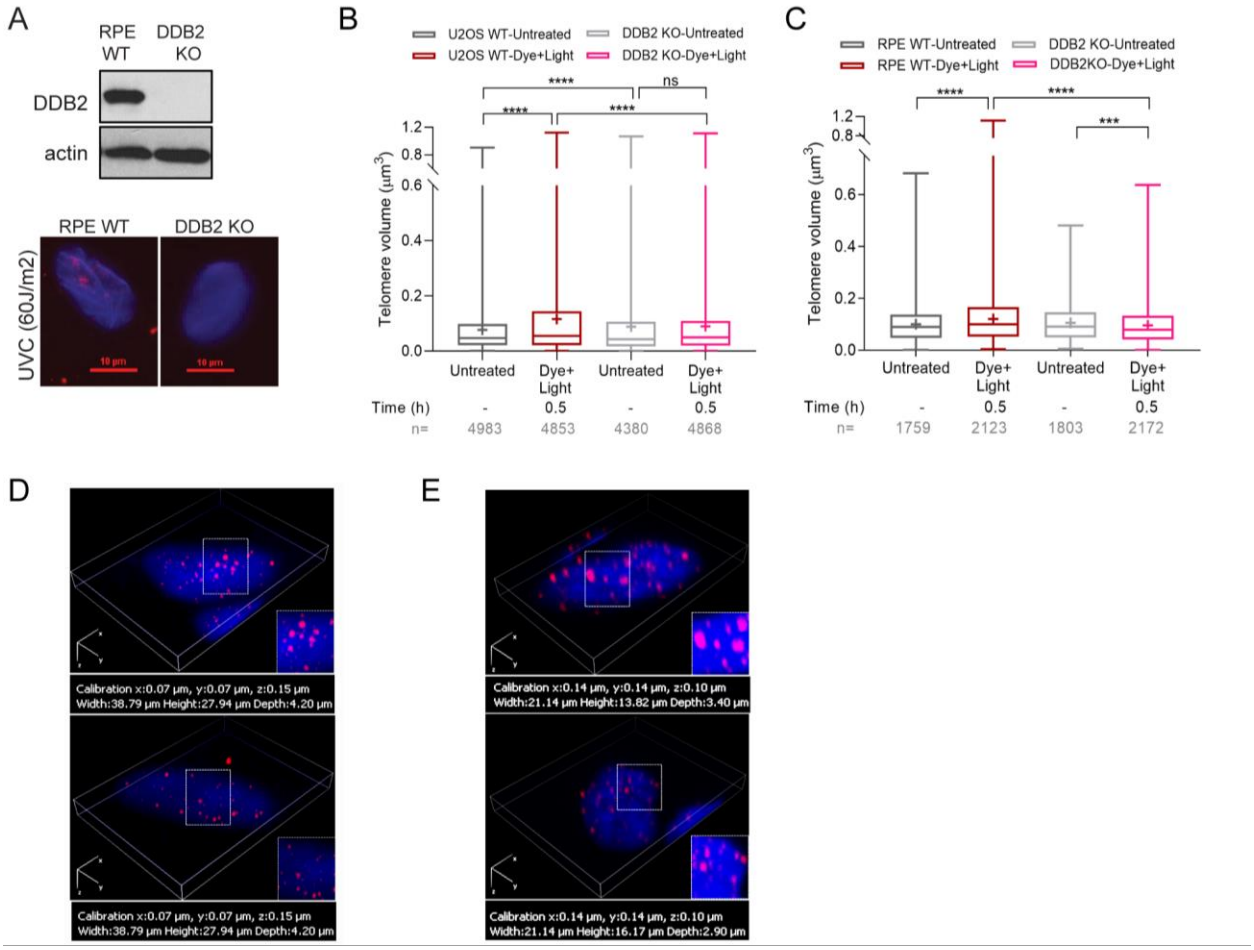

Figure 20: DDB2 mediates chromatin decompaction at sites of telomeric 8-oxoG.

A. Western blot and immunofluorescence for DDB2 in RPE-FAP-TRF1 WT and DDB2 KO cells. B. and C. Telomere volumes were measured in untreated, and cells treated with dye (100 nM, 15 min) plus light (660 nm, 10 min) in WT and DDB2 KO cells. Cells were fixed 30 minutes post treatment. B. U2OS-FAP-TRF1 and C. RPE-FAP-TRF1 cells. D. and E. Representative images of telomere volumes after treatment with dye (100 nM, 15 min) plus light (660 nm, 10 min) in WT and DDB2 KO cells. D. U2OS-FAP-TRF1 and E. RPE-FAP-TRF1 cells. 'n' represents the number of telomeres analyzed for each condition. One-way ANOVA: ***p<0.001, ****p<0.0001.

Appendix E Studying involvement of NER proteins in the repair of oxidative damage

Critical review of studies showing the role that NER proteins play in facilitating repair of oxidative DNA damage, originally published in *Nucleic Acids Research*. Ref. [10]: Kumar, N., S. Raja, and B. Van Houten, *The involvement of nucleotide excision repair proteins in the removal of oxidative DNA damage.* Nucleic Acids Res, 2020. **48**(20): p. 11227-11243.

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SURVEY AND SUMMARY

The involvement of nucleotide excision repair proteins in the removal of oxidative DNA damage

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ABSTRACT

The six major mammalian DNA repair pathways were discovered as independent processes, each dedicated to remove specific types of lesions, but the past two decades have brought into focus the significant interplay between these pathways. In particular, several studies have demonstrated that certain proteins of the nucleotide excision repair (NER) and base excision repair (BER) pathways work in a cooperative manner in the removal of oxidative lesions. This review focuses on recent data showing how the NER proteins, XPA, XPC, XPG, CSA, CSB and UV-DDB, work to stimulate known glycosylases involved in the removal of certain forms of base damage resulting from oxidative processes, and also discusses how some oxidative lesions are probably directly repaired through NER. Finally, since many glycosylases are inhibited from working on damage in the context of chromatin, we detail how we believe UV-DDB may be the first responder in altering the structure of damage containing-nucleosomes, allowing access to BER enzymes.

INTRODUCTION

Reactive oxygen and nitrogen species (ROS/RNS), such as singlet oxygen, superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite, can be generated endogenously by normal cellular metabolism or inflammation, or by exogenous sources such as ultraviolet (UV) or ionizing radiation (IR) (1–3). Oxidation can either directly or indirectly introduce a wide spectrum of base lesions in the DNA (4). Due to the extensive DNA damage caused by oxidation, these lesions have been associated with a large number of human maladies including neurodegeneration, cancer and aging (5).

Some of the most widely studied DNA lesions resulting from oxidation are shown in Figure 1. One of the best characterized oxidative lesions is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoG), the major product produced from the oxidation of guanine. Further oxidation of 8-oxoG results in the formation of spiroiminodihydantoin (Sp) and 5-guanidinohydantoin (Gh). Purine oxidation can also result in the formation of 5′,8-cyclo-purine adducts. An important product of thymine oxidation is thymine glycol (Tg). Cytosine is subject to methylation, resulting in the formation of 5-methylcytosine (5mC). Oxidative removal of 5-methylcytosine (5mC) occurs through an active enzymatic process in which 5mC is oxidized in three steps by a family of TET dioxygenases to form 5-hydroxymethyl-C (5hmC), 5-formylC (5fC) and 5-carboxyC (5caC).

Oxidative base lesions are commonly repaired via base excision repair (BER) pathway (6). BER is initiated after a lesion-specific DNA glycosylase cleaves the glycosidic bond, which frees the lesion, and creates an abasic site (1) (Figure 2). Currently, there are 11 known mammalian DNA glycosylases that can be categorized as monofunctional or bifunctional. Monofunctional glycosylases only possess the ability to break the glycosidic bond between the damaged base and the sugar moiety, resulting in an abasic site, which is processed by AP endonuclease 1 (APE1) to form a 3′OH and a deoxyribose-5′-phosphate (dRP). This dRP is removed by the lyase activity of DNA polymerase β (pol β). Bifunctional glycosylases have an additional AP lyase activity which allows for cleavage of the phosphate backbone, creating a single strand break, leaving a free 5′ phos-

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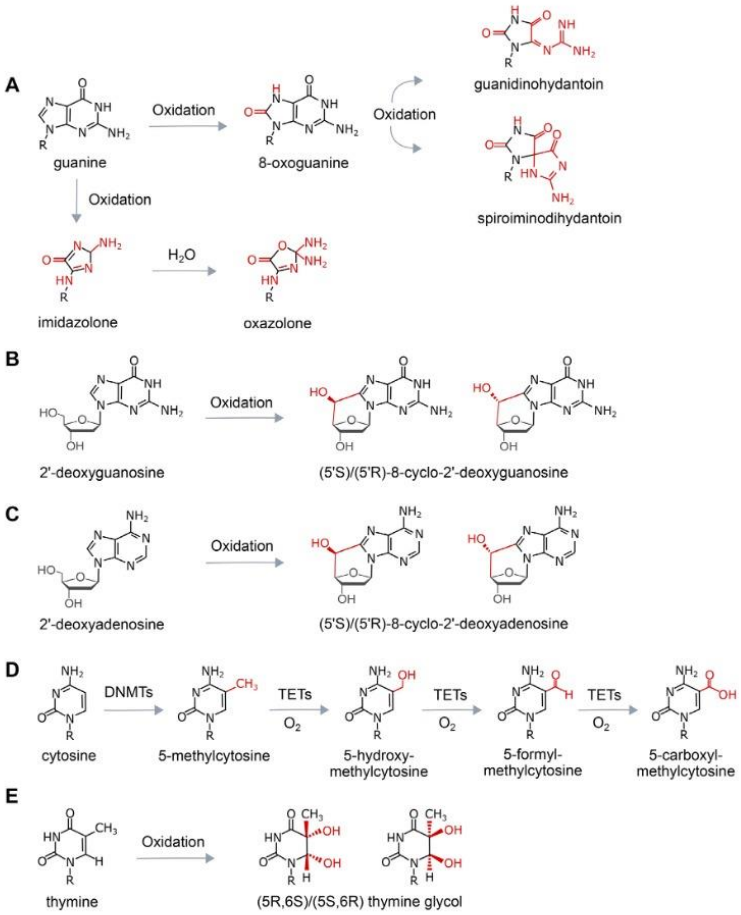


Figure 1. Chemical structures of oxidative lesions formed in DNA. (**A**) Various oxidation products of guanine. (**B**) Formation of cyclic guanosine by oxidation. (**C**) Formation of cyclic adenosine by oxidation. (**D**) Enzymatic oxidative demethylation of 5-methylcytosine. (**E**) Oxidation of thymine to thymine glycol. ROS produces over 100 different types of lesions in DNA, and this figure displays the structures of those damages that are discussed in this review. ROS: Reactive oxygen species; DNMT: DNA methyltransferase; TET: Ten-eleven translocation enzymes.

phate and either a 3′-phospho-α, β-unsaturated aldehyde (3′-PUA) (β-elimination) or 3′ phosphate (β,δ-elimination). APE1 acts on the β-elimination product, while polynucleotide kinase phosphate (PNKP) is required to process the 3′phosphate after β,δ-elimination. The resulting 3′OH is bound by PARP1 which recruits the BER complex consisting of pol β, XRCC1 and DNA ligase. The one base gap is then filled by pol β and the nick in the DNA is sealed by DNA ligase (7). The human 8-oxoG glycosylase (OGG1) is a bifunctional glycosylase responsible for the recognition and removal of 8-oxoG. Like several glycosylases, OGG1 is product inhibited, binding avidly to abasic sites, and turns over slowly in the absence of other proteins such as APE1 (7). Sp and Gh are removed by the actions of the bifunctional glycosylases Endonuclease VIII-like 1–3 (NEIL1–3), which will be discussed in detail in a later section. Tg is removed by the bifunctional glycosylase Endonuclease III-like 1 (NTHL1). 5fC and 5caC are removed by the action of thymine DNA glycosylase (TDG), which is a monofunctional glycosylase. The structures of these glycosylases and their substrates are given in Figure 3.

For more than a decade, studies have provided evidence suggesting a role for NER proteins in the repair of oxidative damage through interactions with BER proteins, reviewed (8–11). NER is the major pathway for the repair of bulky adducts and other helix-distorting lesions, such as UV-induced photoproducts, such as 6–4 photoproducts (6–4 PP) and cyclobutane pyrimidine dimers (CPD) (12). Un-

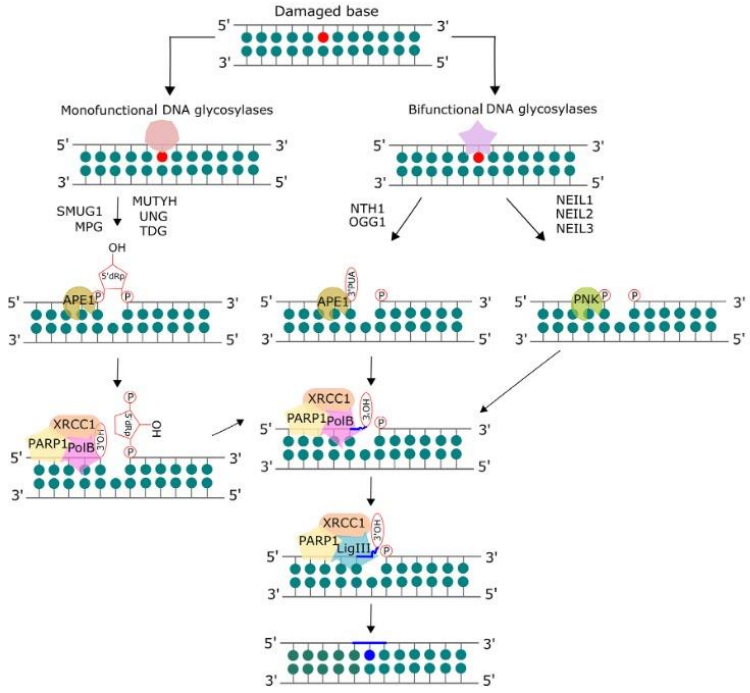


Figure 2. Mammalian base excision repair (BER) pathway. The base lesion is excised by a lesion-specific DNA glycosylase. Monofunctional glycosylases break the glycosidic bond between the damaged base and the sugar moiety, resulting in an abasic site. AP endonuclease 1 (APE1) processes the abasic site to form a 3′OH and a deoxyribose-5′-phosphate (dRP), which is removed by the lyase activity of DNA polymerase β (pol β). Bifunctional glycosylases utilize their AP lyase activity to cleave the phosphate backbone, creating a single strand break, leaving a free 5′ phosphate and either a 3′-phospho-α, β-unsaturated aldehyde (3′-PUA) (β-elimination) or a 3′ phosphate (β,δ-elimination). APE1 acts on the β-elimination product while polynucleotide kinase phosphate (PNKP) is required to process the 3′phosphate after β,δ-elimination. The resulting 3′OH is bound by PARP1 which recruits the BER complex consisting of pol β, XRCC1 and DNA ligase. The one base gap is then filled by pol β and the nick in the DNA is sealed by DNA ligase. Adapted from Kumar *et al.* (8) with permission.

like BER that has a set of glycosylases each tuned to find and process specific altered bases (Figure 3), damage recognition proteins of NER are remarkable in that they have a broad ability to dynamically detect many different structurally and chemically diverse lesions (13). There are two sub-pathways in NER: global-genome NER (GG-NER) and transcription-coupled NER (TC-NER), reviewed in (12,14). These sub-pathways differ in the manner of lesion recognition. In GG-NER, damage recognition proteins scan the entire genome, including heterochromatic, transcriptionally inactive regions, or the non-transcribed strand for damage-induced structural distortions. In contrast, TC-NER is initiated when RNA polymerase (RNAP) stalls at damaged site on the transcribed strand of active genes, in euchromatic regions of the genome. Defects in NER are associated with two important human diseases, xeroderma pigmentosum (XP) and Cockayne syndrome (CS). Damage recognition in GG-NER is initiated by two proteins, UV-DDB and XPC-RAD23B. In response to UV-induced DNA damage, UV-DDB in complex with CUL4 and RBX forms a ubiquitin E3 ligase complex and binds to the chromatin to ubiquitinate histones, making the lesion more accessible to downstream repair proteins in the NER pathway, including XPC-RAD23B. XPC-RAD23B binds with high affinity to the strand opposite to the distorted lesion, which begins the damage verification step of NER. XPC-RAD23B facilitates recruitment of the transcription factor TFIIH. TFIIH is a multi-subunit protein, consisting of 10 proteins, including XPB and XPD, proteins that have DNA helicase folds. When XPD encounters the lesion, its strand opening activity stalls and facilitates the recruitment of XPA, RPA and XPG, collectively known as the pre-incision complex. XPB is believed to act as a translocase to help reel the DNA into the pre-incision complex. XPA and RPA recruit the heterodimeric endonuclease, XPF-ERCC1. Recruitment of XPF-ERCC1 produces an endonucleolytic incision 5′ to the lesion. DNA polymerases δ, ε or κ begin to fill in the repair patch, which stimulates the 3′ endonucleolytic activity of XPG, leading to release of an oligonucleotide of 22–27 nucleotides containing both the lesion and TFIIH. DNA ligase I seals the remaining nick in the repair patch. TC-NER damage recognition is initiated by the pres-

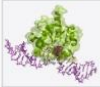
Glycosylase	Name	Substrate	Glycosylase	Name	Substrate
	**SMUG1** Single-strand-specific monofunctional uracil DNA glycosylase 1	U (opp G or A) 5-FU, 5-hU, 5-hmU,		**OGG1** 8-OxoG DNA glycosylase 1	8-oxoG, FaPyG (opp C)
	**MBD4** Methyl-binding domain glycosylase 4	T, U, 5-FU, 5-hmU, εC		**NEIL1** Endonuclease VIII-like glycosylase 1	Tg, FaPyG, FaPyA, 8-oxoG, 5-hU, 5-hC, Sp, Gh, Im
	**UNG** Uracil-N glycosylase	U, 5-FU, 5-hU		**NEIL2** Endonuclease VIII-like glycosylase 2	Same as NEIL1
	**TDG** Thymine DNA glycosylase	T, U, 5-FU, εC, 5-hmU, 5-fC, 5-caC, 5-BrU, Tg,		**NEIL3** Endonuclease VIII-like glycosylase 3	FaPyG, FaPyA, Sp, Gh, 5-hU, 5-hC
	**MPG** Methylpurine glycosylase	3-meA, 7-meG, 3-meG, 7-meG, εA, εG, hypoxanthine,		**NTHL1** Endonuclease III-like 1	Tg, FaPyG, 5-hC, 5-hU
	**MUTYH** MutY homolog DNA glycosylase	A (opp 8-oxoG, C, or G), 2-hA, 8-oxoA			

Figure 3. BER glycosylases, their structures and respective substrates. The glycosylases (green) are bound to DNA (purple) containing a lesion (purple, space-filled). All structures are human except SMUG1 (*Xenopus laevis*), MUTYH (*Geobacillus stearothermophilus*), NEIL2 (*Monodelphis domestica*), NEIL3 (*Mus musculus*), NTHL1 (EndoIII, *Geobacillus stearothermophilus*). PDB: SMUG1 (1OE4), MBD4 (5CHZ), UNG (1EMH), TDG (3UFJ), MPG (1BNK), MUTYH (4YOQ), OGG1 (1EBM), NEIL1 (5ITY), NEIL2 (6VJI), NEIL3 (3W0F), NTHL1 (1ORN). Abbreviations: **U**, uracil; **A**, adenine; **T**, thymine; **C**, cytosine; **G**, guanine; **5-FU**, 5-fluorouracil; **5-hmU**, 5-hydroxymethyluracil; **ε**, etheno; **FaPy**, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine; **8-oxoG**, 8-oxoguanine; **Gh**, Guanidonohydantoin; **Sp**, Spiroiminodihydantonin; **Im**, iminoallantoin; **5fC**, 5-formylcytosine; **5caC**, 5-carboxycytosine; **5-BrU**, 5-Bromouracil; **Tg**, Thymine Glycol; **meA**, 3-methyladenine; **meG**, 3-methylguanine; **5-hC**, 5-hydroxycytosine; **5-hU**, 5-hydroxyuracil; **2-hA**, 2-hydroxyadenine

ence of a stalled RNAP at a lesion site, which facilitates recruitment of Cockayne syndrome proteins (CSB and CSA), and the accessory proteins (UVSSA, XAB2, and HMGN1) to the lesion site on the transcribed strand (15). XAB2 facilitates recruitment of XPA and subsequently TFIIH which intertwines the two NER sub-pathways at the damage verification step. CS patients and a subset of XP patients display signs of neurological degeneration and have been shown to accumulate unrepaired oxidative DNA lesions (16,17). Therefore, it is important to understand any crosstalk which exists between the two repair pathways to gain a better understanding of disease progression.

COULD CYCLOPURINE DEOXYNUCLEOSIDES EXPLAIN NEURODEGENRATION IN XERODERMA PIGMENTOSUM PATIENTS?

5′,8-Cyclopurine deoxynucleotides (cyPu) are endogenous oxidative DNA lesions formed by the reaction of hydroxyl radicals with DNA, and first identified after ionizing radiation (18). These lesions contain damage to both the purine base and the 2′-deoxyribose sugar moiety by forming a covalent bond between the C8 of the base and C5 of 2′-deoxyribose (19). Both cyclo-deoxyadenosine (cdA) and cyclo-deoxyguanosine (cdG) lesions exist as 5′*R*- and 5′*S*-diaestereomers (20) (Figure 1) and cause significant distortions to DNA and therefore, can act as good substrates for nucleotide excision repair (NER) (11). Estimates of cyPu lesions vary, but are generally considered to be less frequent than 8-oxoG, with cdG lesions about an order of magnitude more prevalent that cdA lesions (Table 1).

As mentioned earlier, defects in NER genes can cause the rare disorder xeroderma pigmentosum (XP). About 20% of XP patients exhibit neurological symptoms, which have been posited to be caused by endogenously accumulated oxidative DNA damage (16,17). Work by Lindahl's group supported this hypothesis by demonstrating that a subclass of base lesions formed by γ-irradiation were repaired by normal cell extracts, but not by XP cell extracts (21). The first direct evidence of NER involvement in the repair of cyPu lesions was provided by Kuraoka *et al*. Primer extension assays using mammalian polymerase δ or T7 DNA polymerase were performed to show that both 5′S- and 5′R-cdA can block DNA synthesis by terminating product extension at the cdA site in a 5′-^{32}P-labeled DNA template-primer substrate containing a site-specific 5′S- or 5′R-cdA lesion. Therefore, if left unrepaired, cdA lesions can be highly cytotoxic by blocking DNA replication (22). Moreover, when HeLa cell extracts were incubated with 20 bp cdA substrates, no DNA glycosylase-mediated cleavage was observed suggesting that human DNA glycosylases cannot act on cyPu residues. Finally, the authors used a dual-

Table 1. Oxidative lesions and NER proteins

Lesion	Frequency/10^6 bases *	Reference for lesion frequency	Technique used	Source of DNA	Repaired by	Evidence for NER proteins involved (reference)
8-oxoG	1	(113)	COMET assay	HeLa cells	BER / TC-NER	XPA (67,69,73,75,76), XPC (29,38,67,69,72,73), XPG (67–69), UV-DDB (56), CSA (75), CSB (75)
	1.2	(29)	LC/MS	Human primary keratinocytes		
	4.6	(40)	HPLC-ESI-MS	Rat Liver		
	25**	(114)	LC-MS/MS	Calf Thymus		
	0.35	(115)	NaI extraction; HPLC-EC	Mice Liver		
Gh	0.01–0.07	(116)	LC/MS	Mice Liver and Colon	BER/NER	XPA (83), XPC (83)
Sp	0.01–0.07	(116)	LC/MSfC	Mice Liver and Colon	BER/NER	XPA (83), XPC (83)
8-cyclo-2′dG	2.8 (5′S); 0.7 (5′R)	(29)	GC/MS***	Human primary keratinocytes	NER	XPC (29)
8-cyclo-2′dA	0.1–0.15	(20)	LC/MS; GC/MS***	Calf Thymus	NER	XPA (23), XPC (29), CSA (30), CSB (30)
	0.015–0.03	(117)	^{32}P-Postlabeling Assay	Fetal and postnatal rat liver		
	0.2	(29)	LC/MS	Human primary keratinocytes		
5hmC	247	(118)	Tet-assisted bisulfite sequencing (TAB-Seq)	Human embryonic stem cells	BER	XPC (60)
	1300	(119)	2D-TLC; LC/MS/MS	Mouse embryonic stem cells		
5fC	20	(119)	2D-TLC; LC/MS/MS	Mouse embryonic stem cells	BER	XPC (60)
5caC	3	(119)	2D-TLC; LC/MS/MS	Mouse embryonic stem cells	BER	XPC (60)
Tg	0.01	(120)	Capillary electrophoresis and laser-induced fluorescence detection	human lung carcinoma cells	BER	XPC (60)
Oxazolone	0.02–0.41	(40,114)	HPLC-ESI-MS	Rat Liver	BER	XPC (80)

Abbreviations: 8-oxoG, 8-oxoguanine; Gh, Guanidonohydantoin; Sp, Spiroiminodihydantonin; 8-cyclo-2′dG, 8-cyclo-2′deoxyguanosine; 8-cyclo-2′dA, 8-cyclo-2′deoxyadenosine; 5hmC, 5-hydroxymethylcytosine; 5fC, 5-formylcytosine; 5caC, 5-carboxycytosine; Tg, Thymine Glycol

*Steady state levels of damage found in purified DNA from various sources.

**Commercially available Calf thymus DNA probably contains higher than normal levels of 8-oxoG due to oxidation in purification and processing (114,121)

***GC/MS – method of preparation contributes to high lesion frequency.

incision assay with cell extracts and closed circular DNA substrates harboring a 5′S- or 5′R-cdA lesion to show that the cyPu residues are repaired by excision of a 26–28 bp DNA product. The excision was significantly suppressed in the presence of an XPA antibody, indicating the dependence of repair on the NER pathway. Interestingly, the R form of cdA was repaired more efficiently (∼2-fold) than the S form, however, both diastereoisomers were relatively poor (∼40–150-fold) NER substrates as compared to the 1–3-intrastrand d(GpTpG)-cisplatin crosslink substrate. This study clearly shows that cyPu lesions are removed by NER *in vitro*, and have the ability to cause local helix distortions and block polymerases. Brooks *et al* performed a host reactivation assay (HCR) in Chinese hamster ovary (CHO) cells using a plasmid expressing the luciferase (Luc) gene that contained a cdA lesion on the transcribed strand (23). They showed that a single cdA lesion dramatically decreased the Luc gene expression, suggesting that cdA can act as a strong block to transcription. They also found that repair of cdA lesion on the plasmid was significantly reduced in XPG and ERCC1 mutant cells as compared to wildtype. They further confirmed this result by employing the HCR assay in SV-40 transformed normal (GM00637) and XP-A (XP12BE) cell lines, providing evidence for defective repair of cdA in the XP-A cells. The XP12BE cell line was derived from a XP-A patient (XP2OS) who exhibited severe neurological symptoms (24). Therefore, these studies provide a strong correlation between defective NER and neurodegeneration in XP patients. In this scenario, an obvious prediction would be that NER deficient XP patients would have elevated levels of cyPu lesions in their DNA. Indeed, cyPu lesions have been shown to accumulate over age in the brain tissue of $Xpa^{-/-}$ and $Csb^{-/-}$ mice (25,26), although unlike humans, $Xpa^{-/-}$ mice do not display any neurological abnormalities (27,28). In another study, D'Errico *et al* used X-rays to introduce base lesions in the DNA of normal and XP-C keratinocytes and measured the accumulation of cyPu by HPLC/MS. While both normal and XP-C cells accumulated equal numbers of cyPu lesions after 5Gy of X-rays, XP-C cells were inefficient in the removal of damage over time (29). Similar accumulation of cdA was also observed in CSA deficient (CS-A) fibroblasts treated with X-rays (30). This is consistent with it being a strong transcription blocking lesion as CSA and CSB both recognize DNA damage in the context of transcription. Further studies are required in more XP and CS patients with neurological disorders to establish a direct link between cyPu accumulation and XP neurodegeneration. Despite the low prevalence of XP throughout the world, it would be of interest to set-up a rapid autopsy program to measure cyPu lesions in brain tissues from deceased XP patients.

Whether cdG lesions could be processed by DNA glycosylases was investigated by Pande *et al.* (31). In this study, seven purified glycosylases (bacterial: Fpg, EndoIII, EndoV, EndoVIII; human: NEIL1, NEIL2, and OGG1) failed to incise a 12 or 36 bp duplex containing a S-cdA or a S-cdG lesion. Even at high concentrations of the enzymes (200 fmol), no cleavage activity was observed. They also performed binding assays with the glycosylases and found that at very high concentrations (10–20 pmol), NEIL1 bound to both cdA and cdG substrates as well as undamaged DNA, suggesting that the binding was non-specific. Strikingly, an earlier study had shown that both S- and R-cdA lesions accumulate in $Neil1^{-/-}$ mice (32), suggesting the role of NEIL1 in cyPu repair, although the exact mechanism is still unclear. Furthermore, this study went on to demonstrate that S-cdG was repaired slightly better than S-cdA by NER in human HeLa cell extracts and that the base complementary to the lesion affected the efficiency of repair. They speculated that both base pairing and base stacking are important for the recognition of cyPu lesions in the DNA and that an abnormal Watson-Crick base pairing (e.g. S-cdG:dT) acts as a better substrate for NER. NMR combined with molecular dynamics have proven highly successful in understanding the alterations in the conformation of the DNA helix induced by DNA lesions. It would be of interest to use these techniques to determine the distortions formed on the DNA by the cyPu lesions and investigate the interactions of NEIL1 and other NER recognition proteins with cyPu lesions (33,34).

Recently, for the first time, all four cyPu lesions (5′R-cdA, 5′S-cdA, 5′R-cdG, 5′S-cdG) were examined in the same sequence context by Shafirovich's group and the NER efficiencies were measured by excision assays using HeLa cell extracts (35). In agreement with the study mentioned earlier (22), 5′R-cyPu were repaired more efficiently than 5′S-cyPu. Molecular dynamics (100ns) revealed greater DNA backbone distortions and diminished base stacking in the R form of cyPu as compared to the S form. In cells, DNA lesions are embedded in the nucleosome which can hinder the accessibility of some repair proteins (36–46). Therefore, to explore the effect of CyPu on histone-DNA interactions, Shafirovich *et al.* embedded cyPu lesions in an 'In' (fourth nucleotide on the 5′-side of dyad) and 'Out' (eighth nucleotide facing the aqueous solution environment) rotational setting near the dyad axis in nucleosomes reconstituted with either recombinant histones or histones extracted from HeLa cells (47). In both cases, they made the surprising discovery that cyPu lesions were completely resistant to excision by NER proteins in human cell extracts. This suggests that even though cyPu lesions cause significant distortions to naked DNA duplex, they either do not significantly disturb the DNA-histone interactions at these specific positions or these lesions when embedded in a nucleosome escape detection by NER proteins. It also remains unknown whether these results are relevant in physiological conditions *in vivo*. It would be, therefore, of great interest to extend these studies to living cells, although there are no tools available yet that could be used to specifically introduce cyPu lesions in cells.

As mentioned earlier, NER proteins recognize and repair UV-induced photoproducts, 6–4 PP and CPD. While a 6–4 PP causes a significant distortion in nucleosomal DNA (48), a CPD causes less of a distortion (49). It is well-established that while XPC-RAD23B can recognize 6–4PP, this heterodimer has limited ability to detect CPDs, (50) and in cells recruitment of XPC to sites of CPD in chromatin requires UV-DDB. Furthermore, it is interesting to note that XPC does not efficiently bind DNA configured on a nucleosome (51). On the other hand, UV-DDB has been shown to bind lesions directly in the nucleosome and even shift the nucleosomal register to provide access to more occluded

sites (49,52). Moreover, the binding of UV-DDB seems to precede the activity of ATP-mediated chromatin remodelers (53,54). More recently, as discussed in the last section, our group has demonstrated that the oxidative base damage 8-oxoG, which only causes a mild helix distortion, is recognized by UV-DDB in naked duplex DNA, as well as in living cells (55,56). The relatively mild nucleosome distortion caused by CPD and 8-oxoG is analogous to cyPu. Therefore, it would be interesting to determine the full substrate repertoire of UV-DDB and if UV-DDB is capable of recognizing CyPu and other lesions in the context of nucleosomes.

OXIDATIVE REMOVAL OF 5MC MOIETIES IS STIMULATED BY XPC

5-methylcytosine (5mC) is formed by the addition of a methyl group to carbon 5 of cytosine through the action of a DNA methyltransferase (57). As previously mentioned, during an active enzymatic demethylation process, 5mC is oxidized by TET dioxygenases to 5hmC, 5fC, and 5caC. The latter two lesions are removed by TDG. 5fC, and 5caC are some of the more common oxidative lesions with steady state levels at 20 and 3 lesions per million bases, respectively (Table 1). TDG has been shown to also remove deaminated 5mC (T:G) moieties from DNA (58).

TDG is a monofunctional glycosylase, which as previously mentioned, binds avidly to abasic sites and thus becomes product inhibited. Studies have demonstrated roles for other BER proteins, including NEIL1 and APE1 in facilitating TDG turnover. However, the mechanism of DNA demethylation by TDG in cells remains unclear (59). To this end, Ho *et al* investigated the role of XPC in epigenetic gene regulation through stimulation of TDG. Using an ELISA specific to 5mC, they were able to show XPC-dependent DNA demethylation (60). While these authors reported that XPC plays a role in DNA demethylation, they argued based on a W690S variant of XPC that this activity was independent of XPC's role in NER. This W690S XPC variant, discovered in an XP-C patient, XP13PV, was previously shown to have reduced stability in cells, and cells expressing this variant showed reduced rates of removal of UV-induced photoproducts (61). However, careful analysis of data presented in the Ho *et al.* study indicates that the W609S XPC variant showed reduced stimulation, ∼20% increase of TDG excision compared to TDG alone, of either 5fC:G or 5caC:G. In comparison, WT XPC fully doubled the activity of TDG. These data suggest that reduced damage recognition and/or DNA binding of this W690S XPC variant prevented its stimulation of TDG. To provide further support for the role of XPC in TDG stimulation, the authors performed a ChIP-seq analysis and determined co-enrichment of TDG and the XPC subunit, RAD23B at the promoter region of embryonic stem cells. Additionally, using MeDIP-seq to measure 5mC levels globally in the genome, the authors were able to show reduced DNA methylation in cells overexpressing WT XPC. Single-particle tracking experiments utilizing Halo-tagged TDG and SNAP-tagged XPC, revealed that overexpression of XPC led to a reduction in the length of time Halo-tagged TDG remained bound to DNA. Using shRNA to knockdown XPC expression

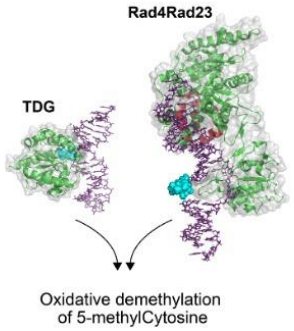


Figure 4. XPC and TDG in oxidative demethylation of 5-methylcytosine. Based on the work by Ho *et al.* (60), XPC works to help turnover TDG, which like other glycosylases, is product inhibited binding tightly to abasic sites. Shown here is the structure of the yeast, XPC homolog, Rad4 (green) Rad23 (red) bound to a DNA duplex (purple) containing a 6–4 photoproduct (blue space-filled), PDB: 6CFI; Human TDG (green) bound to a DNA duplex (purple) containing 2′-fluoro-2′-deoxyuridine (blue space-filled), PDB:3UFJ (122).

the authors demonstrated longer retention of TDG on the DNA. These data support the role of XPC in stimulating TDG activity by facilitating turnover of TDG from the abasic DNA product, (Figure 4). Lastly, the authors determined that TDG stimulation by XPC occurs through interactions between the N-terminus of TDG and the C-terminus of XPC. This study only looked at the role of XPC in TDG stimulation, it would be interesting to determine if any other NER or BER proteins are recruited to the 5mC moiety in response to XPC stimulation. Finally, it should be pointed out that XP-C patients and Xpc-/- mice develop normally and do not appear to have large defect in epigenetic programing during cellular differentiation. Thus, further work is needed on the role of XPC, and NER proteins in the oxidative removal of 5mC.

NER PROTEINS HELP MEDIATE THE REMOVAL OF THYMINE GLYCOL AND 8-OXOG

Guanine oxidation is a well-characterized DNA lesion. ROS acting on guanine results in the formation 8-oxoG, through two subtle modifications on guanine (Figure 1): the addition of an oxo group on carbon 8 and the addition of hydrogen to the seventh position nitrogen (62). These modifications causes the base to rotate from the anti- to the syn-conformation with respect to the deoxyribose moiety around the glycosidic bond causing 8-oxoG to pair with A during replication creating T:A transversions, if left unrepaired. The formation of 8-oxoG lesions in cells is estimated to occur up to 10 000 times per cell per day in humans, with the estimated steady-state levels of about 1–2 8-oxoG lesions/10^6 guanines (63,64) (Table 1). While older literature has referred to this lesion as 8-hydroxy-guanine, this tautomeric form at physiological pH (7.4) is a minor product (65). This relatively high lesion frequency of 8-oxoG (Table 1) coupled with the implications in genome instability emphasize the need for repair pathways dedicated to the

removal of the 8-oxoG lesion. As mentioned earlier, 8-oxoG is commonly removed through base excision repair (BER), through the actions of the DNA glycosylase OGG1. The work by the Mitra laboratory in showing OGG1 is product inhibited and needs the actions of APE1 to facilitate OGG1 turnover, imply the potential for other co-factors outside of BER to stimulate either OGG1 activity or processing of 8-oxoG (7).

The first implications of NER protein involvement in oxidative DNA damage repair was shown using the *Escherichia coli* NER system consisting of the UvrABC complex (66). The authors used a DNA substrate containing a thymine glycol (Tg) lesion to show that UvrABC efficiently recognizes and incises the lesion. This finding was recapitulated in the mammalian system by Sancar and coworkers (67). It has been estimated that the steady-state levels of Tg in mammalian cells are about two orders of magnitude lower than 8-oxoG adducts (Table 1). Using human cell free extracts lacking any one of the XPA-XPG proteins, they were able to show reduced excision of two common oxidative lesions, 8-oxoG and Tg, from damaged DNA substrate (67). The basis of this assay is that a DNA substrate is created by ligating a 5′ ^{32}-P end-labeled oligonucleotide containing an 8-oxoG or Tg moiety into a 139 bp DNA duplex. Dual incisions by XPF/ERRC1 on the 5′ side and XPG on the 3′ side liberates excision products of ∼22–26 bases containing the label. Additionally, they showed using a system of purified proteins the need for a complete NER system containing XPC-RAD23B, XPA, RPA, TFIIH (XPB and XPD), XPG and XPF-ERCC1, in order for proper excision of 8-oxoG or Tg. This work from the Sancar laboratory clearly demonstrates the ability of purified NER proteins to remove oxidative lesions, but did not assess whether there was any interaction between NER and BER proteins or whether NER is an important pathway for their removal in cells. The authors hypothesize the role of NER is to act as a slower alternative pathway for oxidative damage removal by BER, and the loss of this activity in XP patients, contributes to the accumulation of oxidative damage and subsequent neurodegeneration. It is important to point out the ability of the NER machinery to excise 8-oxoG had not been confirmed in any other laboratory or through any other approaches, and it remains to be determined whether NER is a back-up system for the removal of 8-oxoG in the absence of BER.

Following this pioneering work by the Sancar laboratory, which established an *in vitro* role of NER proteins in oxidative DNA damage repair, attention shifted to understanding the roles of specific NER proteins in the repair of 8-oxoG and other lesions induced by oxidative DNA damage. Klungland *et al* began by characterizing the role of XPG in BER of the oxidative lesions, thymine glycol and dihydrouracil. These lesions are excised by the bifunctional DNA glycosylase, NTH1. Using a reconstituted BER system containing hNTH1, APE1, pol β, and XRCC1-DNA ligase III, the Lindahl laboratory was able to show stimulation of NTH1 by XPG (68). Specifically, they were able to show enhanced binding of NTH1 to damaged DNA in the presence of XPG. The authors also looked at the ability of XPG to stimulate OGG1 excision of 8-oxoG but were unable to detect any enhanced OGG1 activity in the presence of XPG. In a later study of oxidative damage repair in melanocytes by Wang *et al.* cells deficient in XPG protein were shown to have decreased repair of hydrogen peroxide (H_2O_2)-mediated oxidative damage, when measured using a luciferase-based host cell reactivation assay (HCR) (69). They were also able to show that cells with defective XPA or XPC proteins showed reduced repair of oxidative damage. This study primarily focused on understanding oxidative DNA damage repair capacity of melanocytes, as melanoma incidence is increased ∼1000-fold in XP patients (70). Wang *et al.* went on to further investigate the role of XPA in oxidative DNA damage repair and showed XPA deficient cells had approximately a 4-fold reduction in oxidative damage repair capacity. While this study showed an apparent involvement of NER proteins in the repair of oxidative DNA damage, it remained unclear the specific roles of XPA, XPC and XPG in this process. The authors hypothesized in the case of melanocytes, the increased presence of melanin which binds to DNA may inhibit the recognition of the damage by both BER and NER proteins. It would be interesting to further investigate whether the levels of melanin prevent recruitment of damage recognition proteins and inhibit subsequent DNA repair.

The Dogliotti lab provided the first evidence of an NER protein, XPC, having a protective role against oxidative stress in human skin cells. Looking at keratinocytes and fibroblasts with a nonsense mutation in the XPC protein, they were able to demonstrate an increased sensitivity to oxidizing damage, such as that from X-rays or potassium bromate, through a colony formation assay (29). While X-rays produce a wide spectrum of DNA lesions including various forms of base damage, single-strand and double-strand breaks, potassium bromide produces primarily 8-oxoG and to a lesser extent other base damages (71). Additionally, D'Errico *et al* demonstrated using HPLC/MS an accumulation and subsequent delayed removal of 8-oxoG from XPC-deficient skin cells. To strengthen support for the role of XPC in oxidative DNA damage repair, they also showed XPC-RAD23B-mediated stimulation of OGG1, the DNA glycosylase responsible for 8-oxoG removal. They were unable to demonstrate XPA stimulation of OGG1, even at high protein concentrations, even though previous studies alluded to a role for XPA in oxidative damage repair (67,69). Furthermore, through far western blot analysis the authors demonstrated a direct binding between OGG1 and XPC-RAD23B, showing XPC enhances the ability of OGG1 to recognize 8-oxoG lesions. The authors did not show a direct interaction between XPC and damaged DNA, indicating its role is possibly to facilitate the turnover of OGG1. In a later study by the Rainbow lab, XPC deficient fibroblasts were shown to have reduced removal of 8-oxoG (72). 8-oxoG was introduced onto the β-galactosidase reporter gene via generation of a singlet oxygen by methylene blue plus visible light. Using this reporter gene, they conducted a host cell reactivation assay (HCR) to investigate the effect of XPC on DNA damage repair. Additionally, while the authors demonstrated pre-treatment of cells with UVC does not change the relative repair rate of 8-oxoG in XPC-deficient cells, pre-treatment with UVC resulted in an approximate 1.5-fold increase in overall repair of 8-oxoG. These data imply a potential role of other proteins induced by UV damage

mediated through p53 stabilization induced gene expression, in the repair of oxidative damage. One such protein may be DDB2.

Taken together these data suggested BER may not work in isolation to remove oxidative damage. However, the specific molecular roles NER proteins may play in the repair of oxidative damage remained unclear. To this end, Parlanti *et al* provided significant insights on the roles of NER proteins, specifically XPA, XPC, CSA, and CSB, on 8-oxoG repair (73). First, using fibroblasts derived from NER deficient mice, they showed reduced repair and increased accumulation of 8-oxoG in XPA$^{-/-}$, XPC$^{-/-}$, CSA$^{-/-}$, CSB$^{-/-}$ and OGG1$^{-/-}$, knockout (KO) cells. Unsurprisingly, the OGG1$^{-/-}$ KO cells showed the greatest reduction in repair and subsequent accumulation of 8-oxoG. However, all of the NER protein MEF KO cell lines also showed a noticeable reduction in the rates of 8-oxoG repair compared to the WT cells with sufficient NER proteins. They also compared repair capacity in both the single and double KO cell lines and were able to demonstrate a more pronounced reduction in repair in the XPA$^{-/-}$/ CSB$^{-/-}$, XPC$^{-/-}$/CSB$^{-/-}$, double KO cell lines, which resembled the repair capacity of the OGG1 $^{-/-}$ cell line. Alternatively, the repair capacities of the XPA$^{-/-}$ and XPC$^{-/-}$ double KO cells resembled that of the single KO cells. These data suggest CSB and OGG1 are involved in the same repair pathway, one that may be different from that of XPC and XPA. However, the exact molecular details of these pathways remain unresolved. This group were also able to recapitulate the results from the mouse experiments in human XPA fibroblasts, by showing decreased repair of 8-oxoG in addition to increased sensitivity to oxidizing agents. Using siRNA targeting OGG1 in the XP12SV40 cell line, an XP-A deficient cell line they were able to show impaired repair of 8-oxoG, when compared to XP-A cells or cells with deficient XPC. It remains unclear how the authors were able to demonstrate impaired repair of 8-oxoG in XPA deficient cells in one study, but failed to show XPA-mediated stimulation of OGG1, the glycosylase mediating the repair of the lesion, in another study (29). These conflicting results further reiterates the ambiguity of the role of XPA in 8-oxoG removal, and other published studies show contrasting results (74). The Spivak and Hanawalt laboratory developed a cutting-edge strategy to investigate the role of XPA in repair of relatively low levels of oxidative damage. Using a comet-FISH (combining single-cell gel electrophoresis with a fluorescence *in situ* hybridization assay), they were able to demonstrate roles for XPA and CSB in the transcription-coupled repair of 8-oxoG (75). Using the ATM gene, they fluorescently labeled the 5′ and 3′ ends using different probes and tracked the increasing distance between the probes after treatment with potassium bromate, as a measure of BER-mediated single-strand break formation. In this way the authors were able to show the repair rates of CSB and XPA deficient cells were comparable to the repair rates of the wild-type non-transcribed strand showing the repair of 8-oxoG is coupled to transcription. To further support the idea of 8-oxoG processing to be coupled to transcription, the authors performed the comet-FISH assay on cells deficient in UVSSA and RNAPII, key proteins in TC-NER, and were able to show that these cells displayed a repair rate similar to that of the wild-type non-transcribed strand. Finally, they showed that in the absence of OGG1, this transcriptional effect of XPA and CSB was lost, suggesting that RNAP is not inhibited by 8-oxoG, but instead by the resulting SSB created by the actions of OGG1 and APE1. The protective role of XPA was further investigated by the Yasui lab, which utilized the TATAM (tracing DNA adducts in the targeted mutagenesis) system to study 8-oxoG lesions into XPA knockout cells (76). Introduction of a single 8-oxoG lesion in cells deficient of XPA had no effect on mutagenesis. Tracks of ionizing radiation can create closely spaced multiple lesions and it was interesting to note that this group was able to show increased mutagenesis in XPA deficient cells with the introduction of multiple 8-oxoG lesions, specifically when the lesions were introduced on the actively transcribed strand.

The Vermeulen lab developed an imaging system to study the roles of NER proteins in 8-oxoG repair. They locally induced 8-oxoG lesions via singlet oxygen by using a photosensitizer and a 405nm laser (38). Using this highly innovative approach, the authors were able to show a difference in the recruitment kinetics of CSB and XPC to damaged sites, with CSB showing slightly enhanced recruitment over XPC ($t_{1/2} = \sim$9 s for CSB versus $\sim$13 s for XPC). CSB and XPC are involved in different sub-pathways of NER, offering an explanation for their different rates of recruitment to damage sites. In support of a role of CSB in transcription-coupled repair of 8-oxoG, they were able to show enhanced CSB recruitment to the transcriptionally active nucleolus, while XPC was seen more in the heterochromatic nucleoplasm, supporting its role in GG-NER. This study while demonstrating the recruitment of CSB and XPC to sites of oxidative damage, did not address whether these proteins are directly involved in the removal of 8-oxoG, either through DNA glycosylase stimulation or in some other step in BER. In a later study, these same authors looked at the role of CSB in 8-oxoG repair by looking at OGG1 recruitment (77). Using the previously described photosensitizer and laser strategy, they induced 8-oxoG lesions and monitored recruitment of CSB and OGG1 to the damage site and demonstrated that CSB recruitment to damage sites is independent of OGG1. These data seem at odds with the previous work by Spivak and Hanawalt who suggested TCR of 8-oxoG can only occur after processing of the lesion to a strand break. Furthermore, the Menoni *et al.* study (77) demonstrated CSB is able to stimulate XRCC1 recruitment to 8-oxoG, in transcriptionally active regions. The authors hypothesize the role of CSB is to aid in the recruitment of XRCC1 and other BER proteins by facilitating chromatin remodeling to aid accessing lesions, especially single-strand breaks. Additionally, it is important to note that APE1 has been shown to interact directly with CSB and is stimulated up to 6-fold in an ATP-independent manner (78). Thus, CSB may provide an important role during transcription-coupled BER by orchestrating both backtracking of stalled RNAP, as well as coordinating downstream BER proteins. Finally, it was shown that CSB deficient cells are hypersensitive to killing by hydroxymethyldeoxyuridine (hmdU) treatment, suggesting a direct role of CSB in SMUG1-mediated removal of this oxidized base from DNA (78).

NER AND BER WORK COOPERATIVELY TO REPAIR OXIDIZED 8-OXOGUANINE LESIONS

The oxidation of guanine can create 2,2-diamino-4-[(2-deoxy-β-D-ery-pentofuranosyl) amino)-5(2H)-oxazolone (Oz), and 8-oxoG. The Oz lesion is normally processed by BER in mammalian cells by the activities of NEIL1 and NTH1 (79). However, using a defined system, Hanaoka and coworkers showed that Oz is also a poor substrate for NER, and that the overall affinity of XPC-RAD23B to a DNA duplex containing Oz was significantly lower than a 6–4 PP containing duplex (80). The 8-oxoG lesion is several orders of magnitude more sensitive to oxidation than the parent guanine moiety resulting in two oxidation products, Sp and Gh, which are less common oxidative lesions, having steady state levels in the range of 0.01–0.07 lesions per million bases (Table 1). These lesions can be removed by BER proteins, however work from several laboratories suggest that NER can also process these lesions. Early studies with the bacterial NER UvrABC system indicated that this enzyme incised a duplex containing several lesions to different extents, 8-oxoG:A the worst (10% completion) with Gh (23%) and Sp (32%) lesions being moderate and amine modified Sp to 62% completion (81). These results suggest that a larger and more distorting lesion is recognized and incised the most efficiently by the bacterial NER proteins.

Work from the Shavirovich and Geacintov laboratories indicated that human NER proteins from cell extracts can perform dual incisions on Sp or Gh lesions embedded in a 135 bp duplex. XPA depletion studies with antibodies to XPA or extracts from XPC$^{-/-}$ fibroblasts failed to produce dual incisions (82). Adding back purified XPC to the latter extract was able to restore NER activity. The same study showed that NEIL1 can also process these lesions in an extract and it was not clear if these two pathways work synergistically or in an antagonistic manner. This question was elegantly explored in living cells by the same group in which they transfected internally ^{32}P-labeled DNA hairpin in which the label was placed near the lesion (83). By transfecting this DNA duplex into human cells, they were able to follow the excision of the oligonucleotide containing the lesion via a NER pathway or direct incision by the activity of NEIL1 initiating BER. They found that compared to a substrate containing a benzo[a]pyrene-dG lesion, Sp or Gh lesions were processed by NER 8- or 6-fold, respectively, less efficiently. Transfection of the Gh substrate into XPA$^{-/-}$ cells failed to produce the characteristic excision product by NER, whereas transfection into NEIL1$^{-/-}$ cells reduced but did not eliminate the BER incision product. Taken together these data suggested to the authors that the amount of XPC and XPA and perhaps their relatively low affinity for Sp and Gh substrates versus 5–10-fold higher levels of NEIL1 with high catalytic efficiency in the cell, probably pushes repair of these substrates into a BER pathway. However, it is not clear whether these transfected DNA hairpins were associated with nucleosomes and what role chromatin structure may play on the processing of Sp and Gh lesions by NER and BER pathways. Finally, to better explore the question of relative affinities of XPC-RAD23B for Sp and Gh lesions and its ability to compete with NEIL1 for these lesions, the same group studied relative binding affinities of XPC-RAD23 and NEIL for 147 bp duplexes containing Sp or Gh lesions using EMSA analysis (84). They found that XPC-RAD23 bound to Sp containing substrate with high affinity (low nM range) and that XPC could effectively compete away NEIL1 binding to these substrates at equal molar concentrations. They then followed NEIL1 incision burst kinetics under single turnover conditions on both substrates in the absence and presence of XPC-RAD23 and found that equal molar concentrations of added XPC-RAD23 greatly reduced the amplitude of the burst phase of NEIL1 cleavage. Together these data strongly suggest that XPC-RAD23 and NEIL1 can directly compete for Sp and Gh lesions. Additionally, it has been demonstrated NEIL2 and NEIL3 can act to remove Sp and Gh lesions (85,86). Further cellular work such as transfecting plasmids carrying these defined lesions into cells and using immunofluorescence to measure the binding kinetics of these proteins will be necessary to better understand if XPC or other NER proteins can bind to these lesions in the cell nucleus.

A NEW ROLE OF UV-DDB IN THE REMOVAL OF 8-OXOG

As previously mentioned, OGG1 is product-inhibited and needs the activity of APE1 to turnover and work on other 8-oxoG lesions (7). Another factor which can impair the activity of BER proteins is the inaccessibility of oxidative lesions in the context of chromatin. DNA glycosylases have been shown to have impaired activity on damage when the lesion is contained within a nucleosome (42–46,87–90). It is important to note, while certain glycosylases such as SMUG1 are completely inhibited, others such as OGG1, AAG or UDG can recognize outward facing lesions and can readily initiate BER (88,90–92). In addition, both NEIL1 and NTH1 have been shown to show reduced activity on Tg substrates embedded in nucleosomes (91,93,94). The issue of lesion accessibility in the context of chromatin is an important factor in NER (36,37). UV-damaged DNA binding protein (UV-DDB), a heterodimeric protein consisting of DDB1 and DDB2, has a demonstrated role in NER for damage recognition in the context of chromatin (95). In response to UV-induced damage, UV-DDB as part of a ubiquitin E3 ligase complex with Cul4A and RBX, ubiquitinates histone H2A to facilitate chromatin remodeling to increase lesion accessibility (55). UV-DDB is known to recognize and bind sites of UV damage, but has also been shown to bind more strongly to a short DNA duplex containing an abasic site, as compared to a DNA substrate containing a CPD, suggesting a possible role for UV-DDB in BER (96,97). In order to directly test this hypothesis, we recently investigated whether UV-DDB plays an important role in initiating the repair of 8-oxoG lesions (56). It should be noted that previous studies missed that UV-DDB was able to discriminate between non-damaged DNA and DNA containing 8-oxoG (96). We have found that the presence of magnesium helps increase damage specificity by greatly decreasing binding to non-damaged DNA duplexes (55,56). EMSA assays conducted in the presence of 5 mM Mg^{2+} showed that UV-DDB preferentially bound abasic sites, CPD and 8-oxoG, with equilibrium dissociation constants,

K_d, of 3.9, 30 and 160 nM, respectively, with high specificity as compared to undamaged DNA (K_d = 1108 nM) (55,56). Using an incision assay, with 8-oxoG and THF as substrates for OGG1 and APE1 specifically, we were able to show that UV-DDB increased the incision activity of both OGG1 and APE1 by 3- and 8-fold, respectively. We then utilized single-molecule fluorescence microscopy of quantum dot (Qdot) labeled proteins and a unique DNA tightrope optical platform to elucidate that UV-DDB increases the turnover and thus the incision activity of both OGG1 and APE1. In this assay DNA containing an abasic site every 2 kb was suspended from poly-Lysine coated 5 micron beads (34) and Qdot labeled OGG1 or APE1 binding to DNA was followed over time in the absence and presence of UV-DDB. We demonstrated that UV-DDB helped dissociate these proteins from the DNA in a concentration dependent manner. Furthermore, by orthogonally labeling UV-DDB and OGG1 or APE1 with different colored Qdots, we were able to show UV-DDB can form transient complexes with OGG1 and APE1 to facilitate their removal from the lesion site. Using an *in vitro* BER reaction, the activity of pol β was measured though the incorporation of radiolabeled dCTP into the gap created by the dual action of APE1 incision and pol β dRPase activity. This assay revealed that addition of UV-DDB increased BER product formation by 30-fold. Additionally, we were able to show that the newly incorporated dCTP could be ligated into full length product, indicating that UV-DDB does not have an inhibitory effect on downstream steps in BER. Lastly, we used a highly innovative chemoptogenetic system to generate site specific 8-oxoG lesions at telomeres (Figure 5). This system consists of a fluorogen activating peptide (FAP) that can bind to a malachite green dye (MG-2I) with high affinity and be excited at a far-red light wavelength (660 nm). When excited, the FAP-MG-2I combination generates singlet oxygen, which is highly reactive and short-lived and can oxidize nearby macromolecules including proteins and DNA. When fused to a telomere binding protein (TRF1), the FAP-MG-2I plus light system generates targeted singlet oxygen and oxidizes telomeric DNA to form almost exclusively 8-oxoG lesions. Using this FAP-TRF1 targeting system, we found that UV-DDB was recruited to telomeric 8-oxoG lesions prior to OGG1, indicating a direct role for UV-DDB as an early damage sensor in chromatin, acting before the initiation of BER (Figure 5).

Two independent studies have highlighted the role of the DDB2 subunit in chromatin decompaction (53,54) and may give insight into how DDB2 could help facilitate BER. In these studies, the authors used cell lines containing heterochromatic lac operator (lacO) regions and found that the lacO array size significantly increased when bound with lac repressor (lacR) tethered to DDB2 (DDB2-LacR) as compared to being bound with lacR alone. The increase in lacO array size is consistent with significant chromatin unfolding. They further showed a similar change in chromatin state triggered by DDB2 at sites of UV damage. Additionally, recruitment of DDB2 was independent of its E3 ligase activity and ATP-driven chromatin remodeling. Based on these data and the inefficiency of OGG1 to act on lesions embedded in nucleosomes (98), we postulate UV-DDB facilitates the recognition of 8-oxoG by BER proteins by first modifying the chromatin architecture at sites of damage, particularly in inaccessible heterochromatic regions, and then stimulates the down-stream processing of the lesion. Future structural and real-time imaging studies should focus on the mechanism of 8-oxoG recognition by DDB2 in reconstituted nucleosomes and in mammalian cells. Future experiments also need to address whether the DDB1-Cul4A E3 ligase is involved in 8-oxoG recognition.

CONCLUSION AND OUTLOOK

Here we have illustrated the cooperativity, and in the case of Sp and Gh lesions, antagonism, between NER and BER proteins during oxidative DNA damage repair, as well as instances where the oxidative lesions are repaired by NER proteins directly. The structure of the oxidative lesion influences the role of NER proteins in the oxidative damage repair response. A comprehensive summary of the involvement of UV-DDB, XPC, CSA, CSB and XPG in the removal of oxidative lesions, specifically 8-oxoG, is presented in Figure 6. Our lab was recently able to demonstrate early recruitment of UV-DDB to sites of 8-oxoG damage, preceding OGG1 recruitment (56), strengthening the argument for interaction between NER and BER pathways. There are 11 mammalian glycosylases which each recognize a range of lesions induced by oxidative damage (6). To this end, current work in our lab is focused on understanding the role of UV-DDB in stimulation of the other mammalian glycosylases beyond OGG1. We have preliminary data suggesting MUTYH activity is stimulated by UV-DDB (56). Additionally, we are examining the role of UV-DDB in the recruitment of downstream NER or BER proteins at oxidative lesion sites to elucidate a role for UV-DDB in facilitating the repair process. The evidence of interplay between the two repair pathways could suggest direct interactions between BER and NER proteins, both on and off DNA, which needs further study by yeast-2-hybrid screens, co-IP and/or biochemical/biophysical methods, such as single-molecule *in vitro* techniques with a purified protein system or cell extracts to examine protein-protein and protein–DNA interactions at a defined lesion site (99). Moreover, these techniques can be used to examine, in real-time, how proteins act in unison to faithfully process DNA lesions. With the advancement of single-particle tracking in living cells and *in situ* labeling of proteins (39–41), and introduction of site-specific lesions (56), we are currently developing methods to monitor the behavior of DNA repair proteins at damage sites in different chromatin states in real time.

Most glycosylases cannot efficiently process lesions embedded in a nucleosome (42–46,87–90). Therefore, additional studies need to be conducted to further understand the molecular mechanisms of lesion recognition, specifically in the context of nucleosomes, chromatin remodeling, and glycosylase activity. Work from the Thoma laboratory, strengthened the argument for a role for UV-DDB in nucleosome remodeling by demonstrating the ability of UV-DDB to modify the register of the DNA on the nucleosome, increasing lesion accessibility (100). The direct role of DDB2 in chromatin decompaction also provides a mechanism of how DDB2 could help facilitate BER (53,54). Thus, DDB2 either acting alone or in conjunction with its asso-

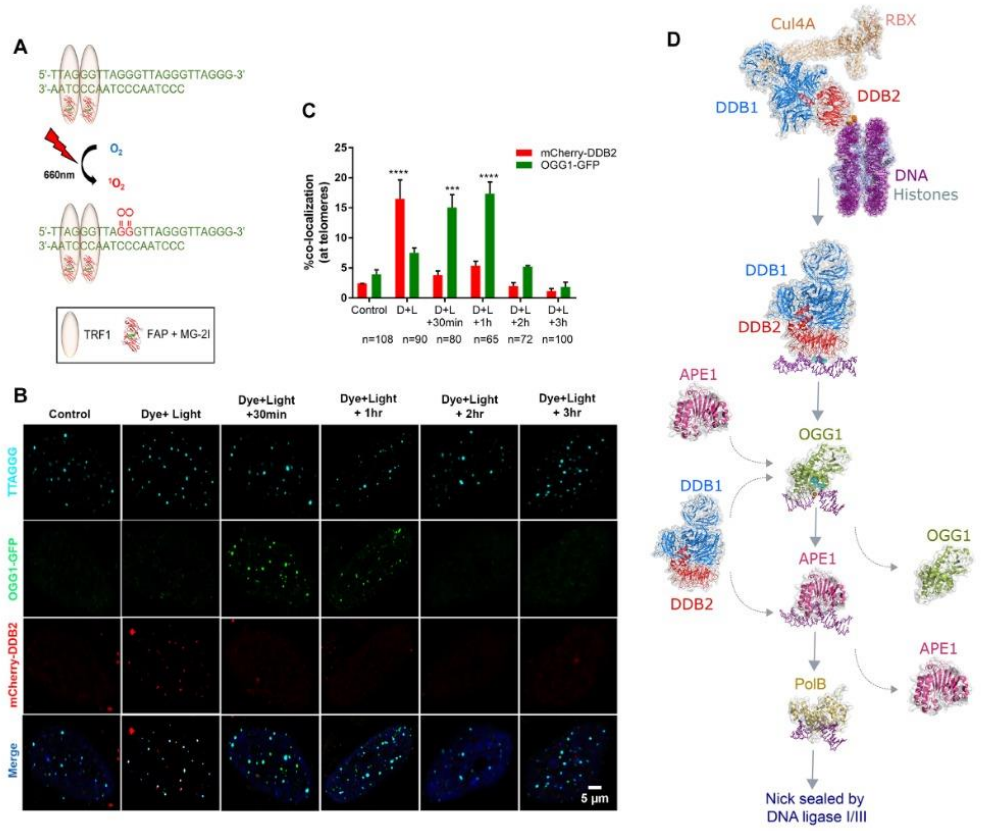


Figure 5. Role of UV-DDB in 8-oxoguanine (8-oxoG) repair. (**A**) A chemoptogenetic approach to introduce 8-oxoG at telomeres. Fluorogen-activating peptide (FAP) is fused to a telomere binding protein (TRF1). In the presence of a malachite green dye (MG2I), the FAP-TRF1-MG2I combination is excitable at far red wavelength (660nm) and generates singlet oxygen. Singlet oxygen reacts with telomeric DNA to form 8-oxoG lesions. (**B**) Immunofluorescence images of mCherry-DDB2 (red) and OGG1-GFP (green) recruitment to and departure from 8-oxoG lesions at telomeres (blue). (**C**) Quantitative analysis of immunofluorescence images in (B). (**D**) Working model of the potential role of UV-DDB in BER of 8-oxoG. Figure adapted from Jang *et al.* (56) with permission. Model of human UV-DDB-CUL4A-RBX bound to a 6–4 photoproduct in the context of a nucleosome, built from PDB codes: 4A0K and 6R8Y (52,123). Human UV-DDB bound to a THF lesion, PDB: 4E54 (124). Human OGG1 bound to 8-oxoG, PDB: 1EBM (125). Human APE1 bound to a 3′ deoxyribose phosphate moiety, PDB: 5DFF (126). DNA polymerase beta bound to gapped and nicked DNA, PDB: 1BPX (127)

ciated E3 ligase activity as part of the DDB1-Cul4A-RBX complex to direct the BER of oxidative damage. Furthermore, it is possible UV-DDB is working cooperatively with other chromatin remodelers, such as PARP1 to increase lesion accessibility (101). As we and other groups have demonstrated, UV-DDB has a high affinity for undamaged DNA, and perhaps the binding of UV-DDB to undamaged DNA alters the chromatin structure and changes gene expression, leading to a modification in DNA repair. Moreover, it would be of note to determine if the rates of 8-oxoG repair are similar throughout the genome. Several groups are currently examining this question and it would appear that the human genome contains hotspots for 8-oxoG formation and possibly repair (102–109). Studying oxidative DNA damage repair *in vivo* has been challenging due to the lack of tools capable of introducing oxidative lesions at defined regions in the nucleus. Several groups including our own have developed tools to overcome this challenge (38,110,111), which can be used to understand differences in repair kinetics in euchromatin and heterochromatin. Perhaps the chromatin state may help dictate whether NER or BER proteins are necessary and sufficient to remove 8-oxoG (112). One hypothesis that needs to be tested is if 8-oxoG occurring in heterochromatin is refractory to BER and needs the additional factors of UV-DDB and XPC to help facilitate chromatin opening. Harnessing our new innovative FAP-dye plus light technology, through direct fusion of FAP to other DNA binding proteins will allow new

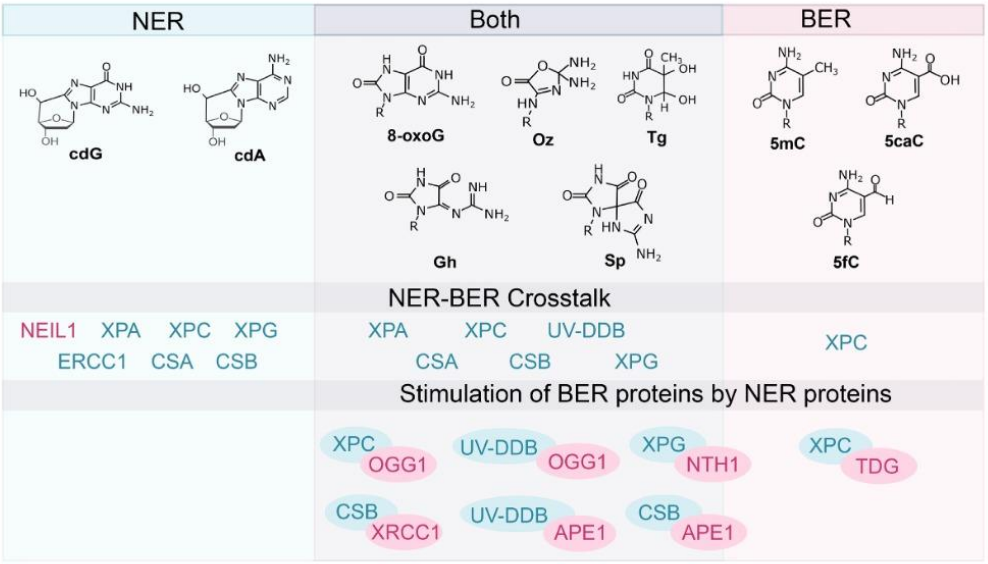


Figure 6. Oxidative DNA damage lesions repaired by BER, NER or both. NER proteins involved in the removal of these oxidative lesions are highlighted. Also depicted above is stimulation of BER proteins, especially glycosylases, by NER proteins. In this schematic, NER refers to both global and transcription-coupled repair. **cdG:** cyclo-deoxyguanosine; **cdA:** cyclo-deoxyadenosine; **8-oxoG:** 8-oxoguanine; **Oz:** Oxazolone; **Gh:** Guanidinohydantoin; **Sp:** Spiroiminodihydantoin; **Tg:** Thymine glycol; **5mC:** 5-methylcytosine; **5fC:** 5-formylcytosine; **5caC:** 5-carboxycytosine.

insights into the repair of 8-oxoG throughout the genome as we attempt to watch DNA repair proteins at high spatial and temporal resolution in living cells. Gaining an understanding of the dynamic process of oxidative DNA damage repair in the context of different chromatin states, should help aid in the development of improved therapies for diseases associated with defects in DNA repair proteins.

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Appendix F Crosstalk between NER and BER

Review of findings highlighting the crosstalk between nucleotide and base excision repair in DNA damage repair, originally published in *Genetics Molecular Biology*. Ref. [12]: Kumar, N., et al., *Cooperation and interplay between base and nucleotide excision repair pathways: From DNA lesions to proteins.* Genet Mol Biol, 2020. **43**(1 suppl. 1): p. e20190104.

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Review Article

Cooperation and interplay between base and nucleotide excision repair pathways: From DNA lesions to proteins

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Abstract

Base and nucleotide excision repair (BER and NER) pathways are normally associated with removal of specific types of DNA damage: small base modifications (such as those induced by DNA oxidation) and bulky DNA lesions (such as those induced by ultraviolet or chemical carcinogens), respectively. However, growing evidence indicates that this scenario is much more complex and these pathways exchange proteins and cooperate with each other in the repair of specific lesions. In this review, we highlight studies discussing the involvement of NER in the repair of DNA damage induced by oxidative stress, and BER participating in the removal of bulky adducts on DNA. Adding to this complexity, UVA light experiments revealed that oxidative stress also causes protein oxidation, directly affecting proteins involved in both NER and BER. This reduces the cell's ability to repair DNA damage with deleterious implications to the cells, such as mutagenesis and cell death, and to the organisms, such as cancer and aging. Finally, an interactome of NER and BER proteins is presented, showing the strong connection between these pathways, indicating that further investigation may reveal new functions shared by them, and their cooperation in maintaining genome stability.

Keywords: Base excision repair, nucleotide excision repair, DNA damage, protein oxidation, UVA light.

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Introduction

It has been suggested that every cell in our body suffers tens of thousands of lesions per day (Lindahl and Barnes, 2000; Tubbs and Nussenzweig, 2017), which if left unrepaired, may lead to mutations, genome instability and cancer. DNA damage can occur from exogenous sources like ultraviolet (UV) light, ionizing radiation (IR), and chemical exposure from pollutants in the air and water. Genomic damage can also be produced from endogenous processes such as replication errors or reactive oxygen species (ROS) from mitochondria or inflammation. Since DNA damage occurs continuously in all living systems, organisms have evolved efficient systems to ameliorate the harmful effects of environmental genotoxicants. Depending on the type of lesion formed in the DNA, six major repair pathways play a key role in maintaining genome stability, these include: direct reversal, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), recombination with two major sub-pathways: homologous recombination (HR) and non-homologous end joining (NHEJ), and interstrand cross-link (ICL) repair which combines features of several pathways including NER and recombination, and is controlled by a wide range of proteins. There are also several dedicated translesion DNA polymerases that allow the replication machinery to bypass specific lesions, at the expense of lowered fidelity (Goodman, 2002). Furthermore, key signaling pathways are controlled by transcription factors like p53 and DNA kinases including ATM, ATR and DNA-PK. Although these pathways have been described to work independently, there are indications that in fact these may interact, in a network for maintaining genome protection. This review

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emphasizes on the interplay between some of the proteins involved in either NER or BER. As for other DNA repair pathways, proteins that participate in NER and BER are also subject to injury, mainly by oxidation, an effect that has been little explored. However, this effect, initially described as an effect of UVA on cells, may interfere on the cells' ability to process DNA damage, adding a new level of complexity in the analysis of NER and BER interplay, as discussed below.

NER consists of a group of proteins that participate in the repair of lesions that cause significant helical distortion in the DNA structure, such as those induced by UV light, environmental mutagens like polycyclic aromatic hydrocarbons (PAHs) and certain chemotherapeutic agents like cisplatin (Wood, 1999; Scharer, 2013). UVC (254 nm) produces mainly cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP), while cisplatin forms intra- or interstrand Pt-adducts. Interestingly, longer wavelengths UVB (280-320 nm) and UVA (320-400 nm), which penetrate the earth's atmosphere, can produce a spectrum of lesions including photoproducts and oxidized bases, removed by NER and as well as BER. NER includes two sub-pathways: global genome NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER operates in the entire genome, including untranscribed regions and silent chromatin, while TC-NER recognizes and repairs bulky DNA lesions in the transcribed DNA strands of active genes only. In GG-NER, XPC-RAD23B acts as the initial damage recognition factor by recognizing destabilized DNA (Sugasawa *et al.*, 1998). UV-DDB is part of a Cul4-RBX1 ubiquitin ligase, which upon UV radiation ubiquitinates DDB2, histones and XPC. While ubiquitinated DDB2 is degraded, XPC shows an elevated DNA binding activity. During the damage verification step of GG-NER, the transcription factor TFIIH is recruited by XPC-RAD23B protein (Sugasawa *et al.*, 2005; Kapetanaki *et al.*, 2006; Wang *et al.*, 2006). TFIIH consists of 10 subunits, including the helicases XPB and XPD that are responsible for opening up the DNA around the lesion (Evans *et al.*, 1997). XPD binding to the lesion facilitates the recruitment of the pre-incision complex (XPA, RPA, XPG) (Wakasugi and Sancar, 1998; Volker *et al.*, 2001; Riedl *et al.*, 2003). Once the second endonuclease ERCC1-XPG is recruited, dual incision by XPG and XPF is initiated and the excision product is released along with TFIIH (Kemp *et al.*, 2012). DNA polymerase (δ/ ε) and ligase I then repair and ligate the gap (Shivji *et al.*, 1995). TC-NER, on the other hand, is triggered by stalled RNA polymerase at a DNA lesion during transcription, causing the Cockayne syndrome proteins (CSB and CSA), and other lesion accessory proteins (UVSSA, XAB2, and HMGN1) to be recruited at the lesion site. With the subsequent recruitment of TFIIH, TC-NER converges with the GG-NER at this step (Fousteri and Mullenders, 2008). Mutations in these NER proteins impair the ability to repair UV damage, causing autosomal recessive disorders including xeroderma pigmentosum (XP) (mutations in XPA-G, XPV) characterized by extreme sensitivity to sunlight and increased risk to skin cancer in exposed areas. About 20-30% of these patients also develop neurodegeneration. Also mutations in CSA and CSB, affecting only TC-NER, result in Cockayne syndrome (CS), with patients presenting developmental impairment and neurodegeneration, related to premature aging (Marteijn *et al.*, 2014; Menck and Munford, 2014; Karikkineth *et al.*, 2017).

BER is a dedicated pathway that removes a wide range of chemically altered bases (Svilar *et al.*, 2011; Wallace, 2014; Bauer *et al.*, 2015; Thapar and Demple, 2017; Whitaker *et al.*, 2017) (Figure 1). This type of damage typically results from spontaneous reactions in the cells (deamination, oxidation and methylation), metabolic by-products (ROS) and exogenous sources like alkylating agents (methyl methane sulfonate), ionization radiation (IR), X-rays, and pollutants, including cigarette smoke. Due to its redox potential, guanine is the most susceptible base to oxidation, forming mainly 8-oxoguanine (8-oxoG). This lesion is highly mutagenic and if not repaired, can pair with adenine, causing a G:C to T:A transversion. One of the earliest steps in the repair of base lesions is lesion recognition and removal by DNA glycosylase. In the case of 8-oxoG, this is mediated by a dual functional glycosylase, 8-oxoG glycosylase (OGG1) which first removes the damage through hydrolysis of the glycosidic bond, creating an apurinic/apyrimidinic (AP). This abasic site is acted on by a weak lyase activity of OGG1 causing cleavage 3' to the abasic site. OGG1 has higher affinity for the abasic site and is therefore product inhibited, and needs the action of an AP endonuclease (APE1), to help turn the enzyme over and cleave, leaving a 5' deoxyribose-phosphate moiety generating a one base pair gap (Hill *et al.*, 2001). Some models of BER show poly(ADP)-ribose polymerase (PARP1) activation during this transient nick and gap phase, which, through the production of poly(ADP)ribose, helps recruit the remaining DNA repair factors, XRCC1, a scaffold protein, DNA polymerase beta and DNA ligase I. During repair this gap is filled in by DNA polymerase beta, and ligated by DNA ligase I or III.

While both BER and NER pathways have been conventionally associated with specific substrates, growing evidence shows a significant cooperation between these two repair mechanisms, and has recently been reviewed (Melis *et al.*, 2013; Limpose *et al.*, 2017; Shafirovich and Geacintov, 2017). The relevance of this potential interaction includes the fact that NER deficient (XP and CS) patients may develop developmental and neurological symptoms, related to premature aging, that can be due to endogenous lesions, such as DNA damage induced by oxidation, which are normally considered substrates for BER. Thus, understanding BER and NER interplay may help us to better understand the causes for the symptoms of premature aging found in these patients and even for the normal aging process. In fact, certain types of DNA damaging agents can re-

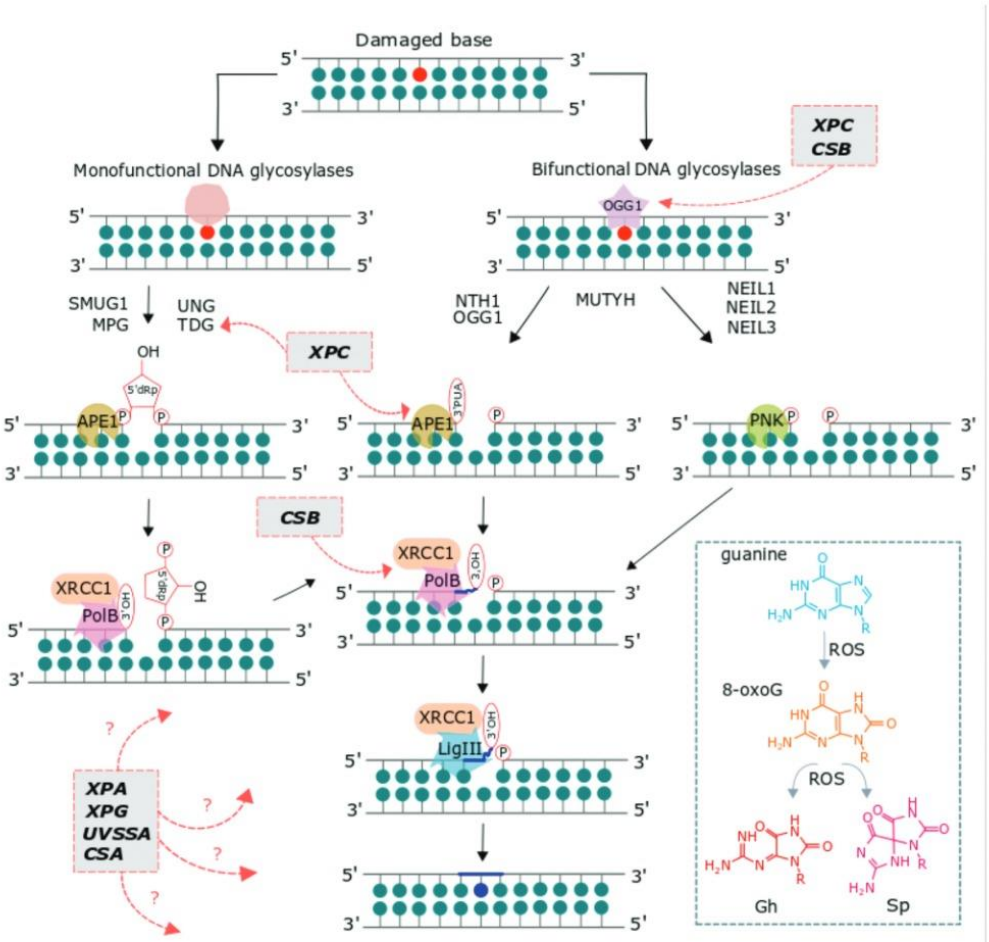

Figure 1 - Mono- and bi-functional DNA glycosylase-initiated short-patch base excision repair (BER) in mammalian cells. The process consists of these main steps: Excision of the base lesion, incision by an AP endonuclease, end processing, gap filling and ligation. Insert shows the common oxidative lesions repaired by BER: 8-oxoguanine (8-oxoG), guanidinohydantoin (Gh), spiroiminodihydantoin (Sp). Grey boxes (red dashed outline) indicate the involvement of NER (XPA, XPC, XPG, CSA, CSB and UVSSA) proteins in BER.

sult in a spectrum of DNA lesions that are handled by different DNA repair pathways, thus it is expected that proteins and enzymes from different pathways may cooperate to remove specific types of DNA damage. Moreover, these enzymes may also be injured by oxidation affecting, as shown by UVA irradiation, a component that also generates a mixture of DNA lesions that involves both NER and BER. This review discusses some of the more recent advances made in understanding the interplay between these two pathways by discussing specific DNA lesions and the proteins that recognize and remove them. The additional effects of oxidation of proteins related to these pathways by UVA exposure also interfere with the cells' capacity to process the different types of DNA lesions, with possible biological consequences such as carcinogenesis. This is also reviewed, focusing on the effects on NER and BER, and proteins that act on both pathways.

Oxidatively generated base damage recognized by NER pathway

Cooperative interactions in processing 8-oxoG

A number of base modifications are recognized by the BER pathway (Figure 1), but one of the most common and

well-studied lesions is 8-oxoguanine (8-oxoG). As described above, 8-oxoG is processed by OGG1 through BER, although recent studies show that other proteins and sub-pathways may partner in this process. One of the earliest experiments suggesting an involvement of NER proteins in the repair of oxidized base damage was an *in vitro* study from the Sancar laboratory. They showed that cell free extracts from human cell lines either lacking or containing mutated NER proteins (XP-A, XP-B, XP-C, XP-D, XP-F and XP-G) had markedly reduced ability to excise two major oxidized base damage, 8-oxoG and thymine glycol (TG), as part of an excision oligonucleotide consistent with NER (Reardon *et al.*, 1997). They went on to show complete NER system reconstituted with purified XPA, RPA, TFIIH (containing XPB and XPD), XPC-HHR23B, XPG, and ERCC1-XPF proteins, were necessary and sufficient to excise 8-oxoG or TG. While these studies indicated that NER proteins are capable of acting on two common oxidized bases, whether NER proteins had a direct role in BER by interacting with BER proteins or intermediates was uncertain. The authors suggested that perhaps NER is a relatively slow back-up system for BER.

D'Errico *et al.* (2006) provided the first direct evidence that XPC plays a role in the protection against oxidative stress. They demonstrated that keratinocytes and fibroblasts with mutations in XPC were extremely sensitive to potassium bromate and ionizing radiation. Using LC/MS and HPLC-ED, they were able to show the accumulation of 8,5'-cyclopurine 2'-deoxynucleosides and slow removal of 8-oxoG and 8-oxoA, respectively, in cells lacking XPC. Biochemical assays with purified proteins showed stimulation of DNA glycosylase OGG1 by XPC-HR23B and western blots showed that purified XPC-HR23B interacted directly with OGG1. Unlike XPC, at the concentrations surveyed, XPA was not capable of stimulating OGG1. This study indicates that XPC-HR23B facilitated recognition of 8-oxoG in an OGG1-dependent BER. It is interesting to note that XP-C patients, in addition to high skin cancer rates, also have a higher incidence of internal cancer development (Giglia *et al.*, 1998; Hollander *et al.*, 2005; Sarasin *et al.*, 2019). Thus, reduced kinetics of oxidatively generated DNA damage might be a major contributor to these internal cancers. Moreover, oxidatively generated base damage are also associated with increased risk of neurodegenerative diseases (Chen *et al.*, 2012; Liu *et al.*, 2017). While XP-A, XP-B, XP-D and XP-G patients may show neurodegeneration symptoms, XP-C patients show no signs of neurological defects. Thus, it is possible that XPC might be acting as a cofactor in the repair process, therefore its loss alone does not display major effects. In a separate study by Kassam and Rainbow (2007), methylene blue plus visible light (photoactivated MB, which generates singlet oxygen) was used to produce 8-oxoG in an adenovirus-encoded β-galactosidase (β-gal) reporter gene, and a host cell reactivation (HCR) assay was used to demonstrate that human cells deficient in XPC showed lower HCR as compared to WT cells, supporting a role for XPC in the processing of 8-oxoG (Kassam and Rainbow, 2007). Similarly, XP-A and XP-C NER deficient cells were found to be more sensitive to photoactivated MB, compared to NER proficient cells (Berra *et al.*, 2013). Problems dealing with the oxidized base damage, in XP-A and XP-C cells, were confirmed with observations of cell cycle delay (increased G2/M arrest) and genotoxic stress (H2AX phosphorylation). These results confirm NER proteins participate in the processing of oxidatively generated base damage, although which type of lesion (including 8-oxoG) is involved was not clear.

In order to better understand the potential roles of XPA and XPC in the removal of oxidized bases, Parlanti *et al.* (2012) went on to study the rates of 8-oxoG removal, as measured by HPLC–ED, in mouse embryo fibroblasts (MEFs) derived from NER ($Csb^{m/m}$, $Csa^{-/-}$ $Xpa^{-/-}$ $Xpc^{-/-}$ and combinations of these) and/or $Ogg1^{-/-}$ deficient mouse mutants following treatment with the oxidizing agent, potassium bromate. While $Ogg1^{-/-}$ deficient mutant cells displayed a dramatic deficiency in the rate of 8-oxoG removal, NER deficient mutants ($Csb^{m/m}$, $Csa^{-/-}$ $Xpa^{-/-}$ $Xpc^{-/-}$) also displayed reduced rates of removal as compared to WT MEFs. Furthermore, $Csb^{-/-}$ $Xpa^{-/-}$ and $Csb^{-/-}$ $Xpc^{-/-}$ double mutants were more deficient in repair as compared to the single mutants, and very similar to the deficiency observed in $Ogg1^{-/-}$ MEFs. On the other hand, $Xpc^{-/-}$ $Xpa^{-/-}$ double mutant did not show slower repair kinetics as compared to the single mutant MEFs, suggesting that XPC and XPA function through the same pathway, while CSB is OGG1-dependent, but XPA/XPC independent. These mouse experiments were confirmed in human XP-A primary fibroblasts that were more sensitive to potassium bromate as compared to WT fibroblasts. Furthermore, SV40-transformed XP-A deficient cell line (XP12SV40), in which OGG1 was knocked down with siRNA, showed slower 8-oxoG repair kinetics than either the XP-A cells alone or when XPC was knocked down. Whether this enhanced repair of 8-oxoG through the action of XPA, XPC, and CSB is mediated through canonical BER is unclear. Why XPA did not stimulate OGG1 activity in their previous study, but a deficiency in XPA showed a slower rate of 8-oxoG remains to be reconciled. Also, the involvement of these proteins could vary in the context of chromatin accessibility. Finally, it is interesting to note that the $Xpa^{-/-}$/$Xpc^{-/-}$ and $Csb^{m/m}$/$Ogg1^{-/-}$ double mutant mice are viable and do not show evidence for neurodegeneration (Friedberg and Meira, 2006; Laposa *et al.*, 2007).

XP-G deficient cells were also found to be sensitive to the treatment with photoactivated MB, indicating that XPG protein, and thus NER, participate in the processing of oxidized base damage (Soltys *et al.*, 2013). This was observed for cells from a severely affected patient, with neurological problems, carrying an *XPG* mutation that completely abrogates the protein. The increased sensitivity was also confirmed by HCR of plasmids treated with photoactivated MB. Interestingly, two different *XPG* missense

alleles, from patients with no neurological symptoms (but with XP typical increased frequency of skin tumors), showed sensitivity to UV-light induced DNA damage, but not to oxidized base damage induced by singlet oxygen. These results indicate that XPG protein might participate on the removal of UV-induced lesions by NER, with an independent function for oxidatively generated base damage, and defects on this latter function is in fact relevant for the induction of neurological symptoms in XP-G patients.

Cellular imaging of 8-oxoG processing involving CSB and XPC

Menoni *et al.* (2012) used a novel imaging tool to study the role of XPC and CSB in the repair of oxidized base damage in living cell. By using a photosensitizer Ro 19-8022 and 405 nm laser light, they were able to generate localized oxidized base damage in specific regions of the nucleus. XPC-GFP and CSB-GFP both were seen to be recruited to the sites of damage. CSB appeared to be recruited faster than XPC, possibly due to different intrinsic mobility or chromatin binding properties. Indeed, they reported that CSB was prominently recruited in the nucleolus (possibly due to high transcription activity) and XPC accumulated more densely in the heterochromatic region, consistent with their roles in TC-NER and GG-NER of UV-induced photoproducts, respectively. Interestingly, they reported, but did not show the data, that neither XPB nor XPA was recruited to the damage site even after 5-10 minutes of damage induction. These data suggesting that CSB and XPC recruitment was independent of subsequent steps in NER is in contrast to the work by Parlanti *et al.* (2012) who showed that both XPA and XPC might facilitate 8-oxoG removal.

In a more recent study by Vermeulen's group, the role of CSB in 8-oxoG repair was further elaborated (Menoni *et al.*, 2018). Using the live-cell imaging approach described above, it was shown that OGG1 recruitment to the damage site was independent of CSB, but the recruitment of the BER scaffolding protein XRCC1 was stimulated by CSB in a transcription-dependent manner. It is possible that as a chromatin remodeler, CSB helps XRCC1 loading under certain circumstances, perhaps in transcribed genes or at specific genomic regions that are not accessible to the downstream BER proteins.

Comet-FISH assay reveals an involvement of XPA, CSB, and UVSSA in TCR of 8-oxoG

As noted above, the role of XPA in the processing of 8-oxoG adducts has been controversial, and contrasting studies have been published. In an elegant tour-de-force study, Guo *et al.* (2013) combined a single-cell electrophoresis (Comet assay) with fluorescence *in situ* hybridization (FISH) and established the involvement of XPA and CSB preferentially in transcription-coupled 8-oxoG removal. For these experiments, 5'- and 3'-ends of the ATM gene were labelled with different fluorescent probes. The increase in the distance between the probes after damage was an indication of single strand breaks. The repair rates of transcribed and non-transcribed strands in CS-B and XP-A cells were similar, indicating that they played a role in TCR of 8-oxoG. They also showed that elongating RNAP II and UVSSA were necessary for this process consistent with TCR. The authors speculated that after initial recognition and incision by OGG1 and APE1, the single stranded DNA formed causes a block to transcription, recruiting the TCR proteins to continue repair. This model is consistent with the work by Vermeulen's group cited above. XPC, since it is involved in GG-NER, was not investigated in this study.

XPC also stimulates a thymine specific DNA glycosylase

Spontaneous deamination of C or 5-methylC creates dU-G and T-G mismatches which are processed by uracil DNA glycosylase (UDG) family and thymine DNA glycosylase (TDG), respectively. XPC-HR23B was shown to stimulate TDG activity in an in vitro nicking assay (Shimizu *et al.*, 2003). While XPC, itself, did not have any effect on nicking the G/T mismatch oligonucleotide, it stimulated TDG activity in a dose dependent manner, probably promoting enzymatic turnover of TDG. XPC also stimulates OGG1 binding to damaged DNA, and a weak interaction between the proteins was obtained from far western analysis (Parlanti *et al.*, 2012). Finally, Melo *et al.* (2016) showed a correlation between XPC deficiency and OGG1/ APE1 expression levels, and a physical interaction between XPC and APE1 using co-immunoprecipitation. These studies are summarized in Tables 1 and 2.

Oxidized guanine lesions are excised more efficiently by competing BER than NER pathways

The base damage, 8-oxoG is susceptible to further oxidation, leading to the formation of spiroiminodihydantoin

Table 1 - Oxidative lesions recognized by NER factors.

Lesions	Protein involved	References
8-oxoG and TG*	NER proteins	Reardon *et al.* (1997)
8-oxoG	XPC-CSB (TC-BER)	Menoni *et al.* (2012)
8-oxoG	CSB (TC-BER)	Menoni *et al.* (2018)
8-oxoG	XPA, CSB and UVSSA	Guo *et al.* (2013)
8-oxoG	XPC/XPA	Parlanti *et al.* (2012)
Guanine lesions	NER proteins	Shafirovich *et al.* (2019)

*8-oxoG and thymine glycol

Table 2 - Protein interactions between BER and NER.

Protein-protein interaction	References
XPC-HR23B and TDG	Shimizu *et al.* (2003)
XPC and OGG1	D'Errico *et al.* (2006)
XPC and APE1/OGG1	Melo *et al.* (2016)

(Sp) and 5-guanidinohydantoin (Gh), which are recognized by the DNA glycosylase NEIL1 (Luo *et al.*, 2000; Niles *et al.*, 2001; Hailer *et al.*, 2005; Krishnamurthy *et al.*, 2008; Zhao *et al.*, 2010).

Very recently, Shafirovich *et al.* (2019) examined the excision of these lesions in intact human cells and the relative contribution of BER and NER in the processing of these lesions. In this study, an internally labelled hairpin substrate containing these lesions were transfected into HeLa cells. DNA was isolated at different time points and run on a PAGE gel. The BER activity was determined by the presence of a 65nt incision product, while the presence of a 24-30nt excision product indicated NER activity. The hairpins with both Gh and Sp lesions exhibited BER, as well as NER activity, suggesting a competition between these two pathways in repair. Addition of unlabeled hairpin with a known BER substrate 5-OHU caused significant reduction in the BER product, but an increase in the NER product. This suggests that the participation of these two pathways depends on the local concentration of the recognition factors that recognize and bind to the same lesions in a competitive manner.

Bulky DNA lesions recognized by BER pathway

BER protects cells against Pt-adducts

Platinum-based drugs are most widely used for the treatment of cancer (Kelland, 2007). The three approved drugs for treatment are: cisplatin, oxaliplatin and carboplatin. These drugs form platinum adducts by either covalently linking two nucleotide residues on the same DNA strand (intrastrand crosslink) or from opposite strands (interstrand crosslink- ICL). Left unrepaired, ICLs cause cytotoxicity by blocking transcription and replication (Huang and Li, 2013). Although this damage is largely repaired by NER, Kim *et al.* (2015) showed that APE1 protective role against damage caused by ICLs. Using a slot-blot assay and an antibody against 1,2-Pt-(GpG) DNA adducts, they demonstrated that reducing the expression of APE1 by siRNA inhibited the repair of cisplatin adducts. This inhibition was restored by adding back APE1 with repair activity, but not the redox signaling function. Furthermore, altering APE1 expression affected the expression levels of two NER proteins, RPA and XPA, suggesting an interaction between these two pathways. However, it should be pointed out that cisplatin exposure is also known to induce ROS production (Marullo *et al.*, 2013), therefore explaining the involvement of BER proteins in the repair process, and APE1 expression may also help to protect repair proteins from oxidation (see below). Therefore, more studies are required to unravel the exact role and interplay between BER and NER proteins in the repair process of cisplatin induced DNA damage.

Slyskova *et al.* (2018) recently used a CRISPR/Cas9 screen to determine which proteins and pathways are involved in the repair of oxaliplatin and cisplatin induced adducts. These drugs covalently bind to DNA and form crosslinks, mainly Pt-GpG (60–65%) and Pt-ApG (25–30%), along with monoadducts (2%). They showed that the proteins involved in TC-NER and/or BER were essential in protecting cells against the cytotoxicity of oxaliplatin and cisplatin. Using fluorescence recovery after photobleaching (FRAP), they showed evidence for the recruitment of CSA, CSB, and XRCC1 at localized ICLs, generated by 8-methoxypsoralen+UVA, in living cells. Additionally, the recruitment of these proteins was found to be transcription dependent, as the recruitment was suppressed by blocking RNAP elongation using flavopiridol. Finally, by knocking down OGG1 and XPA, they were able to determine that the recruitment of XRCC1 was BER dependent, but NER independent. Measurement of H_2DCFDA fluorescence was used to validate that platinum drugs generate oxidatively generated damage, necessitating the presence of BER proteins. The oxidized base damage could cause an accumulation of BER intermediates like abasic sites and single-strand breaks that contribute to the transcription block, along with the ICLs. These data might help explain the presence of TC-NER proteins, CSA and CSB, but not GG-NER proteins, XPC or DDB2. Apart from acting on the adducts directly, it is possible that CSB is recruited to these oxidized base damage in a transcription-dependent manner, to recruit XRCC1, as described previously (Menoni *et al.*, 2012, 2018).

DNA glycosylase NEIL1 binds and excises psoralen-induced monoadducts and interstrand crosslinks

Using a combination of excision assays, cell survival assays, and *in vitro* BER assay, Couve-Privat *et al.* (2007) showed that NEIL1 and APE1 deficient cells are sensitive to 8MOP+UVA. There was no further increase in sensitivity when both these proteins were depleted together, suggesting that they function via the same pathway. Additionally, these proteins are able to excise psoralen monoadducts, but not ICLs, in duplex DNA (Couve-Privat *et al.*, 2007). Specifically, NEIL1 cleaves the monoadduct generating 3'-phosphate termini, that is removed by APE1. Finally, by reconstituting BER *in vitro,* they show that NEIL1 and APE1 can repair psoralen monoadducts in a pol-β dependent manner. To further elucidate the role of NEIL1 in the repair of psoralen ICLs, the group used a three-stranded DNA structure with an unhooked ICL, which is a physiological representative of an ICL lesion, after being acted upon by endonucleases. They show that NEIL1 could excise this substrate and catalyze an *in vitro* BER reaction, indicating multiple modes of action of NEIL1 in psoralen adduct repair (Couve *et al.*, 2009). Another study by the same group looked into the role of NEIL1 and NEIL3 in the repair of ICL repair intermediates like the three- and four-stranded DNA structures, generated via FANCM mediated replication fork bypass and demonstrated that both glycosylases participate in the repair (Martin *et al.*, 2017). In a contrasting study by McNeill *et al.*

(2013) it was shown that NEIL1 was recruited specifically to ICLs but not monoadducts. In living cells, when treated with trioxsalen, NEIL1 recruitment was not affected in the presence of an antioxidant, N-acetyl-L-cysteine (NAC), indicating different mechanisms of recruitment to oxidative damage and ICLs respectively. Interestingly, NEIL1 was recruited and dispersed within 8 minutes post irradiation, while XPC was seen until 60 minutes after damage induction, suggesting that the glycosylase was not being recruited as part of the XPC complex. Moreover, NEIL1 deficient cells were resistant to psoralen + UVA damage, and had a faster rate of psoralen removal, hinting on a negative role of NEIL1 in the repair of ICLs. The authors theorize that the contradicting results could be a result of variation in techniques or using trioxsalen versus 8-methoxypsoralen for damage induction. While 8-MOP produces about 20% monoadducts, trioxsalen generates < 2%. Future studies are required to understand these discrepancies in more detail. These studies are summarized in Table 3.

Repair proteins as target of UVA light-induced oxidative stress:

UVA light forms a mixture of photoproducts and oxidatively generated base damage:

Ultraviolet (UV) light is a well-known DNA damaging agent, and most of the organisms on this planet are exposed to it via sunlight, with important pathophysiological consequences such as skin carcinogenesis and photoaging. While the ozone layer shields the surface of the earth from harmful UVC (100-280 nm) light, more than 90% of UVB (280-320 nm) and UVA (320-400 nm) reach the earth's surface. UVA light plays an important role in sunlight-induced DNA damage, as it corresponds to 95% of sunlight UV component, and penetrates deeper in the human skin because of its longer wavelength.

UVA light induces a mixture of different types of DNA lesions, including photoproducts and oxidized bases, which are then repaired by both NER and BER, providing an interesting model to investigate the DNA repair capacity of these pathways (Ravanat *et al.*, 2001; Sage *et al.*, 2012; Schuch *et al.*, 2017). Pyrimidine dimers, such as CPDs and 6-4 PPs, are formed through direct photon absorption by DNA bases (Schuch *et al.*, 2009; Cortat *et al.*, 2013). Evidence for the role of UVA light in causing direct DNA damage was demonstrated as early as in 1973, by observing the formation of CPDs in the genome of *Escherichia coli (*Tyrrell*,* 1973*)*. More recently, the biological relevance of pyrimidine dimers (CPDs) induced by UVA was observed in Chinese hamster cells and in human skin (Douki *et al.*, 2003; Mouret *et al.*, 2006). Although 6-4PPs ($t1_{/2}$ ~2-4 hrs) are removed by NER at a significantly faster rate compared to CPDs ($t_{1/2}$ ~ 24 hrs) , they were also detected upon UVA light exposure in DNA repair deficient cell models (Schuch *et al.*, 2009; Cortat *et al.*, 2013). It is important to mention that 6-4 PP undergo Dewar isomerization, to form a new photoproduct, which is repaired by NER. In fact, upon exposure to sunlight, UVA radiation converts the 6-4 PP into Dewar PP (Clingen *et al.*, 1995; Perdiz *et al.*, 2000; Douki *et al.*, 2003; Douki and Sage, 2016).

UVA light also induces DNA damage by mechanisms that involve oxidative stress, generated as a result of irradiation. Intracellular ROS can be generated through photosensitization reactions caused by endogenous chromophores absorbing UVA-light, including DNA, urocanic acid, pophyrins, flavins, melanin and their precursors and metabolites (Emri *et al.*, 2018). These photosensitized molecules, normally in the triplet state, can either react directly with DNA (type I reaction) or transfer their energy to molecular oxygen, to form $^1O_2$ and subsequently generate ROS (type II reaction). In both cases, the result may be DNA oxidation (Di Mascio *et al.*, 1990; Halliwell and Aruoma, 1991; Evans *et al.*, 2004). ROS may also be generated as a delayed response to irradiation, probably due to the activation of cellular enzymes, such as NADPH oxidases and cyclooxygenases (Valencia and Kochevar, 2008; Birch-Machin and Swalwell, 2010). UVA-induced ROS can generate a variety of modifications, including 8-oxoG, abasic sites, single and double strand breaks and crosslinks (Cadet *et al.*, 2005; Schuch *et al.*, 2017).

UVA induces both direct and indirect DNA damage, repaired by NER and BER, respectively. Mutational effects by UVA are typically due to lesions induced by direct DNA absorption (pyrimidine dimers). Most of the published studies have reported C > T changes at dipyrimidine sites (Robert *et al.*, 1996; Ikehata *et al.*, 2003; Agar *et al.*, 2004; Kappes *et al.*, 2006), which is similar to UVC and UVB induced mutagenesis (Brash *et al.*, 1987; Douki *et al.*, 2003; Kappes *et al.*, 2006; Herman *et al.*, 2014). Interestingly, this type of mutation has been detected in nonmelanoma (Giglia-Mari and Sarasin, 2003), as well as, melanoma skin cancers (Greenman *et al.*, 2007; Pleasance *et al.*, 2010). However, the participation of UVA light-induced oxidative stress in these mutagenic and damaging processes cannot be completely ruled out. Cells have a broad array of antioxidant mechanisms, which provide the initial defense to minimize the oxidation of proteins, DNA and other biomolecules. Human skin has elaborate enzymatic and non-enzymatic defenses against ROS, such as the superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH)/glutathione peroxidase systems. The transcription

Table 3 - Bulky lesions recognized by BER.

Lesions	Protein involved	References
Pt-adducts	APE1	Kim *et al.* (2015)
Pt-adducts	OGG1/XRCC1	Slyskova *et al.* (2018)
ICLs	NEIL1	McNeill *et al.* (2013)
		Couvé-Privat *et al.* (2007)
		Couvé *et al.* (2009)
		Martin *et al.* (2017)

factor, Nrf2 (NF-E2-related factor 2) coordinates the activation of several genes whose products participate in the cellular response to the oxidation of biomolecules. Further, studies have shown that Nrf2 plays a protective role in keratinocytes and fibroblasts against the damaging effects of UVA-induced DNA lesions (Hirota *et al.*, 2005; Tian *et al.*, 2011).

UVA light interferes with DNA repair as a consequence of protein oxidation

Apart from the antioxidant systems, DNA repair mechanisms act as a protection barrier against UVA-induced lesions. As commented earlier, NER generally repairs lesions that cause significant distortions in the DNA molecule, such as CPDs and 6-4 PPs, whereas BER repairs ROS-induced small base covalent modifications. Therefore, interplay between these two processes would be important to deal with the variety of damage induced after UVA-irradiation. Work on UVA has revealed that proteins and lipids are also affected by ROS, and, interestingly, proteins involved in DNA repair are highly sensitive to oxidation. Studies show that UVA-light in the presence of photosensitizers caused extensive protein oxidation, affecting DNA damage removal by NER (Peacock *et al.*, 2014), as well as BER (Gueranger *et al.*, 2014). Thus, protein oxidation may be a direct consequence of UVA irradiation increasing the mutation risk by sunlight (McAdam *et al.*, 2016). Confirming these observations, studies from our lab showed that protein oxidation by UVA irradiation also affects the ability of human cells to replicate their genetic material, probably due to translesion synthesis (TLS) and NER being affected in irradiated XP-V cells (Moreno *et al.*, 2019ab). Curiously, previous work reported that UVA-induced singlet oxygen leads to DNA replication arrest independently of cell cycle checkpoints activation, probably due to a transient decrease of dNTP pool, probably not related to the oxidation of DNA repair proteins (Graindorge *et al.*, 2015). This suggests that UVA light-induced oxidative stress has a greater contribution in impairing proteins that participate in DNA repair and replication pathways than in inducing direct damage to DNA. Interestingly, the use of antioxidants strongly protected the cells from the damaging effects of UVA-light, justifying the use of antioxidants in sunscreen creams. The hope is that the antioxidants would not only reduce cell killing effects of sunlight, but also reduce mutagenesis and skin cancer risk, by improving NER-mediated removal of the mutagenic photoproducts.

Various proteins linked to DNA repair are targets for oxidation by UVA light (Karran and Brem, 2016), but other genotoxic agents that induce oxidative stress have also been reported to promote inhibition of DNA repair. XPA and XPE proteins (from NER) were shown to be directly affected by the oxidative stress caused by arsenic (Grosskopf *et al.*, 2010; Zhou *et al.*, 2015). Arsenite also damages PARP1, causing inhibition of poly(ADP)-ribosylation and thereby, interfering with BER (Ding *et al.*, 2009). PCNA, a key replication and repair protein is also damaged by singlet oxygen generated from UVA activated photosensitizer generating an oxidative crosslink between two subunits, involving a histidine residue in the intersubunit domain (Montaner *et al.*, 2007). OGG1, a central glycosylase for 8-oxoG repair (BER) in human cells was inhibited by oxidative stress induced by cadmium (Bravard *et al.*, 2006) or by the inflammatory cytokine TNF-alpha (Morreall *et al.*, 2015). Moreover, OGG1 was inhibited by 6-thioguanine (6-TG) activated by UVA light (Gueranger *et al.*, 2014). Partial inactivation of MUTYH, Ku70 and Ku80 proteins due to 6-TG and UVA light was shown to also compromise BER and NHEJ repair activities (Gueranger *et al.*, 2014). UVA and photosensitizers also oxidized XRCC3 protein, impairing homologous recombination (Girard *et al.*, 2013). Most of these cases of protein oxidation have been related to the oxidation of cysteines, sensitizing the cells to DNA damage by affecting the DNA repair pathways. Of special interest is the oxidation of RPA in human cells caused by photosensitizers and UVA light (Gueranger *et al.*, 2014; Guven *et al.*, 2015). RPA is the main protein that stabilizes single strand DNA (ssDNA) and has central roles in DNA repair processes (such as NER and BER) and replication of undamaged and damaged templates (Cimprich and Cortez, 2008; Lukas *et al.*, 2011; Jones and Petermann, 2012) . Surprisingly, it has been demonstrated that RPA is the main limiting factor for NER, after UVA irradiation with photosensitizers. This was shown by measuring NER capacity *in vitro* with extracts from cells that were treated with 6-TG and UVA light, where supplementing or overexpression of RPA recovered NER activity (Gueranger *et al.*, 2014; Guven *et al.*, 2015). In fact, oxidation of RPA by UVA and photosensitizer seems responsible for a decrease in the cell's ability to remove CPD, 6-4PP and 8-oxoG, thus affecting badly NER and BER (Guven *et al.*, 2015).

RPA is also an important player in DNA damage responses (DDR), where it accumulates and stabilizes ssDNA, recruiting checkpoint and other DNA repair proteins to the damage site. RPA in ssDNA is also a signal to PCNA ubiquitination which is the main regulator of the TLS pathway (Ghosal and Chen, 2013). Therefore, oxidative stress not only impairs RPA protein, but it can also destabilize the signaling of pathways that are necessary for the removal of and/or tolerance to different types of DNA damage (Figure 2). Disruption of such important pathways that control DNA damage may be an aggravating factor for people with DNA repair deficiencies such as XP. As cells from these patients are more sensitive to DNA damage, their use has been proposed to better understand the effects of UVA-light in human cells (Schuch *et al.*, 2017). In fact, evidence that protein oxidation due to UVA-light may aggravate XP cells' phenotype has been obtained from NER and pol eta deficient cells (Cortat *et al.*, 2013; Moreno *et al.*, 2019a). Pol eta (XP-V) deficient cells are able to repair bulky DNA lesions such as CPDs induced by UVC light but

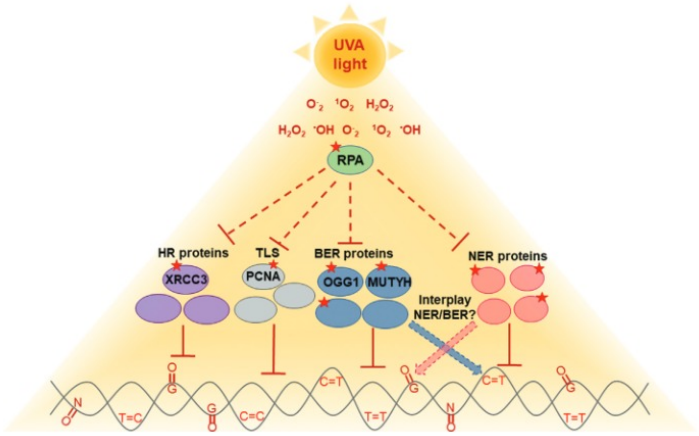

Figure 2 - UVA light induces mixture of photoproducts and oxidized base damage in the DNA, as well as, ROS production. As result of UVA-induced ROS production, protein oxidation has been gaining attention because it can also damage DNA repair proteins, which acts on both NER and BER. Thus, UVA irradiation causes decreased repair capacity of target lesions of both pathways, which may be the result of RPA oxidation, or the oxidation of other NER and BER proteins. RPA impairment may further compromise other DNA repair or tolerance pathways, such as homologous recombination (HR) and translesion synthesis (TLS). Red stars represent proteins known to be target of oxidative stress.

have impaired NER when these lesions are induced by UVA light, probably due to protein oxidation. The use of antioxidants protected UVA-irradiated cells, improved CPD removal, as well as the ability of these cells to replicate their damaged DNA (Moreno *et al.*, 2019a). Moreover, the lack of pol eta and other TLS proteins has been reported to impair NER due to the recruitment of RPA to TLS site (Auclair *et al.*, 2010; Tsaalbi-Shtylik *et al.*, 2014), and the limiting effect of this protein may be even stronger in conditions of oxidative stress. Finally, BER proteins have not been yet evaluated in this context, but as target of oxidation, this pathway may also be affected by UVA-light. Therefore, protein oxidation is not only an important cancer risk factor for XP patients, but also for the normal population.

NER and BER interactome analysis

To further understand the interplay between the NER and BER pathways, and scrutinize the underlying interaction between their associated proteins, we conducted a systems biology analysis. We listed the proteins that play major roles in both processes, including all their divisions (i.e., TC and GG-NER, and monofunctional and bifunctional DNA N-glycosylases for BER). By employing the metasearch engine STRING 11 (https://string-db.org/) (Szklarczyk *et al.*, 2017), we prospected a protein-protein interaction (PPI) network composed of 32 proteins related to NER and 23 associated to BER. The initial network created in STRING was used as input in the software Cytoscape 3.6.1 for manipulation (Shannon *et al.*, 2003). Additionally, aiming to identify the most topologically relevant nodes in the PPI network, we employed the Cytoscape plug-in CentiScaPe 2.2 (Scardoni *et al.*, 2009) for degree and betweenness centrality analysis. Degree calculates the number of interactions of each node, and nodes with above average degree values are called "hubs". Betweenness calculates the number of shortest paths that go through each node, and these nodes with above average scores are named "bottlenecks". Hence, the hub-bottlenecks (HB) nodes are the most topologically relevant nodes and retain critical regulatory roles within the cell, being classified as "bridges" between biological processes and key molecular modulators (Yu *et al.*, 2007; Pang *et al.*, 2016). Figure 3 portrays the crosstalk between NER and BER and Table S1, lists all interactions between the NER and BER processes from the network.

Clearly, the PPI network reveals a very high number of interactions (229) among the proteins of the two pathways. Nevertheless, the NER pathway appears with more intragroup connections than the BER process (432 and 73 connections, respectively). This maybe due to the fact that BER have many DNA damage recognizing proteins that act more independently one from the other.

Some proteins did not show any inter-pathway connection, been only associated with their own repair mechanisms, these proteins were: (i) NEIL1, NEIL2, MBD4, SMUG1 and PNKP for BER; but none for NER. These aspects should be taken lightly. The lack of intergroup interaction does not necessarily mean that they do not participate in other repair mechanisms, only that they are not the major inter-pathway integrators. For example, MBD4 is a multidomain protein with four different protein regions with a role in the apoptotic pathway, while TDG is related to epigenetic modulation of embryonic development (Sjolund *et al.*, 2013). The proper interpretation is that those

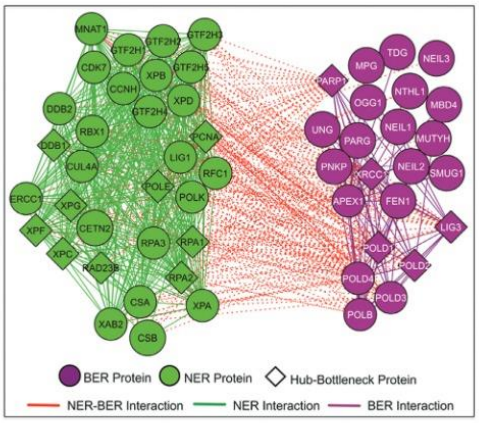

Figure 3 - PPI Network depicting the major players in both NER and BER. High connectivity between the two pathways can be observed in the network, which is composed of 55 proteins (32 from NER, 23 from BER) and 734 edges. The parameters used in the STRING software for the *Homo sapiens* organism, were: (i) medium confidence score of 0.4; (ii) "expression", "databases", "*neighborhood*" and "co-expression" interaction sources enabled; (iii) only queried proteins on the 1st shell; and (iv) no interactions on the 2nd shell. The centralities analyzed were node degree and betweenness, where 16 HB were identified.

proteins, when it comes to the interplay between NER and BER, are not the major bridges between the two pathways.

With the exception of the DNA polymerase δ subunits, PARP1 has one of the highest number of connections in the NER group, interacting with almost all proteins and being one of the top nodes in terms of intergroup connectivity (29 out of 39 connections), besides being an important HB. The HBs present in the network were DDB1, XPC, XPF, XPG, RAD23B, RPA1-2, POLE and PCNA for NER, and POLD1-2, LIG3, XRCC1 and PARP1 for BER. It is expected that proteins such as POLE, POLD1-2, LIG3 and PCNA appear as HB, due to their broad and pivotal role in genome replication and maintenance. Additionally, the appearance of RPA and DDB1 as HB is also not surprising, taking into consideration that both proteins are widely associated to different DNA repair pathways, cell cycle, replication, among others (Fanning *et al.*, 2006; Zou *et al.*, 2006; Iovine *et al.*, 2011). Most of the other HB proteins that show strong intergroup connections are discussed above for their participation on both BER and NER, but it is interesting to mention the high level of connections (on both pathways) of LIG3 (22 intergroup interactions out of a total of 33 connections) an XRCC1 (23 intergroup out of 35 connections).

Conclusions

DNA repair pathways have been classified as they were discovered, and, in general, they are considered to perform independent and different functions. This is the case for NER and BER, which are normally related to the removal of bulky or modified base lesions, respectively. On the other hand, agents that cause DNA damage, typically generate different types of lesions, that may require different DNA repair pathways to maintain genome stability. Although many efforts have been made to understand the interplay between NER and BER proteins, we know relatively little of these connections. We presented current data on the action of NER proteins on oxidatively damaged DNA, and the role of BER proteins in the protection to agents that form bulky DNA lesions. There is no consensus on the participation of specific proteins in this interplay. The oxidation of repair proteins, mainly RPA, promotes impairment of both NER and BER, adding a new level of complexity to this intricate question. By evaluating the known interactions among NER and BER proteins, the interactome, presented in Figure 3 tells us that there are many connections that are still poorly understood and how they affect these two pathways remains to be elucidated. Understanding this dynamic interplay at specific types of lesions, might prove important in unraveling the underlying mechanisms of carcinogenesis, aging, and neurodegeneration.

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Conflict of interests

The authors declare that they have no conflict of interest.

Author contributions

NK and NCM wrote the first draft of the manuscript and had equal contribution to this review, BCF analyzed the interactome data and wrote the related item, BVH and CFMM helped to define themanuscript items and edited to entire manuscript. Both also supervised the study and contributed funding resources. All authors read and approved the final version.

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Supplementary material

The following online material is available for this article:
Table S1 – All BER X NER inteactions found in the interactome.

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Appendix G Role of UV-DDB in DNA damage recognition

Review discussing UV-DDB's role in maintaining genome stability, originally published in *DNA repair*. Ref. [110]: Beecher, M., et al., Expanding molecular roles of UV-DDB: Shining light on genome stability and cancer. DNA Repair, 2020: p. 102860.

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Expanding molecular roles of UV-DDB: Shining light on genome stability and cancer

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ABSTRACT

UV-damaged DNA binding protein (UV-DDB) is a heterodimeric complex, composed of DDB1 and DDB2, and is involved in global genome nucleotide excision repair. Mutations in *DDB2* are associated with xeroderma pigmentosum complementation group E. UV-DDB forms a ubiquitin E3 ligase complex with cullin-4A and RBX that helps to relax chromatin around UV-induced photoproducts through the ubiquitination of histone H2A. After providing a brief historical perspective on UV-DDB, we review our current knowledge of the structure and function of this intriguing repair protein. Finally, this article discusses emerging data suggesting that UV-DDB may have other non-canonical roles in base excision repair and the etiology of cancer.

1. Introduction

Life has evolved a series of pathways to remove specific types of DNA damage. Nucleotide excision repair (NER) is dedicated to the removal of a wide variety of helix distorting lesions, including: ultraviolet (UV) photolesions, cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP); bulky adducts formed by chemical carcinogens, such as polycyclic aromatic hydrocarbons; and certain DNA lesions formed from chemotherapeutic agents, like cisplatin. NER consists of two sub-pathways: global genome NER (GG-NER) and transcription-coupled NER (TC-NER). GG-NER detects and repairs DNA damage occurring in the entire genome, while TC-NER recognizes and repairs lesions in the transcribed DNA strand of active genes, reviewed in [1] and [2], respectively.

GG-NER of UV-induced photoproducts is initiated by the UV-damaged DNA binding protein (UV-DDB), which is a heterodimeric protein consisting of DDB1 (127 kDa) and DDB2 (48 kDa). UV-DDB is part of a larger complex containing cullin-4A/4B and RBX1 that possess E3 ligase activity, and associates with chromatin in response to UV radiation [3,4], see Fig. 1. UV-DDB ubiquitinates histones to destabilize the nucleosome, thereby allowing downstream repair proteins, such as XPC, to access the lesion [5,6] (Fig. 1). Defects in NER proteins are associated with a rare autosomal recessive disorder called xeroderma pigmentosum (XP), characterized by extreme sensitivity to sunlight-induced skin pigmentation changes and increased risk of skin cancer [7]. In particular, mutations in *DDB2* are associated with xeroderma pigmentosum complementation group E (XP-E) [7].

After reviewing early experiments characterizing UV-DDB, this article explores structure and function studies that have given molecular insights into how UV-DDB participates in NER. We then describe a new non-canonical role of UV-DDB in base excision repair [8]. Finally, we describe provocative studies suggesting how loss of DDB2 may function in cancer development.

2. Historical perspective of UV-DDB: evidence for its canonical role in NER

2.1. Early characterization of UV-DDB

Chu and Chang, in 1988, used electrophoretic mobility shift assays (EMSA) to analyze proteins in HeLa cell extracts that bind avidly to UV-irradiated DNA [9]. They reported two slowly migrating protein-DNA complexes with high affinity to damaged DNA as compared to undamaged DNA. These bands were observed in extracts from all XP

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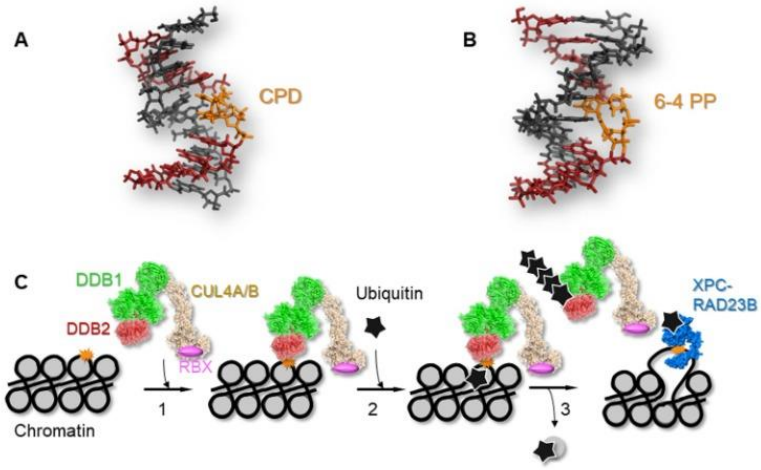

Fig. 1. Canonical role of UV-DDB in global genome nucleotide excision repair of UV-induced photoproducts. Two major UV-induced photoproducts, (A) cyclobutane pyrimidine dimer (CPD), PDB code: 1N4E; and (B) 6-4 pyrimidine-pyrimidone product, (6-4PP), PDB code: 3EI1. (C) UV-DDB (DDB1, green; DDB2, red) forms a complex with CUL4A (tan) and RBX (pink) to form an E3 ubiquitin ligase. Step 1, the UV-DDB-CUL4A-RBX complex- E3 ligase (PDB code: 4A0K, [54]) recognizes UV-induced photoproducts (orange) in the context of chromatin. Step 2, the E3 ligase ubiquitinates (black star) histone H2A. Step 3, the ubiquitinated nucleosome is evicted, and DDB2 is auto-poly-ubiquitinated decreasing its binding affinity for the UV-photoproduct, [30], allowing the recruitment of XPC (blue), which is mono-ubiquitinated stabilizing its interaction with the UV-photoproduct.

complementation groups (A-G), except XP-E. The absence of these bands in XP-E was corrected by the addition of extracts from other XP groups, indicating that this factor was specifically involved in recognition of UV damage. Similar studies were performed by Protic and Levine at about the same time [10]. Interestingly, a comparable DNA binding factor was purified from human placenta in the mid-1970s by Feldberg and Grossman [11]. In the early 1990s, the UV-damaged DNA binding factor, which was later shown to consist of DDB1 and DDB2, was purified from primate cell extracts [12–15]. However, there was considerable heterogeneity in the activity of these preparations.

A defect in UV-DDB binding activity in XP-E provided correlational, but not causal evidence, therefore the Linn and Hoeijmakers laboratories investigated the ability of purified UV-DDB to correct the DNA repair defect in XP-E cells by microinjection [16]. Using an unscheduled DNA synthesis (UDS) assay, labeled thymidine incorporation was quantified after exposing XP-E cells (with and without injected UV-DDB) to UVC. Injection of UV-DDB protein corrected the repair deficiency in two XP-E patient cells (XP2RO and XP82TO) by ~2-fold. This stimulation of UDS was not observed in cells from other XP groups, providing direct evidence that loss of UV-DDB causes repair defects in XP-E cells [17,18]. Shortly after, Linn's laboratory isolated the human genes encoding *DDB1* and *DDB2* subunits and revealed that *DDB2* is mutated in XP-E cells Chu and colleagues further studied UV-DDB in hamster cells [19], in which, similarly to XP-E cells [7,20], the UV-DDB binding activity is deficient and inactive DDB1 is expressed. Consequently, transfection of hamster cell lines with the human *DDB2* cDNA led to activation of DDB1, resulting in DNA binding activity of UV-DDB.

2.2. DDB2 mRNA is regulated by p53

The loss of the human tumor suppressor gene *p53* is one of the most common genetic alterations (41 %) in human cancers [21]. In human cells, the transcription factor, p53, is stabilized in response to DNA damage, which in turn induces transcription of downstream genes, leading to cell cycle arrest and apoptosis [22]. Interestingly, like XP-E cells, $p53^{-/-}$ human cells are deficient in GG-NER of UV photoproducts [23,24]. Hwang and Chu compared $p53^{+/+}$ and $p53^{-/-}$ human fibroblast lines to demonstrate that p53 is required to induce *DDB2* mRNA levels after UV and ionizing radiation (IR) [25]. The increase in DDB2 protein levels after UV damage was observed between 16–24 hours, consistent with a gradual accumulation of stable p53 protein. In contrast, DDB1 levels were not dependent on p53. The human *DDB2* gene was found to have a p53 binding site on the 5′-UTR sequence [26]. Surprisingly, this regulatory region is not conserved in mice, suggesting that the mouse *Ddb2* gene is not p53-dependent. Consistent with this notion, when treated with UV light, neither WT nor $p53^{-/-}$ mouse fibroblast lines displayed an increase in *DDB2* mRNA levels. Furthermore, most mouse cells express very low levels of DDB2 and consequently have much reduced GG-NER of CPD compared to human cells. As Hanawalt and colleagues have pointed out, since the regulation of GG-NER in mice and humans is fundamentally different, it is important to be cautious when using mouse models to recapitulate certain human cancer conditions [27].

2.3. Contrasting studies suggest differential recognition of CPD and 6-4PP by UV-DDB

Several *in vitro* and *in vivo* studies show differential stimulation of CPD and 6-4PP repair by UV-DDB [25]. While XP-E patients are deficient in GG-NER of CPDs, they also display delayed repair of 6-4PP [28]. Hence, UV-DDB might partially be required for 6-4PP repair in certain parts of the genome, thereby rendering studies in live cells or in reconstituted nucleosomes an essential step in delineating the action mechanism of UV-DDB.

To better understand the role of UV-DDB in the different repair rates for CPD and 6-4PP, the Ford and Yasui laboratories established CPD or 6-4PP specific photolyase-expressing cell lines in XP-A cells, allowing them to determine the relative affinity of UV-DDB for either of these two photoproducts in cells [29]. By exposing cells to UVC through a 3-micron filter (local UV damage) and reactivating a photoproduct specific photolyase, each type of lesion could be observed in isolation. Using this technique, it was observed that while DDB2 recognized both CPD and 6-4PP, XPC, a damage verification protein in GG-NER, only appeared to recognize 6-4PP. Overexpression of DDB2 enabled CPD recognition by XPC, suggesting that for XPC to recognize CPDs embedded in chromatin, UV-DDB must process the nucleosome, as shown by Lan et al. [30]. Another *in vivo* study compared normal and XP -E fibroblasts after local UV damage induction to show a delay in the removal of 6-4PP by ~50 % in XP-E cells and a delay in the recruitment of other NER factors to the lesion, indicating that UV-DDB partially stimulates 6-4PP repair in cells [31]. However, no difference in 6-4PP repair between normal and XP-E cells was observed when UVC was exposed globally to the entire plate of cells. This suggests that UV-DDB can enhance 6-4PP repair when the number of lesions is sufficiently

low. It was calculated that a 30 J/m^2 local UV exposure induces roughly the same number of lesions as UV-DDB molecules (~10^5 [13]), while a global UV exposure of the same dose to the entire plate of cells induces 7-fold more 6-4PPs and therefore exceeds the number of UV-DDB molecules.

2.4. UV-DDB is an E3 ligase and ubiquitinates histone H2A

Cellular experiments demonstrated a puzzling finding of UV-DDB levels after UV-induced DNA damage. Rapic-Otrin et al. demonstrated that UV-DDB associates tightly with chromatin, and only DDB2 is concomitantly degraded within 3 h of UV exposure in a dose-dependent manner [32]. They also showed that in XP-E cells with mutated DDB2, which binds poorly to UV-irradiated DNA, degradation of DDB2 does not occur. Further studies with a proteasome inhibitor, NIP-L3-VS blocked degradation of poly-ubiquitinated DDB2. This treatment also helped stabilize p53 by inhibiting MDM2-mediated degradation of p53. Other DNA damaging agents, such as IR did not show depletion of DDB2, but due to IR-induced stabilization of p53, DDB2 expression was induced as early as 12 h after damage and was stably and significantly increased at 24 and 48 h after IR. Finally, this study showed that UV-DDB interacts with the histone acetyl transferase, p300, *via* DDB1. Sugasawa and coworkers, in a study published in 2005, confirmed that UV-DDB is a ubiquitin E3 ligase, consisting of cullin4A (CUL4A) and Roc1 (RBX) that auto-ubiquitinates itself and also ubiquitinates XPC [33]. They went on to show that while mono-ubiquitinated XPC is stabilized at sites of UV-induced DNA damage, the binding of UV-DDB is destabilized, due to poly-ubiquitination of DDB2. This work led to the concept that UV-DDB needs to poly-ubiquitinate itself to allow proper hand off of UV-induced photoproducts to XPC during GG-NER, Fig. 1C [4,34,35]. Both CUL4A and CUL4B have been shown to interact with UV-DDB and support ubiquitination of H2A [36].

In a series of experiments, Lan et al. showed that UV-DDB-CUL4B E3 ligase can specifically bind to mononucleosomes containing UV damage and mono-ubiquitinate histone H2A and H3 [30]. Mono-ubiquitinated H2A at Lys119 and Lys120 helps facilitate the destabilization of nucleosome containing UV-induced photoproducts, as mutating these residues to Arg prevented the dissociation of poly-ubiquitinated DDB2 from the UV damage containing nucleosome. Finally, they showed that while UV-DDB can mono-ubiquitinate H3, H3 ubiquitination is not necessary for UV-DDB-mediated destabilization of the nucleosome.

2.5. UV-DDB-dependent regulation of NER

During the purification and characterization of proteins necessary and sufficient to reconstitute NER on naked DNA, Wood and coworkers found that UV-DDB stimulated the incorporation of radiolabeled nucleotides into UV damaged plasmids by around 2-fold, suggesting that UV-DDB might play "an accessory, but not a core role in NER" [37]. Using a fully reconstituted NER system consisting of seven purified proteins and 136 base pair (bp) DNA substrates containing either a CPD or 6-4PP lesion, Matsunaga and coworkers demonstrated that UV-DDB could stimulate excision of CPD by 5-17-fold in a reaction containing 571 fmol of UV-DDB, but only displayed a ~2 fold stimulation for 6-4PP even when used at a significantly reduced amount (0.57–5.7 fmol) [38]. Interestingly, higher concentrations of UV-DDB were inhibitory for 6-4PP excision, probably due to UV-DDB's higher affinity for 6-4PP as compared to CPD, which could form abnormally tight complexes and potentially block subsequent steps in the repair process. Furthermore, using the locally induced UV damage method (described above), the authors showed that FLAG-tagged p48 (DDB2) translocated to the UV-irradiated regions immediately after irradiation, suggesting that DDB2 participates in the early steps of repair. This recruitment was observed even in cells lacking other NER components (XP-A, XP-C and XP-F cells), strongly supporting the idea that UV-DDB is upstream of other DNA repair enzymes and that its binding acts as a "first responder" at UV photoproducts. Seidman and colleagues showed that UV-DDB was also an early responder at sites of monoadducts caused by angelicin plus 365 nm light, but not at psoralen crosslinks [39]. This result is also quite interesting, indicating that UV-DDB might have a broader substrate repertoire than previously identified, which may include some forms of chemical base damage.

Hoeijmakers and Vermeulen's group have pioneered the use of fluorescence recovery after photobleaching (FRAP) to study UV-DDB in response to UV damage in cells [40]. This was done by photobleaching a strip spanning the nucleus and monitoring the fluorescence recovery in a region of untreated and UVC-treated cells. Using this method, they determined that the binding kinetics of mCherry-tagged DDB1 is similar to DDB2 or CUL4A, demonstrating that these proteins are recruited to the UV damage sites as one large complex. Moreover, when either DDB2 or CUL4A was depleted by siRNA, UV-induced immobilization of DDB1 at the damaged region was dependent on DDB2, but not on CUL4A. Finally, they showed that the UV-induced immobilization of DDB1 is observed for up to two hours in XP-A and XP-C cells, and by four hours is reduced to that of non-irradiated cells, suggesting that this dissociation of DDB1 is not due to repair progression, but is due to the degradation of DDB2 by the 26S proteasome.

To further characterize the regulation of NER by UV-DDB, Sugasawa and colleagues used FRAP to monitor XPC-GFP in the presence or absence of DDB2 [41]. Presence of UVC abrogated the recovery of fluorescence, signifying that XPC-GFP is trapped on UV lesions. Furthermore, overexpression of DDB2 reduced the fluorescence recovery of XPC, suggesting that DDB2 either facilitates recruitment of XPC to lesions or allows it to remain longer at the lesions. To examine this, they used a 266 nm laser to locally induce UVC damage and showed that recruitment of XPC-GFP was delayed in the absence of DDB2. It is important to note that this study was performed using low doses of UVC (5–10 J/m^2), therefore the number of lesions generated was probably lower than the number of UV-DDB molecules. At higher doses of UVC in which the initial photoproduct density exceeds the number of UV-DDB molecules, the repair process might occur in two phases: a fast phase, stimulated by UV-DDB and a slower phase, corresponding to direct binding of the lesion by XPC. However, XPC has been suggested to be at lower concentrations in the cell than UV-DDB [42], and repair kinetics could be altered by DNA condensation into heterochromatin.

2.6. DDB2 can directly change the core histone density at UV-induced DNA lesions

A number of chromatin remodelers have been shown to play an important role in the regulation of nucleotide excision repair (reviewed in [43]). Dantuma and colleagues identified a novel role for DDB2 in unfolding of higher order chromatin structures at the sites of UVC damage [44]. Using a LacR-tagged DDB2 construct in various cell lines consisting of integrated LacO arrays, it was shown that tethering of LacR-DDB2 can significantly reduce density of GFP-tagged H1, H2A and H4. Furthermore, the decondensation of chromatin by DDB2 was independent of DDB1-CUL4A E3 ligase activity and dependent on ATP hydrolysis, indicating that ATP-dependent chromatin remodeling factors might be involved in the process. Also, this process required poly (ADP-ribose) polymerase (PARP1) activity, which has been linked to chromatin remodeling in the context of double-strand breaks [45,46].

Using FRAP in cells stably expressing SNAP-tagged histone 3.3, Polo and Almouzni demonstrated that DDB2 is necessary and sufficient for changing the histone density at locally induced UV damage sites, causing a local redistribution of SNAP-H3.3 [47]. Knockdown of chromatin remodeling factors, ALC1 and INO80, did not affect the histone dynamics, suggesting that DDB2 binding is upstream of any chromatin remodeling activity. Furthermore, in contrast to the previous study discussed above [44], redistribution of histone H3.3 at sites of UV damage was PARP1-independent. How PARP is mechanistically linked to chromatin remodeling during NER is still unclear and requires further

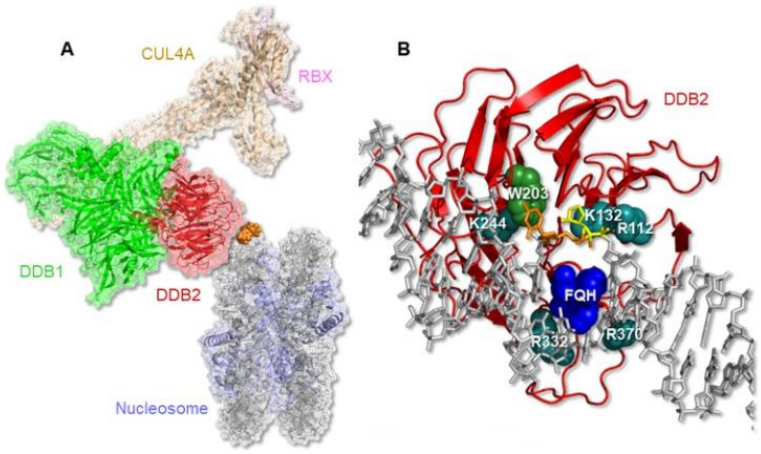

Fig. 2. Structure of UV-DDB. (A) Molecular model of UV-DDB-CUL4A-RBX complex bound to a nucleosome. DDB1 (green); DDB2 (red); CUL4A (tan); RBX (pink); histones (blue); nucleosome DNA (gray); 6-4 photoproduct (orange). Built from PDB codes: 4A0K and 6R8Y, [54] & [61]. (B) Damage recognition interface of DDB2 (red ribbon) with tetrahydrofuran, THF (yellow)-containing DNA (gray) with adjacent 3′ base flipped out (orange). This flipped out base is stabilized by W203 (green) and an FQH knuckle (dark blue) fills the void made by the two flipped out bases. Important salt contacts with positively charged amino acids (cyan) with phosphate backbone are shown. K244 when mutated to E causes XP-E. PDB codes: 4E54 & 4E5Z, see [49].

investigation, see also Section 4.1 below.

3. Molecular Architecture of UV-DDB: clues to damage recognition

3.1. Structure of UV-DDB

Human DDB1 and zebrafish DDB2 lacking the *N*-terminus were co-crystalized with either a 14 bp DNA containing a 6-4 photoproduct or a 16 bp DNA containing an abasic site analog, tetrahydrofuran (THF), and their structures were resolved to 2.8 and 2.3 Å, respectively, by Thoma and coworkers [48], Fig. 2A & B. Later, Yeh and coworkers solved a co-crystal structure of full-length human UV-DDB bound to a THF in a 25 bp DNA that was resolved to 2.85 Å [49]. This new structure revealed two interesting features. First, the *N*-terminal 66 residues of DDB2, folded into three helical segments as a triangular paddle. Second, the crystal revealed a dimer of UV-DDB $(DDB1\text{-}DDB2)_2$ bound to two separate DNA helices. This study also used electron microscopy (EM) and atomic force microscopy (AFM) to show that UV-DDB binds DNA both in a monomeric (DDB1-DDB2) and a dimeric $(DDB1\text{-}DDB2)_2$ form, the latter containing two DNA molecules in each complex. Together these UV-DDB structures from both laboratories revealed extensive contacts between DDB2 and the DNA strands around the damaged site and provided important clues on how UV-DDB can recognize different types of damage.

DDB2 is organized as a seven-bladed WD40 β−propeller in which the hairpins of repeats 4–7 make extensive contacts around the damaged site. Both structures showed that the 6-4 photoproduct and the THF with the 3′ adjacent base were flipped into a hydrophobic pocket in DDB2 and the 3′ base was stabilized by a stacking interaction with Trp203, Fig. 2 B. These flipped out bases leave a two-base gap in the DNA duplex, which is filled by Phe334 (F), Gln335 (Q), and His336 (H) that form a beta-hairpin knuckle-like structure that is inserted through the minor groove. The structures also revealed an extensive set of salt-bridges between the Arg112, Lys132, and Lys244 on the damaged strand, and the Arg332 and Arg370 contacting the non-damaged strand, Fig. 2B, also see Movie 1. While the Thoma structure revealed a 40° bend at the damaged site, the Yeh structures and AFM analysis did not reveal such a sharp bend for UV-DDB complexes [49,50].

3.2. XP-E mutations give insights into DDB2 function

Of the 15 confirmed patients with XP-E, eight amino acid changes, including several frameshift/truncation mutants, altered splice mutants and deletions affecting both alleles, have been discovered, Table 1, Fig. 3, and Movie 2. Strikingly, an XP-E patient (XP82TO) was shown to have a Lys244 to Glu mutation and this single variant was shown to be sufficient to cause loss in photolesion recognition and subsequent repair [51]. As discussed below (Section 3.5), this mutant causes increased sliding on the DNA [50]. Several other DDB2 variants from XP-E patients (Arg239Ile, Asp307Tyr, Thr305Asn, Pro357Leu) map to the DDB2-DNA interface and would be expected to decrease or eliminate UV-DDB DNA binding activity. One compound mutation (a heterozygous mutation with different mutations on each allele), Asn349Δ/Leu350Pro, as well as the Arg273His variant are localized within the DWD box motif, mutations of which impair the interaction with DDB1, thus rendering the DDB2 directed ubiquitination inactive [52,53].

3.3. Structural aspects of UV-DDB-CUL4-RBX E3 ligase

Thoma and coworkers have solved the molecular architecture of UV-DDB bound to CUL4-RBX (CRL4) which forms a $CRL4^{DDB2}$ E3 ligase [54]. There are over 200 different CRLs in human cells that fall into five distinct families (CRL1,2,3,4A/B, and 5). CUL4A/B containing CRLs belong to a family of E3 ubiquitin ligases which regulate a wide range of cellular processes through dedicated substrate receptors (DCAFs). $CRL4^{CSA}$ and $CRL4^{DDB2}$ help target this ubiquitin E3 ligase to DNA during TC-NER and GG-NER, respectively.

3.4. Cop9 signalosome as a chaperone for UV-DDB

In the absence of DNA damage, cellular $CRL4^{DDB2}$ is bound to the COP9 signalosome complex (CSN) [55], see a UV-DDB interactome in Fig. 4. The CSN is an eight subunit isopeptidase complex that through its CSN5 subunit, proteolytically removes a ubiquitin-like moiety, NEDD8 from the cullins. NEDD8 modification serves as a regulator of cullin activity. When NEDD8 is attached to CUL4, its E3 ligase is in an active mode. However, when bound by CSN complex, UV-DDB-CUL4 is rendered inactive through deneddylation. Structural studies by the Thoma laboratory revealed that DNA binding by DDB2 antagonizes its interaction with CSN3, helping to displace the CSN complex and allowing continued neddylation and activation of the E3 ligase [56]. These structural studies confirmed the earlier work by Nakatani and coworkers who showed that CSN differentially regulates $CRL4^{CSA}$ and $CRL4^{DDB2}$ during nucleotide excision repair [57]. Knockdown of CSN5 with siRNA in BJ1 fibroblasts decreased GG-NER by 55 % and TC-NER by 43 %. Despite this pioneering work on these complexes in 2003, it is

Table 1
Characterization of XP-E causing mutations.

Patient / Cell line	Allele 1 Amino Acid	Allele 2 Amino Acid♦	Cancer	UDS	HCR	Biochemistry	Cellular Imaging
XP1GO	Thr305Asn		BCC, SCC, M [20]	NR	Reduced [20]	NR	NR
XP37BE[&]	Arg273His		BCC, SCC [20]	NR	Reduced [20]	*DDB1 and Cul4A not detected by co-IP [91,92]	No binding to local UV damage; *Part of the DDB-CUL4A-ROC complex [42]
XP66BE[&]	Arg273His		M [20]	NR	Reduced [20]	*DDB1 and Cul4A not detected by co-IP [91,92]	No binding to local UV damage; *Part of the DDB-CUL4A-ROC complex [42]
XP408BE/ GM01389/ GM01646	Leu350Pro	Asn349del	BCC, SCC, M [20]	50 % [51,93]	Reduced [20]	EMSA, no binding activity (E) [51,93]; DDB1and CUL4A not detected by co-IP [51,94];no mono-Ub-H2A on chromatin after UVC [94]	No binding to local UV damage; Fails to recruit DDB1 and Cul4A [44]
XP2RO/ GM02415[$]	Arg273His		BCC [20,95]	40-60 % [51,93,96]	NR	EMSA, no binding activity (E) [97] *DDB1 and Cul4A not detected by co-IP [91,92]	No binding to local UV damage; *Part of the DDB-CUL4A-ROC complex [42]
XP3RO/ GM02450[$]	Arg273His		BCC [20,95]	40-60 % [51,96]	NR	*DDB1 and Cul4A not detected by co-IP [91,92]	No binding to local UV damage; *Part of the DDB-CUL4A-ROC complex [42];
XP82TO	Lys244Glu		None (as of 2011) [20,95]	44 % [51,96]	NR	EMSA, no binding activity (E) [28,93,97] ; Partial binding activity detectable (P) [91]; no histone ubiquitination in nucleosome [91]	No binding to local UV damage [44,91]; Slides on DNA in the absence of Mg^{2+} [50]
XP23PV	Leu235_Lys341del		BCC [20,51]	65 % [51,93]	NR	EMSA, no binding activity (E) [51,93]	No UV-induced chromatin decondensation [44]
XP25PV	Asp307Tyr No change		BCC, SCC [20,51]	50 % [51,93]	NR	EMSA, no binding activity (E) [51,93]; DDB1 not detected by co-IP [51]	No binding to local UV damage; No recruitment of DDB1 and Cul4A [44]
XP27PV	Lys244X Trp236Valfs10 Leu235_ Lys341del		BCC, SCC, M [20,51]	48 % [51]	NR	EMSA, no binding activity (E) [51,93];	NR
Ops1	Arg313X		BCC, SCC, M [20,28,95]	99–138 % [28]	NR	EMSA, no binding activity (E) [28] DDB1 and Cul4A not detected by co-IP [91]	NR
XP115BR	Met383fs		None (as of 2016) [7]	~50 % [7]	NR	NR	NR
XP105BR	Pro357Leu	Arg239Ile	BCC, SCC, M [7]	~50 % [7]	NR	NR	NR
XP98BR	Trp54X		BCC, SCC, M [7]	~50 % [7]	NR	NR	NR
XP100BR	Splice		BCC [7]	~50 % [7]	NR	NR	NR

Abbreviations: **BCC,** Basal cell carcinoma; **SCC**, Squamous cell carcinoma; **M**, Melanoma, **UDS,** Unscheduled DNA synthesis assay; **HCR,** Host cell reactivation; **Biochemistry** includes electrophoretic mobility shift assays (EMSA), western blotting and Co-IP; **E:** Cell extracts; **P:** Purified protein; **Cellular Imaging** examines both recruitment to local damage and FRAP experiments. **NR**: not reported.
&: Siblings; **$:** Second cousins; ♦empty boxes for allele 2 amino acid indicate homozygous mutation; * contrasting results from biochemistry and cellular studies.

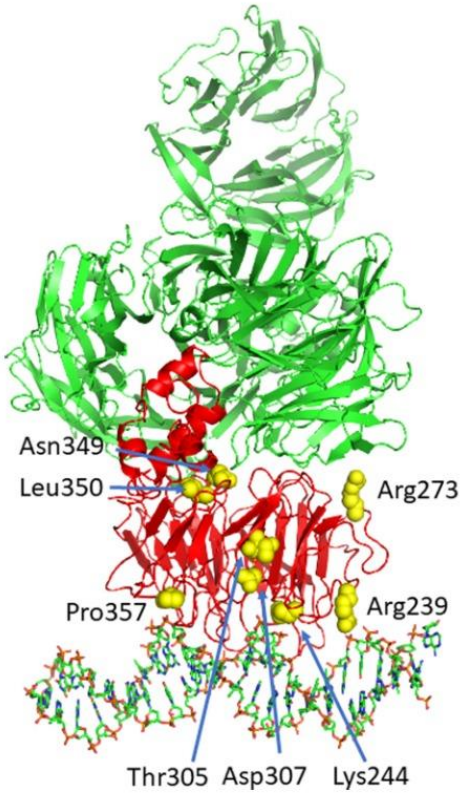

Fig. 3. Positions of mutated amino acids in DDB2 that cause XP-E. Shown on the structure of DDB2 (red) are amino acids (yellow) that are altered from point mutations shown in Table 1. All other mutations resulting in frameshifts, stop codons and large deletions are not shown on this structure. Note that patient GM01389 apparently showed a compound mutation in one allele involving a Leu350Pro substitution and deletion of Asn349 in the other allele. Thus, both amino acids are shown in yellow in the DDB2 structure.

still not clear why removal of a negative regulator of these two E3 ligases paradoxically slows both forms of repair, rather than enhances repair. It is possible that the COP9 signalosome helps chaperone spurious DNA binding by DDB2, which has high affinity for non-damaged DNA. In this regard, it is interesting to note that van Driel and coworkers used FRAP analysis to show that the 3D diffusion of EGFP-labeled CUL4A and EYFP-labeled DDB2 were consistent with a complex of ~ 500 kDa, strongly supporting that this complex is being bound by the COP9 signalosome [42].

3.5. Single-molecule analysis of UV-DDB reveals 3D searching and dimerization on DNA

As described above, early work on UV-DDB damage recognition using EMSAs indicated a major band consistent with a heterodimeric complex of DDB1 and DDB2 bound to DNA, but a more slowly migrating band is often observed at higher molecular weights consistent with either multiple DNA binding events or the formation of two UV-DDB complexes binding together. In order to investigate this phenomena in greater detail, Ghodke in our group examined UV-DDB interaction on defined substrates by two single-molecule approaches, AFM and fluorescence microscopy of quantum-dot labeled proteins on a DNA tightrope optical platform [50,58]. AFM provides volume data on protein-DNA complexes in such a way that the volume is directly proportional to the molecular weight of the complex and therefore the oligomeric state [58]. Using AFM, Ghodke was able to show that both WT-UV-DDB and a DDB2-Lys244Glu variant were able to dimerize forming $(DDB1\text{-}DDB2)_2$ complexes on DNA in which they bound to two DNA molecules. This Lys244Glu variant of DDB2 found in patient XP82TO was previously shown to bind weakly to DNA [51], see also Table 1 and Fig. 3. Using a fluorescence microscopy imaging platform consisting of UV-irradiated lambda DNA suspended on 5 μm poly-L-lysine coated beads and Quantum-dot labeled UV-DDB, Ghodke followed association and dissociation events of both WT and DDB2 Lys244Glu variant UV-DDB in real-time. He found that while WT UV-DDB performed a 3D search, rapidly binding and releasing from DNA without sliding (see Movie 3), the XP-E mutant, variant Lys244Glu slid on DNA and was able to form dimers (see Movie 4). The dissociation rates of WT UV-DDB consisted of time intervals of ~ 1, 10, and 100 s. This led to the concept that UV-DDB uses "conformational proofreading" in which binding to a damaged site helps to alter the conformation of both the DNA and the protein to increase stability and therefore, the length of the interaction of UV-DDB on DNA [59]. The Lys244Glu variant was not able to bind specifically to damage sites and showed little or no long-lived pausing on DNA. Thus, it would appear that the loss of a single positively charged amino acid residue interacting with DNA is sufficient to cause UV-DDB to slide on DNA. As described below in Section 5, we have found that WT UV-DDB has limited linear diffusion on DNA in the presence of Mg^{2+}.

3.6. Interaction of UV-DDB with nucleosomes containing site-specific lesions

Based on Lan et al. [30], and later work by Kurumizaka, Sugasawa and Thoma [60], UV-DDB was found to bind specifically to nucleosomes containing DNA photoproducts and was proposed to be the first responder to UV-induced DNA damage in chromatin. In a major tour-de-force study, Thoma and coworkers used cryo-EM to examine UV-DDB's interactions with a nucleosome containing defined damage sites at various positions along the nucleosome [61]. Due to the inherent wrapping of the DNA around the histone octamer, it might be expected that lesions pointing more inward would not be accessible to UV-DDB. However, UV-DDB was shown to shift the DNA register, altering the nucleosome architecture as much as by three base pairs to allow access to occluded sites.

4. Roles of post-translational modifications (PTM) in regulating UV-DDB-mediated repair

4.1. PARylation

UV-DDB is known to interact with a number of proteins during GG-NER, including the histone acetyl transferase p300 and the Cul4A-RBX1 complex that ubiquitinates histones (H2A, H3, H4), DDB2 and XPC in response to UV radiation [34]. Additionally, Mullenders and colleagues identified a novel role for PARP1 in PolyADP-ribosylation (PARylation) of DDB2 and subsequent recruitment of SWI/SNF chromatin remodeler, ALC1 to UV damaged DNA [62]. DDB2-associated proteins were analyzed by chromatin immunoprecipitation and mass spectrometry, revealing that PARP1 bound both DDB1 and DDB2. Consistent with the PARylation of DDB2, rapid recruitment of GFP-ALC1 to sites of UV lesions was observed and found to be DDB2-dependent. Furthermore, PAR was detected by immunofluorescence at sites of UV damage and colocalized with TFIIH. Although DDB1 was also shown to bind to PARP1, whether it is PARylated or acts in association with PARP1 was not pursued and requires further investigation.

Using FRAP, it was observed that DDB2 had a prolonged retention time on the DNA in the absence of Poly(ADP-ribose) glycohydrolase

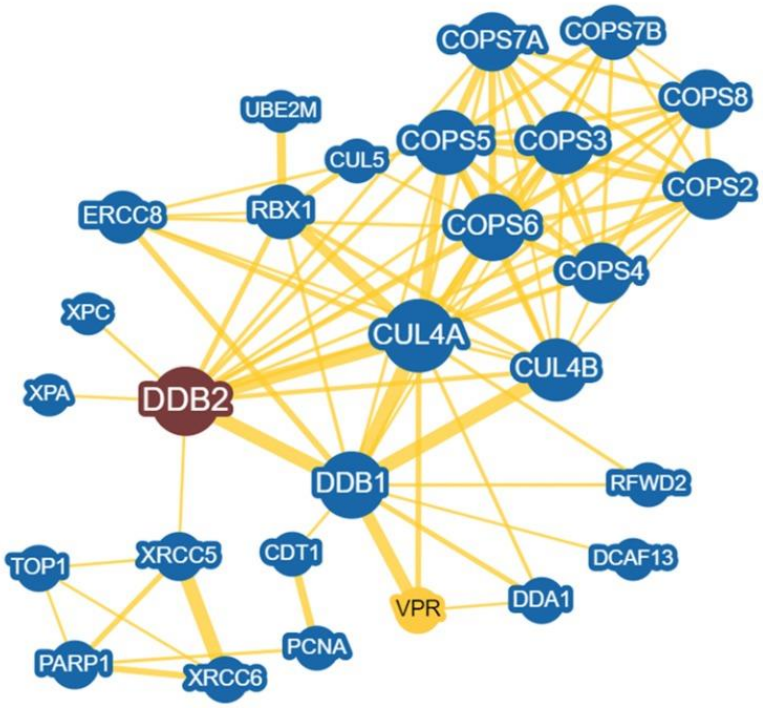

Fig. 4. DDB2 interactome prepared in BioGRID. Human DDB2 was selected and the minimum evidence was set to 5. The interactome network centered on DDB2 (red) was created using an arbor layout. Nodes in blue = 28 are DDB1 and/or DDB2 interacting proteins in human cells. Some low interacting nodes were trimmed out of the network. These interactors are shown in yellow for direct physical evidence. Greater node size represents increased connectivity and thicker edge sizes represent increased evidence supporting the association. VPR node is in yellow and is a major HIV protein that interacts with DDB1 to help direct the ubiquitination and degradation of key cellular proteins to aid in HIV infection.

(PARG), suggesting that PARylation might stabilize the protein at the lesion site. Moreover, inhibition of PARP activity resulted in suppressed PARylation but increased ubiquitination of DDB2, indicating that PARylation may prevent DDB2 auto-ubiquitination and subsequent degradation. In contrast to the previous study, PARP1 inhibition led to extended retention of DDB2 on the damaged DNA as measured by immunoblotting [63]. It would be interesting to determine whether PARylation of UV-DDB helps prevent auto-ubiquitination and whether PARP inhibition causes more rapid degradation of UV-DDB through the 26S proteasome. This study also verified that DDB2 is PARylated after UV damage using dot blots and further showed that XPC recruitment is delayed in the absence of PARP1-mediated PARylation of DDB2 [63].

4.2. SUMOylation

Small ubiquitin-related modifiers (SUMOs) can regulate a variety of cellular processes including transcriptional regulation, signal transduction and maintenance of genome integrity by causing rapid changes in protein-protein interactions. SUMO is covalently attached to proteins through a cascade similar to that of ubiquitination. Using co-immunoprecipitation, Iijima and colleagues showed that DDB2 is SUMOylated post-UV damage and PIASy (protein inhibitor of activated STST proteins) was the major SUMO E3 ligase involved [64]. Furthermore, knockdown of PIASy impaired the repair of CPDs, but not 6-4PP, as measured by ELISA. Future cellular studies showing a DDB2-dependent recruitment of SUMO to sites of UV damage will help clarify this PTM of UV-DDB in NER.

To this end, another study using immunoprecipitation showed DDB2 SUMOylation after UV damage and further screened the DDB2 sequence to identify the sites of SUMOylation [65]. Three lysine residues (Lys5, Lys77 and Lys309) were identified and mutated to arginine. Lys309Arg completely abolished the DDB2 modification upon UV damage. Moreover, when expressed in cells, this mutant was deficient in the removal of CPDs as well as in the recruitment of XPC as compared to the WT, indicating that the SUMOylation at Lys309 is functionally significant.

5. Novel role of UV-DDB in the repair of oxidative DNA damage

Our genomic DNA is constantly exposed to oxidative stress in the form of reactive oxygen species (ROS) from mitochondria and pro-inflammatory processes, or from exogenous sources like UVA/B and IR. This can lead to direct oxidation of the DNA bases such as 8-oxoguanine (8-oxoG). Repair of this lesion is initiated by 8-oxoG glycosylase (OGG1), which removes the oxidized base through hydrolysis of the glycosidic bond, creating an apurinic/apyrimidinic (AP) site. This is recognized by an AP endonuclease (APE1), which incises the damaged strand, leaving a single nucleotide gap with a 3′−OH primer. DNA polymerase β (pol β) synthesizes DNA from the 3′−OH end. DNA ligase III and x-ray cross-complementing factor 1 (XRCC1) help ligate and seal the nick [66].

The earliest evidence of recognition of AP sites by UV-DDB was shown by Chu and colleagues using an electrophoresis DNA binding assay [67]. A 148 bp DNA probe was end-labeled with ^{32}P and damaged by UV to induce CPD and 6-4PP. Unlabeled DNA containing AP sites was used as a competitor. Partially purified UV-DDB from HeLa cell extracts was run on a gel along with the DNA. While the DNA containing AP sites was able to inhibit UV-DDB binding to UV damaged DNA, the affinity was 17-fold lower for the AP sites.

More direct evidence of recognition of AP sites by UV-DDB was established separately by Fujiwara [68], as well as Wood and colleagues by using recombinant purified UV-DDB and DNA substrates with a site-specific CPD, 6-4PP and AP site [69]. The latter group observed a 6-fold higher affinity for CPD, 83-fold higher affinity for 6-4PP and a 46-fold

Table 2
Magnesium increases the binding specificity of UV-DDB.

Substrate	No Mg^{2+}	5 mM Mg^{2+}	Fold Difference
37 bp duplex	K_d^1 (nM)	K_d^1 (nM)	
THF	0.9 ± 0.2	3.9 ± 0.5	4.3
CPD	4.5 ± 0.3	30.4 ± 2.4	6.8
8-oxoG:C	35.3 ± 2.1	159.6 ± 12.4	4.5
undamaged	42.3 ± 2.6	1108 ± 95.5	26.2

CPD, cyclobutane pyrimidine dimer (thymine-thymine); THF, tetrahydrofuran. 1, K_d = equilibrium dissociation constant determined by EMSA from three separate experiments.

higher affinity for an AP site as compared to undamaged DNA. A mismatch substrate was also tested with a 50-fold higher affinity as compared to undamaged DNA. This broad substrate specificity is probably because UV-DDB does not detect the damage site directly but recognizes helix-distorting lesions that can be flipped into the binding site of DDB2 (Fig. 2B).

Recently, our lab has established a novel role for UV-DDB in the repair of oxidative DNA damage [8]. Using a combination of biochemical, single-molecule and cellular studies, we demonstrated that UV-DDB can recognize and aid in the repair of 8-oxoG lesions. We further showed that when the EMSA are done in the presence of Mg^{2+} in the binding buffer, gel, and running buffers, UV-DDB has enhanced specificity for both abasic sites and 8-oxoG. As shown in Table 2, while Mg^{2+} greatly diminishes binding to a non-damaged 37 bp duplex by 26-fold from 42 nM to 1100 nM, Mg^{2+} only decreased binding to abasic site and 8-oxoG:C base pairs by 4-5-fold, greatly enhancing the specificity window of UV-DDB. We next showed that UV-DDB stimulated OGG1 and APE1 activity on 8-oxoG:C and abasic site containing substrates by ~3-fold and ~9-fold, respectively. Using a single-molecule DNA tightrope assay where damaged DNA is suspended between 5 μm poly-L-lysine coated beads and quantum-dot labeled purified proteins are observed in real time [58], UV-DDB was found to undergo limited linear diffusion in the presence of Mg^{2+} in the flow cell as compared to strict 3D searching on DNA with little or no linear diffusion in the absence of Mg^{2+}, Movie 5. We also showed that UV-DDB could form transient complexes with OGG1 and APE1 on the damaged site [8]. Moreover, UV-DDB facilitated the dissociation of these proteins from the damaged site, suggesting that UV-DDB can help turnover OGG1 and APE1 from the abasic site. Also, hTERT-immortalized fibroblasts that had greatly reduced DDB2 expression were more sensitive to potassium bromate, an oxidizing agent that forms predominantly 8-oxoG lesions in the DNA.

Finally, a novel chemoptogenetic approach was used to target 8-oxoG lesions specifically to the telomeres [70,71]. This system uses a telomeric repeat-binding factor 1 (TRF1) fused to a fluorogen activating protein (FAP) that binds avidly to malachite green (MG-2I) dye. This TRF1-FAP-dye combination, when excited by far red wavelength (660 nm) generates singlet oxygen, forming 8-oxoG lesions in telomeric DNA. Using this technique, we demonstrated that UV-DDB is recruited to 8-oxoG lesions immediately after damage. Moreover, OGG1 co-localizes with UV-DDB, but is recruited more slowly, suggesting that UV-DDB may be the first responder to base damage within chromatin. Based on these data and structural studies by Thoma's group, we propose that UV-DDB plays two roles in the repair of 8-oxoG, Fig. 5: 1) UV-DDB recognizes the lesion directly in the nucleosome and aids in the recruitment of downstream proteins; 2) UV-DDB helps turnover OGG1 and APE1 from the abasic site to allow for coordinated and unperturbed repair. Future studies will characterize the downstream repair pathway that is being regulated by UV-DDB in 8-oxoG repair. Furthermore, it will be interesting to adapt this approach to follow the formation and repair of 8-oxoG lesions in different regions of the genome and analyze the potential differences in repair kinetics depending on the chromatin structure. Finally, since this study also demonstrated that UV-DDB stimulated MUTY removal of A across from 8-oxoG, it will be interesting to study whether UV-DDB can stimulate the nine other known mammalian glycosylases during BER [72].

6. Provocative role of DDB2 in cancer

6.1. Internal cancers in XP patients and potential role of DDB2 in human cancers

While XP patients show extremely high levels of skin cancer, 14 of the 726 XP patients had brain, breast or respiratory track neoplasms [73]. More recent analysis of 31 patients indicated that four of them also displayed elevated levels of internal cancers, including T-lymphoblastic leukemia, myelodysplasia, kidney adenocarcinoma, cervical cancer and thyroid adenocarcinoma [74]. Furthermore, several XP patients were reported to die from their internal malignancies [75]. Certain complementation groups also show neurological disorders, suggesting a potential role of NER proteins in the removal of oxidative DNA lesions [7]. The number of patients reported with XP-E is extremely rare. While internal cancers have been found in XP patients, only one XP-E patient in the NIH series [described in reference 20] has shown an internal cancer – papillary thyroid carcinoma (Kenneth Kraemer, personal communication). Since thyroid cancers are not infrequent in the general population, it is not clear whether thyroid cancer in this XP-E patient is due to the loss of DDB2 activity. Additionally, XP-E patients develop a larger number of skin lesions, including melanoma, despite having later onset of skin cancers compared to XP patients in other complementation groups [7,76]. The Cancer Genome Atlas (TCGA) data indicates that endometrial, cervical and breast cancer patients with higher levels of *DDB2* mRNA expression show significantly better long-term survival (Fig. 6A).

6.2. DDB2-deficient mice are more prone to UV-induced skin cancer and die prematurely due to spontaneous internal cancers

Four independent studies have reported increased cancer risk and tumor formation in DDB2-deficient mice [77–80] exposed to UV light. A protective role for DDB2 against tumor formation was first shown in 2005 by Raychaudhuri and coworkers in which all sixteen DDB2 knockout mice developed skin tumors after 38 weeks of UVB irradiation compared to only two of fifteen wildtype and two of sixteen heterozygotes [80]. This was confirmed by Linn and coworkers using sample sizes of 40–77 for wildtype, heterozygote and DDB2 knockout mice treated with a dose of 2500 J/m^2 of UV-B five times a week for up to 35 weeks [78]. These DDB2 knockout mice not only developed skin tumors within 13 weeks of treatment compared to 19 weeks in heterozygotes and wildtype mice, but also developed a significantly larger number of skin tumors with an average of 13 in the knockouts as compared to 0.3 in the heterozygotes and 0.08 in the wildtype mice [78]. In a similar study, the Mullender laboratory demonstrated that mice with increased DDB2 expression in skin keratinocytes (Lys14-DDB2) had increased tumor-free survival and smaller tumor sizes compared to wildtype and heterozygote mice when exposed to 500 J/m^2 of UVB [79]. To provide more impactful results, it would have been interesting to show that knocking out XPA in these genetic backgrounds completely abrogated the effect of DDB2 overexpression in the avoidance of UV-induced skin cancer, which would demonstrate a direct role of DDB2 in enhanced GG-NER.

In addition to DDB2's role in cancer protection when exposed to UV irradiation, DDB2 also plays a remarkable role in protecting against spontaneous internal tumors in mice, Fig. 6B & C [77]. DDB2 knockout (KO) mice were shown to have lower overall survival and developed spontaneous tumors with a tumor-free survival of 25.3 months compared to 28.5 or 33.4 months for their heterozygote or wildtype counterparts. These mice also had a higher tumor incidence of 46 % compared to 37 % for heterozygotes and 25.6 % for wildtype. The

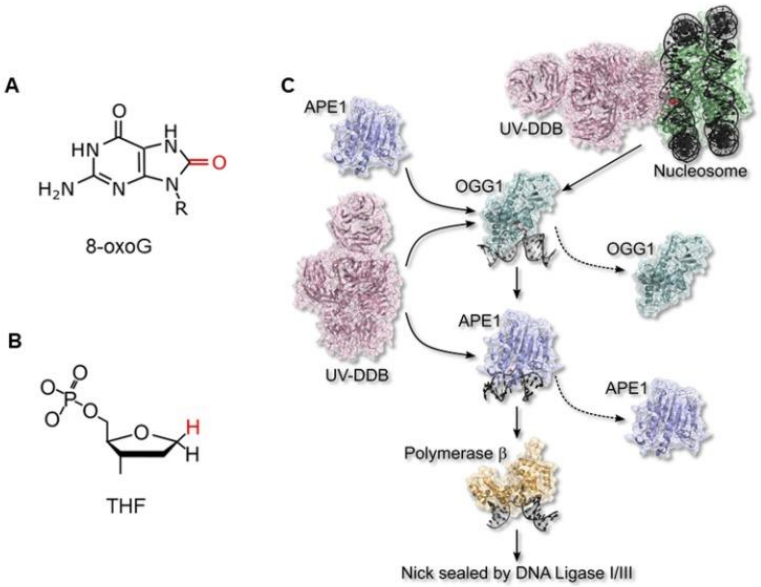

Fig. 5. New non-canonical role of UV-DDB in base excision repair (BER) of oxidative lesions. (A) 8-oxoG, a major form of base damage induced by oxidative stress. (B) Tetrahydrofuran (THF), a stable abasic site analog. (C) A working model of how UV-DDB stimulates BER removal of 8-oxoG. For details see Section 5 and ref [8]. UV-DDB is the first responder at 8-oxoG sites in nuclear DNA. Biochemical and single-molecule evidence showed that UV-DDB stimulates OGG1 removal of 8-oxoG by 3-fold and APE1 by 8-fold through UV-DDB-mediated dissociation. UV-DDB stimulated the DNA polβ gap filling step of BER *in vitro* by 30-fold. Figure adapted from [8].

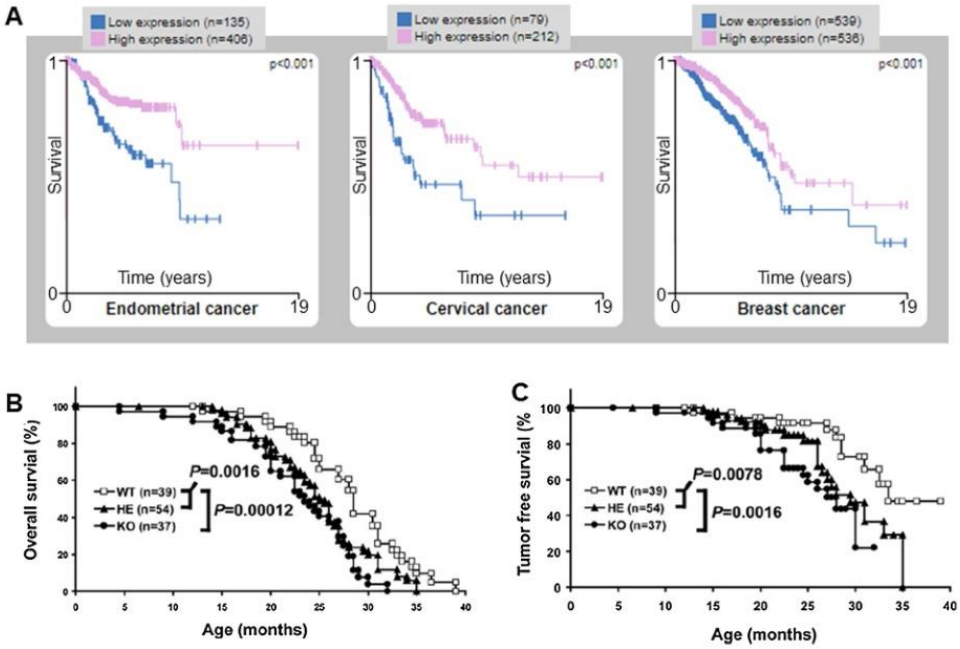

Fig. 6. Association of decreased *DDB2* mRNA expression with increased cancer risk. (A) The Human Protein Atlas was queried for *DDB2* under the Pathology tab. The prognostic summary reached high significance for patient survival in three cancer types: endometrial, cervical, and breast. Low mRNA expression (blue) indicated a poor prognosis as compared to high (pink) mRNA expression levels of *DDB2*. Other cancers sites showing a similar trend using Kplot.com [98], included, kidney renal clear and papillary cell, liver, lung, sarcoma, stomach, and thyroid. Linn and colleagues showed that DDB2 heterozygous and knockout mice have decreased overall survival (B) and tumor-free survival (C). From [77] with permission.

spontaneous tumors included adenocarcinoma of the lung and mammary gland as well as several forms of sarcoma [77]. Together these studies suggest a direct role of DDB2 in delaying internal cancers that arise spontaneously in mice. These mice data combined with our recent observation for a role of UV-DDB in stimulating base excision repair of oxidative lesions suggest that perhaps ROS arising from pro-inflammatory conditions may be sufficient to drive tumors in DDB2-deficient mice. In this regard, it would be of interest to look at the mutation signatures in the spontaneous tumors of DDB2 KO mice.

6.3. The effect of Cul4A knockout on DDB2 in cancer prevention

As described earlier, DDB2 is regulated by Cul4A/B ubiquitin E3 ligase. Auto-ubiquitination of DDB2 with multiple ubiquitin chains causes degradation by the 26S proteasome, potentially constraining UV-DDB's ability to promote GG-NER. Hence, contrary to the established role for the $CRL4^{DDB2}$ in GG-NER (Fig. 1C), it might be speculated that abrogating Cul4A ubiquitin ligase may increase DDB2 stability and enhance DNA repair, leading to cancer protection. This hypothesis was directly tested in Cul4A knockout mice, which exhibited elevated CPD repair and delayed proliferation [81]. Additionally, 13 out of 14 Cul4A knockout mice when exposed to UVB irradiation remained cancer-free compared to their 19 wildtype counterparts that all had squamous cell carcinoma within 48 weeks [81]. This study indicates that DDB2's tumor suppression is restricted by Cul4A ubiquitin ligase and that targeting Cul4A ubiquitin ligase may serve as a novel therapy to increase DNA repair capacity.

6.4. Potential tumor suppressor: cell survival and cancer protection

It has been indicated that DDB2 may have a wider role beyond GG-NER and act as a tumor suppressor, such that its deficiency results in increased cancer risk and tumors as described above, also recently reviewed [82]. One way DDB2 may act as a tumor suppressor is through promotion of apoptosis in damaged cells. To this end, Linn and coworkers reported a higher viable cell count from mouse embryonic fibroblasts (MEFs) isolated from DDB2 knockout mice compared to their heterozygote and wildtype mice counterparts, up to 120 h post-UVC irradiation [78]. They also observed a significant decrease in caspase-3 activity in these knockout MEFs post-UVB irradiation. However, there are opposing results suggesting that DDB2 does not play a direct role in apoptosis. In 2005, Mullenders and coworkers showed that enhanced DDB2 expression in mouse dermal fibroblasts resulted in no significant change in apoptotic activity compared to knockout and wildtype mice after exposure to UVC irradiation [79]. The lack of apoptosis in this latter study could be due to a number of factors including: the small dose of UV irradiation, 8 J/m^2 of UVC, that may not have yielded sufficient lesions to see distinct differences between the knockout and wildtype cells; the differences in cells tested; and the different techniques employed for caspase-3 [78,79].

6.5. Potential oncogene in breast cancer

In contrast to TCGA data, which indicates that increased *DDB2* mRNA expression is correlated with higher survival for breast cancer patients, Becuwe and coworkers describe DDB2 as a possible oncogene in breast cancer [83]. They found that knocking down DDB2 in MCF7 cells resulted in reduced colony formation by ~2-fold and overexpressing DDB2 in MDA-MB231 cells resulted in increased colony formation by ~3.7-fold. Additionally, DDB2 knockdown MCF7 cells had delayed cell cycle progression in flow cytometry experiments. Although these data may suggest DDB2 acts as a potential oncogene in breast cancer, future work should seek to confirm these findings in other breast cancer cell lines.

6.6. Tumor formation and metastasis

Additional experiments in mice have suggested that loss of DDB2 facilitates metastatic colon cancer [84,85]. Specifically, mice injected with HCT116 colon cancer cells deficient in DDB2 had larger tumor masses than those injected with HCT116 cells with wildtype DDB2 [84]. Similarly, it was demonstrated that DDB2 knockout mice treated with azoxymethane/dextran sulfate, a colon cancer carcinogen, had a higher abundance of tumors and a larger tumor mass compared to wildtype animals [85]. Staining of tumors in DDB2 KO mice indicated reduced expression of RNF43, a suppressor of Wnt signaling and displayed increased *Cdx1* mRNA expression, a Wnt target gene. Furthermore, CHIP-seq data suggested that DDB2, perhaps with DDB1, binds to the upstream regulatory region of the RNF43 promoter and its knockdown in HT-29 colorectal cancer resulted in decreased ubiquitination of the Wnt receptor, thereby increasing Wnt signaling [85]. More experiments are necessary to confirm DDB2's role in Wnt signal regulation, such as UV-DDB's binding affinity to the promoter sequence of the *Rnf43* gene.

In addition to DDB2's role in tumor formation, independent of its E3 ligase function, DDB2 has been suggested to inhibit epithelial to mesenchymal transition (EMT) in colon cancer [84]. Interestingly, there is a negative correlation between the grade of colorectal cancer and expression of DDB2 and E-cadherin, an epithelial cell marker. This was further investigated by examining the morphology and mesenchymal/epithelial markers in HCT116 cells expressing normal or low levels of DDB2: DDB2-deficient cells had mesenchymal-like features such as elongated morphology, increased vimentin, a mesenchymal marker, and decreased E-cadherin expression. Similar findings were demonstrated in a metastatic colorectal cancer cell line, SW620 compared to an early stage colorectal cancer cell line from the same patient, SW480 [84]. Furthermore, when colorectal cancer tumors expressing normal or low levels of DDB2 were transplanted or injected into mice and examined after 4 weeks, there was a higher incidence of lung and liver metastasis in mice that received DDB2-deficient cells [84]. These findings possibly indicate a role for DDB2 in suppressing metastasis *via* inhibition of EMT. Future studies should examine mice for metastatic sites in other organs and over longer lengths of time. Since cancer cells often use EMT to transition to metastasis, DDB2's role in EMT may suggest a function of DDB2 in preventing metastasis.

7. Outlook

UV-DDB, as part of CUL4-RBX E3 ligase, plays an essential role in the removal of UV-induced photoproducts in the context of chromatin during global genome NER. Recent work from our lab suggests that UV-DDB plays a non-canonical role in BER for the removal of 8-oxoG by the stimulation of OGG1 and APE1 [8]. Mammalian cells contain 11 glycosylases and it will be of great interest to determine whether UV-DDB can stimulate these enzymes during the removal of other forms of base damage [72]. More studies are needed to understand the complete damage repertoire of UV-DDB both in naked DNA and in the context of nucleosomes. Since UV-DDB has high affinity for undamaged DNA, it is possible that its binding to non-damaged sites in the genome affects chromatin structure and influences gene expression of critical cellular genes that are altered during cellular transformation. The increase in spontaneous tumors in DDB2 knockout mice [77,80], and their high sensitivity to UV-induced skin tumors [78], as well as the more surprising sensitivity to the azoxymethane/DSS model of colon cancer [85], needs to be studied in greater detail. If UV-DDB aids in the removal of alkylation damage in chromatin, then it is possible this latter result is due to a decrease in the removal of potentially mutagenic methylated bases. Many DNA glycosylases appear to be inhibited in their ability to remove damaged bases in the context of nucleosomes [86–90]. Since UV-DDB has been found to bind to abasic sites embedded in nucleosomes in any orientation [60] and also alter the

register of DNA lesions on nucleosomes [61], it is possible that UV-DDB binding directly to damage sites or through its associated E3 ligase activity may work to facilitate the removal of damaged bases during BER. The future holds great promise for investigation of this remarkable protein.

Note added in proof

While this manuscript was under consideration, three new cases of XP-E were reported. Two Chinese patients with the same homozygous deletion in *DDB2* resulting in a complete loss of the protein (Yang R, Kong Q, Duan Y, Li W, Sang H. *BMC Med Genet.* 2020 Mar 30;21(1):67.)

The second case resulted from a mutation found in a youth in Brazil resulting in a terminal truncation of the protein at residue 335 (Santiago KM, Castro LP, Neto JPD, de Nóbrega AF, Pinto CAL, Ashton-Prolla P, Pinto E Vairo F, de Medeiros PFV, Ribeiro EM, Ribeiro BFR, do Valle FF, Doriqui MJR, Leite CHB, Rocha RM, Moura LMS, Munford V, Galante PAF, Menck CFM, Rogatto SR, Achatz MI. *J Eur Acad Dermatol Venereol.* 2020 Apr 1.)

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dnarep.2020.102860.

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