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The Study of XPG Domains that Regulate the Late Steps of Nucleotide Excision Repair

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Adebanke Fidelia Fagbemi

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

Orlando D. Schärer, Ph.D. – Dissertation Advisor

Professor, Departments of Pharmacological Sciences and Chemistry

Arthur P. Grollman , M.D. – Chairman of Defense

Distinguished Professor, Department of Pharmacological Sciences

Daniel Bogenhagen, M.D.

Professor, Department of Pharmacological Sciences

Dale Deutsch, Ph.D.

Professor, Department of Biochemistry and Cell Biology

Carlos de los Santos, Ph.D.

Professor, Department of Pharmacological Sciences

This dissertation is accepted by the Graduate School

Lawrence Martin

Dean of the Graduate School

Abstract of the Dissertation

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The human nucleotide excision repair (NER) pathway resolves a range of lesions in DNA including those caused by harmful UV light, environmental mutagens, and agents of chemotherapy. The pathway involves the concerted action of over 30 proteins that recognize the damage, excise it within an oligonucleotide, and fill in the resulting gap, restoring the DNA to its original form. The importance of NER is evident in impaired patients who suffer from a range of diseases with symptoms such as high predisposition to skin cancer, extreme sensitivity to sunlight, and neurological and developmental abnormalities. While the early steps in NER involving the damage recognition and incision steps are well understood, a lot remains to be learned about the later steps including the transition from dual incision to repair synthesis, and finally ligation of the DNA.

XPG is the endonuclease that makes the 3' cut on the damaged DNA strand during dual incision. The protein has been suggested to play an important role in regulating the late steps of NER, as it interacts with the replication and repair synthesis factor PCNA. In this thesis the roles of four domains of XPG in the late steps of NER were analyzed. These domains include a previously identified PCNA-interacting domain (PIP-C), a new putative PCNA-interacting domain (PIP-N), a ubiquitin-binding motif (UBM), and the nuclease active site. The nuclease active site mutant (E791A) supported uncoupled incisions as well as partial repair synthesis, suggesting that the incisions could be ordered with the 5' incision occurring before the 3' incision, and that XPG itself is involved in regulating the transition from dual incision to repair synthesis. Additionally, the PIP-N and UBM domains were found to be important for damage removal, as well as assembly and disassembly of late factors of NER. The UBM mutant displayed a phenotype similar to the E791A mutant, suggesting that an interaction of XPG with ubiquitin is important to trigger its nuclease activity. This work reveals that the timing of the XPG incision plays an important role in regulating the late steps of NER, and that interactions with PCNA and ubiquitin are important elements of this regulation.

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LIST OF ABBREVIATIONS

(6-4)PP:	(6-4)photo-products
BER:	base excision repair
CAF-1:	chromatin assembly factor 1
CS:	Cockayne Syndrome
DAPI:	(4’-6’-diamino-2-phenylindole)
ERCC:	excision repair cross complementing
FEN1:	flap endonuclease 1
IF:	immunofluorescence
LUD:	local UV damage
NER:	nucleotide excision repair
PCNA:	proliferating cell nuclear antigen
PIP:	PCNA-interacting peptide
RFC:	replication factor C
RPA:	replication protein A
ssDNA:	single-stranded DNA
TFIIH:	transcription factor IIH
TLS:	translesion synthesis
TTD:	trichothiodystrophy
UBM:	ubiquitin-binding motif
UDS:	unscheduled DNA synthesis
UV:	ultraviolet
XAB2:	XPA binding protein 2
XP:	Xeroderma pigmentosum

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CHAPTER 1

Introduction

Adapted from the review titled "Regulation of endonuclease activity in human nucleotide excision repair" by Adebanke F. Fagbemi, Barbara Orelli and Orlando D. Schärer, accepted in the DNA Repair special issue (in press).

Abstract

Nucleotide excision repair (NER) is a DNA repair pathway that is responsible for removing a variety of lesions caused by harmful UV light, chemical carcinogens, and environmental mutagens from DNA. The NER pathway involves the concerted action of over 30 proteins that sequentially recognize a lesion, excise it in the form of an oligonucleotide, and fill in the resulting gap by repair synthesis. ERCC1-XPF and XPG are structure-specific endonucleases responsible for carrying out the incisions 5' and 3' to the damage respectively, culminating in the release of the damaged oligonucleotide. The introductory chapter of the thesis reviews the recent work that led to a greater understanding of how the activities of ERCC1-XPF and XPG are regulated in NER to prevent unwanted cuts in DNA or the persistence of gaps after incision that could result in harmful, cytotoxic DNA structures.

Introduction

Maintenance of the integrity of the genetic material or deoxyribonucleic acid (DNA) within living organisms is vital for the survival and evolution of all living species. The DNA is involved in the control of every aspect of the physiology of an organism and codes for the proteins that run all processes within it. DNA in living organisms, however, is under constant attack from both endogenous agents produced as a result of metabolic activities within the cell, such as reactive oxygen species [1, 2], as well as exogenous agents of damage such as UV from sunlight, ionizing radiation, or environmental mutagens [3, 4]. Damage to the DNA, if left unrepaired, interferes with normal cellular processes such as replication and transcription, and can lead to cell death by apoptosis, or, in the long run, introduce mutations in the genome that ultimately result in carcinogenesis [3].

Five main DNA repair pathways have evolved to deal with the different types of lesions: direct repair, base excision repair, nucleotide excision repair, mismatch repair and double-strand break repair by non-homologous end joining or homologous recombination [1, 4, 5]. Most of these repair processes involve removal of a lesion within the DNA by excision, often carried out by endonucleases that incise the DNA near a lesion. This thesis is focused on the nucleotide excision repair (NER) pathway, and specifically on the structure-specific endonuclease XPG, which makes the 3’ incision in NER. Regulation of the function of the

structure-specific endonucleases in NER is important to prevent unwanted cuts within the genome. Furthermore coordination of transition from one step of the pathway to the next is also of significance to prevent the occurrence of dangerous intermediates and will be addressed in this thesis.

Nucleotide Excision Repair

The pathway responsible for resolving bulky modifications of the DNA resulting from ultraviolet (UV) light or a variety of environmental chemicals is nucleotide excision repair (NER) [6, 7]. A number of genetic disorders resulting from mutations in NER genes, xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD), underscore the biological importance of this pathway [8]. Among these, XP is the prototypical DNA repair disorder and XP patients are extremely photosensitive due to their inability to repair UV-induced lesions and have a high predisposition to tumors in the skin, and, in severe cases, develop neurological disabilities (Figure 1) [8, 9]. These patients are classified into eight complementation groups (XP-A through XP-G, so named for the respective mutated gene, and XP-V for the variant form). The phenotypic manifestations of CS and TTD are more complex and involve severe developmental and neurological abnormalities [8], which are not caused by defects in NER, but are likely due to a mild impairment of transcription.

Figure 1: **Images of two Xeroderma pigmentosum patients displaying skin abnormalities resulting from sun exposure.** Xeroderma pigmentosum, a disease resulting from impaired NER, is characterized by extreme sensitivity to sunlight and subsequent high susceptibility to tumors in the skin and other exposed organs such as the eyes and tongue, as shown in the pictures [10, 11].

The NER pathway is highly versatile and complex and has the ability to process a myriad of different lesions [6]. Versatility in substrate recognition is accomplished by over 30 different proteins that sequentially assemble at sites of damage to recognize the lesion, excise it in the form of a 24-32-nucleotide-long stretch of ssDNA, fill in the gap using the non-damaged strand as a template and ligate the nick (Figure 2) [6, 12].

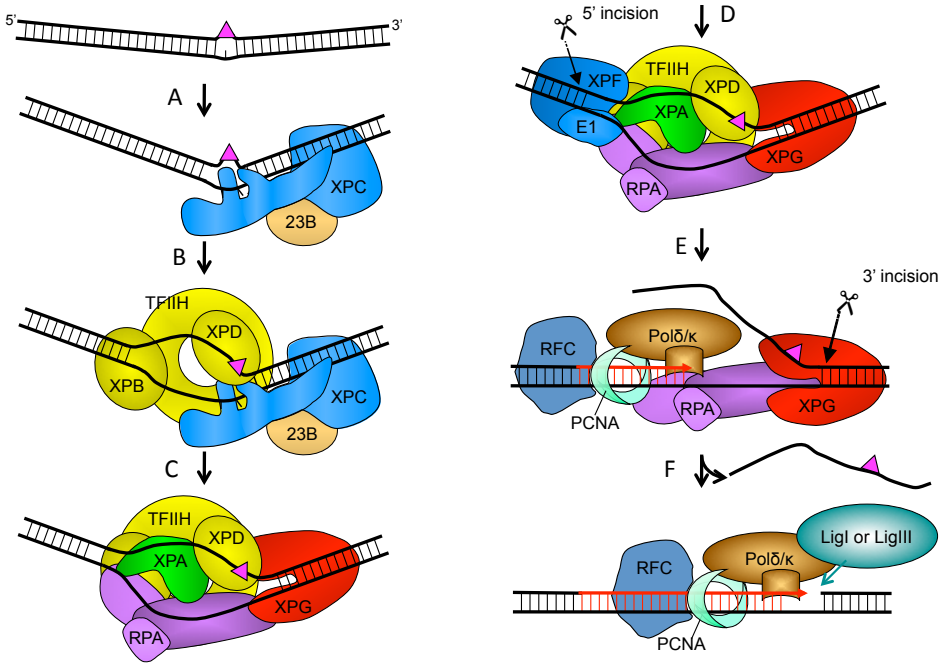

Figure 2: **Model for the nucleotide excision repair (NER) pathway.** (A) A bulky DNA lesion is recognized by XPC-RAD23B, which binds to the undamaged strand of DNA, allowing for the recruitment of TFIIH (B). The lesion is verified by XPD resulting in the recruitment of XPA, RPA, and XPG leading to formation of the preincision complex (C). ERCC1-XPF is recruited to the preincision complex through interaction with XPA leading to DNA incision 5' to the damage (D), which produces a free 3'-OH group available for inititation of repair synthesis by the replication machinery, and in turn triggers 3' incision by XPG (E). Repair synthesis is completed and DNA ligase IIIα or ligase I seals the nick to complete the process (F).

This chapter focuses on a key step in NER, the excision of the damaged oligonucleotide by two incisions made 5' and 3' to the lesion by the two structure-specific endonucleases ERCC1-XPF and XPG, respectively. This dual incision represents the first irreversible step in the pathway, formally resulting in the formation of a ssDNA gap of about 30 nucleotides. Since persistence of a 30-nucleotide gap or an incision in the absence of DNA damage is a possible deleterious side effect of this dual

incision reaction, it is crucial that the activity of ERCC1-XPF and XPG is tightly regulated. We will discuss emerging evidence that the timing and order of the incisions and the careful synchronization with repair synthesis is a built in feature of the NER pathway to prevent unwanted cuts within the genome.

Damage recognition and preincision complex formation primes the endonucleases for incision

Two sub-pathways of NER lead up to damage excision and they make use of pathway-specific factors and differ in their mode of damage recognition. Transcription-coupled NER (TC-NER) functions on actively transcribed strands, involves recognition of the lesion through stalling of RNA-Polymerase II, and additionally involves the recruitment of CSA, CSB and XAB2 factors to the damage prior to recruitment of core NER factors [13]. The second pathway, global genome NER (GG-NER) removes lesions throughout the genome, and is better understood at a mechanistic level. GG-NER is initiated by XPC-RAD23B, which recognizes the thermodynamic destabilization of a DNA duplex induced by a lesion. To achieve this, XPC-RAD23B does not interact with the lesion itself, but rather binds to DNA with single-stranded character opposite the lesion (Figure 2A) [14-16]. XPC then recruits the ten-subunit complex TFIIH, which contains two helicase subunits. It is thought that XPB pries apart the two DNA strands [17], while XPD tracks along the DNA with a 5'-3' polarity

stalling as it encounters the lesion ensuring the presence of a chemical alteration in the DNA (Figure 2B) [18, 19].

The result of TFIIH activity is an opened DNA structure [20, 21] that leads to the recruitment of the next three factors, XPA, RPA, and XPG, and formation of a stable preincision complex (Figure 2C). XPA is a small 273 amino acid protein originally thought to be the damage recognition factor. It was later found to have high affinity to kinked rather than damaged DNA, leading to the hypothesis that it interacts with an intermediate DNA structure in NER subsequent to damage recognition [22, 23]. XPA additionally interacts with several NER factors, including RPA [24], TFIIH [25, 26] and ERCC1 [27, 28]. It is therefore thought that XPA helps in positioning the different factors in the complex to allow dual incision to occur accurately. RPA is a trimeric protein that binds single-stranded DNA and has essential functions in replication and recombination [29]. Each RPA trimer has a preferred binding site of 30 nucleotides, which is strikingly similar to the size of the excised fragment in NER. Current models therefore place RPA on the non-damaged strand of DNA (Figure 2C-E), where it helps to position the two endonucleases, while protecting the non-damaged strand following incision [12]. At the same time, XPG is recruited to the damage region by interaction with TFIIH independently of XPA and RPA, to complete the formation of a stable preincision complex, resulting in the simultaneous release of XPC-RAD23B [30-32]. As we will discuss in more detail below, recent studies

suggest that the catalytic activity of XPG is not revealed at this point [21, 33] and XPG fulfills a structural role in stabilizing the pre-incision complex generating an open-stable complex [34].

The last factor to join the preincision complex is the second endonuclease, ERCC1-XPF, which is recruited through interaction with XPA [28, 35]. Following its arrival, a number of catalytic steps are triggered (Figure 2D-F): dual incisions 5’ and 3’ to the lesion by XPF and XPG, respectively [33]; repair synthesis filling in the gap by DNA polymerases δ, ε, or κ and associated factors [36] and finally, sealing of the nick by DNA ligase I or IIIα [37], restoring the DNA to its original undamaged state. The order and regulation of these catalytic steps, with an emphasis on the role of ERCC1-XPF and XPG is discussed below.

XPG is a latent endonuclease with structural and catalytic roles in NER

XPG is a FEN1 family nuclease member with a unique spacer region that regulates its function in NER

XPG belongs to the FEN1/XPG family of endonucleases, possessing the N- and I-nuclease domains that are highly conserved within the family (Figure 3A). Crystal structures of the family-members, T4 RNase H [38], T5 5’-exonuclease [39] and FEN1 [40, 41] suggested that

the N- and I-domains together form the nuclease core. In XPG, mutation of several conserved acidic active site residues, including D77, E791, and D812 abolished catalytic activity of the protein [42, 43]. However, XPG differs from FEN1 in that it contains a much larger (600 amino acid) spacer region that separates the N and I domains (Figure 3A) [44]. The spacer region is predicted to be highly disordered, has no known structural motifs, and is highly acidic. The spacer region mediates multiple protein-protein interactions, including with TFIIH [31, 45, 46], and RPA [47] and is therefore required for the recruitment of XPG to sites of NER [48].

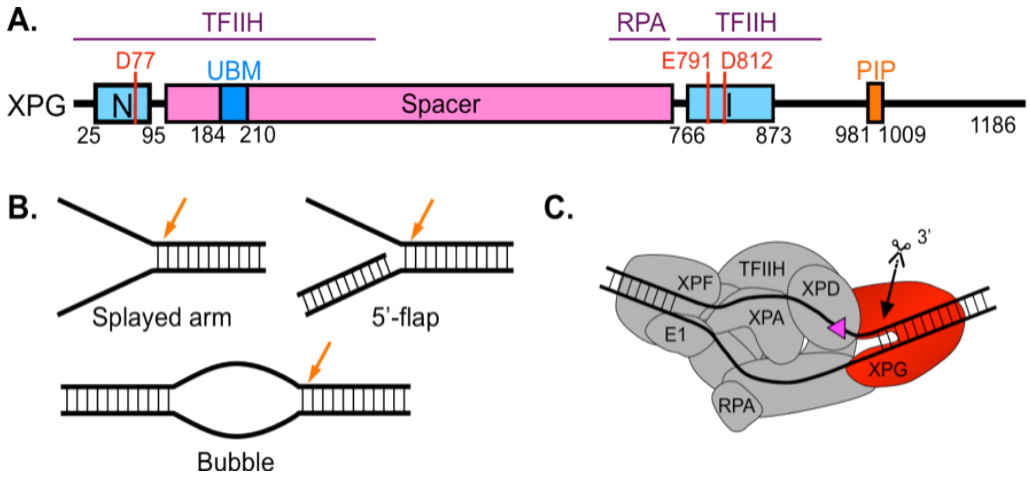

Figure 3: **The structure-specific endonuclease XPG.** (A) The primary structure of XPG shows the 1186 amino acid protein with its active site made up of the N and I regions (light blue) separated by the 600 amino acid spacer region (pink). Active site residues that were functionally analyzed (D77, E791, and D812) are shown in red. Regions for interaction with TFIIH, RPA, and PCNA (PIP) and the ubiquitin-binding motif (UBM) are indicated. (B) XPG cleaves 5' flap, splayed-arm and bubble substrates at ss/dsDNA junctions with 5' overhangs (orange arrows), and (C) makes the 3' incision in NER.

The spacer region of FEN1 is only about 70 amino acids long and forms a flexible helical loop. Biochemical and structural studies have suggested that the DNA threads through the loop and that the loop needs to undergo a structural rearrangement for FEN1 to become catalytically active [40, 49, 50]. It is likely that the XPG spacer region undergoes a similar rearrangement as it has been shown that XPG has distinct requirements for binding and cleaving DNA substrates [51]. Interestingly, replacing the spacer region of FEN1 with that of XPG generated a FEN1-XPG hybrid protein with biochemical characteristics of both proteins, able to cleave bubble substrates which are not processed by FEN1, while also showing a preference for double-flap over single-flap substrates, typical of FEN1 and not XPG [52]. Intriguingly, the FEN1-XPG hybrid protein was able to partially complement the NER activity in XPG-deficient cell lines, demonstrating that the spacer region has important roles in mediating substrate specificity and protein-protein interactions in NER. Other studies have attributed this effect of a contribution of the spacer region to the binding of bubble structures [53].

XPG is recruited to NER complexes through interaction with TFIIH

In the course of NER, XPG makes dynamic interactions with several other proteins in the NER pathway including RPA [47, 54], PCNA [55] and TFIIH [31, 45, 46, 48, 56]. The strongest interaction is the one with TFIIH [31], and is required for the recruitment of XPG to NER complexes [57]. The interaction between XPG and TFIIH seems to occur

through at least two different domains, located in the N-terminal/spacer region as well the C-terminal region of XPG [45]. Interestingly, the interaction of XPG and TFIIH does not only seem to be necessary to localize XPG to sites of repair, but also to complete NER, likely by ensuring that XPG is properly positioned to catalyze the 3' incision reaction. In line with this thinking, a protein corresponding to the patient allele of XPG with deletion of residues 225-231 (found in XP/CS patient XPCS1BD) had reduced affinity for TFIIH and was unable to restore UV sensitivity to XP-G cells, despite having normal nuclease activity [46]. Nonetheless, the XPGΔ225-231 protein colocalized with sites of UV damage initially, but its association within the NER preincision complex was unstable, not allowing for proficient NER. Observations that certain patient mutations in XPB and XPD subunits of TFIIH that result in NER deficiencies lead to a delayed recruitment of XPG to sites of UV damage underscore the complexity of the interaction between XPG and TFIIH [56].

Possible roles of XPG in TC-NER, transcription and the repair of oxidative damage

The biological complexity of XPG function is evidenced by the fact that patients with XPG deficiency may suffer from XP or combined XP/CS phenotype. Point mutations that primarily affect the nuclease of XPG result in an XP phenotype, while truncation mutations lead to an XP/CS phenotype, indicative of important protein-protein interactions at the C-terminus of the protein [58, 59]. The other factors that are also associated

with the XP/CS phenotype are the XPB and XPD subunits of TFIIH [8]. In line with this observation, a fraction of XPG appears to be stably associated with TFIIH, where it appears to support a role of TFIIH in receptor-mediated transcription (Table 1) [60, 61]. Furthermore, a role of the *S. cerevisiae* XPG homolog Rad2 in RNA polymerase II-mediated transcription has been demonstrated more directly [62]. Significantly, these transcription-related functions of XPG and Rad2 are independent of its nuclease function. The mechanisms by which XPG functions in TC-NER are the subject of active research and discussion and remain to be fully elucidated [53, 63-65]. Additionally, XPG is thought to have other roles in maintaining genome stability, including the modulation of removal of oxidative damage by base excision repair (BER) and possibly other pathways (Table 1) [66-68].

Pathway	Function(s)	Interacting Partners	References
GG-NER	Preincision complex formation; 3' incision	RPA, TFIIH, PCNA	[31, 45-47, 55, 69]
TC-NER	TC-NER complex assembly; 3' incision	TFIIH, PCNA, RNA Pol II, CSB	[53, 63, 64]
Transcription	?	RNA Pol II, TFIIH	[53, 60, 62]
BER	Stimulation of DNA glycosylase, other roles (?)	NTH1	[66, 67]

Table 1. Known functions of the XPG protein

ERCC1-XPF, the last factor arriving in the preincision complex, initiates dual incision

Properties, functional domains and structures of ERCC1-XPF

ERCC1-XPF forms an obligate heterodimer in mammalian cells [70] and *in vitro*, and the two proteins are unstable in the absence of each other [71, 72]. Both ERCC1 and XPF were originally cloned by complementation of UV sensitive rodent cell lines [73, 74] and by sequence homology to the yeast Rad1 gene [70]. ERCC1 and XPF dimerization occurs through two helix-hairpin-helix (HhH) motifs found at the C-termini of both proteins (Figure 4) [75, 76]. The HhH motifs are structurally similar to those found in other DNA binding proteins and also contribute to DNA binding in ERCC1-XPF [77-80]. Another important DNA binding region is believed to be the N-terminus of XPF, which bears the same structure as the helicase domain of the archeal superfamily 2 (SF2) helicases, but lacks the residues necessary for ATP hydrolysis [81, 82].

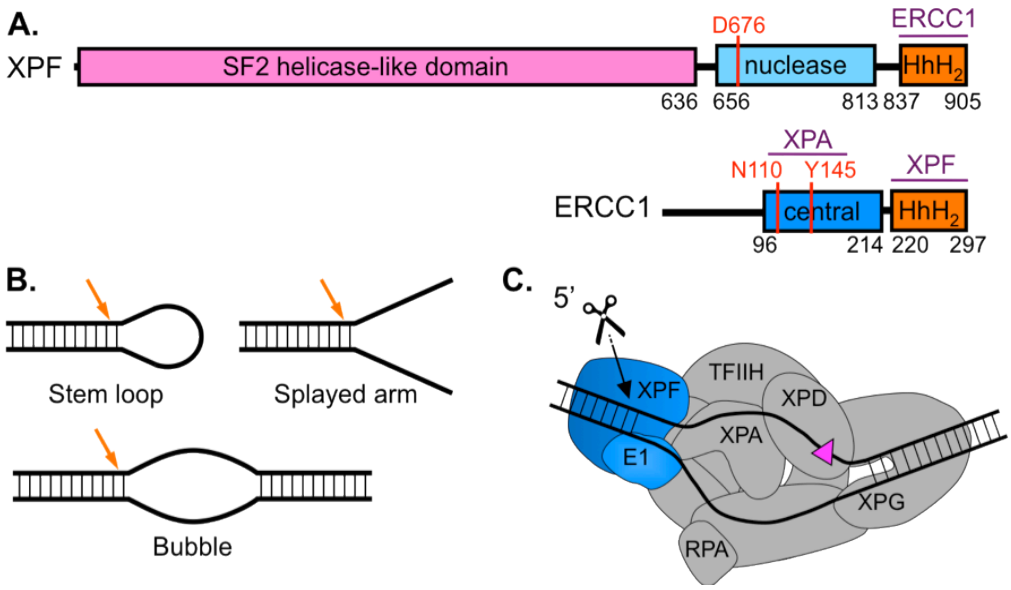

Figure 4. The structure-specific endonuclease ERCC1-XPF. (A) The primary structure of ERCC1-XPF shows the two members of the heterodimer that interact through their helix-hairpin-helix regions (orange). The nuclease active site XPF is indicated in light blue and the active site residue D676 in red. The central domain of ERCC1 (blue) interacts with XPA with the residues that mediate this interaction highlighted in red. The helicase-like domain of XPF (pink) belongs to the SF2 family of helicases, but has a defective ATP binding site. Interaction regions for heterodimer formation and with XPA are shown. (B) ERCC1-XPF cleaves stem-loop, splayed-arm and bubble substrates at ss/dsDNA junctions with 3' ssDNA overhangs (red arrows), and (C) makes the 5' incision in NER.

The first indication that ERCC1-XPF carries out the 5' incision in NER was provided by studies of the yeast homologs Rad1-Rad10, which were found to cleave ss/dsDNA junctions with 3' ssDNA overhangs [83]. An equivalent role was subsequently shown for the human ERCC1-XPF protein [70, 74, 84]. Subsequently, work from our laboratory revealed that the nuclease active site of the heterodimer resides within the XPF subunit of the complex, and contains the highly conserved V/IERKX$_3$D signature motif [85, 86]. The cluster of acidic residues allows the coordination with

metal ions necessary to catalyze the incision in the DNA strand, and mutations of key residues affect nuclease, but not DNA binding activity.

Intriguingly, it was suggested that ERCC1 has evolved through gene duplication from the XPF gene in lower eukaryotes, as XPF forms a homodimer in archaea [75]. Indeed, the crystal structure of the ERCC1 central domain revealed a high structural homology with the nuclease domain found in archeal orthologs of XPF [77, 87]. However, the groove that harbors the catalytic residues in XPF is rich in aromatic and basic amino acids in ERCC1 implicating a role in DNA or protein binding.

An Interaction with XPA is essential for recruitment of ERCC1-XPF to NER complexes

The ERCC1-XPF heterodimer interacts specifically with XPA, and this interaction is necessary for functional NER. The first evidence of this interaction came from yeast two-hybrid and pull-down assays [27, 88], which revealed that amino acid residues 91-119 of ERCC1, and 75-114 of XPA are necessary for the interaction and for NER to take place [27]. Three highly conserved glycine residues, G72, G73, and G74 of XPA were found to be necessary for the interaction and deletion of these residues resulted in loss of the ability of the XPA protein to restore UV resistance to XP-A cells [88]. More recent studies revealed that a small peptide of XPA (residues 67-80) is necessary and sufficient for mediating the interaction with ERCC1 [28]. Structural studies revealed that this peptide, which is

unstructured in solution, undergoes a transition to assume a turn made up of G72-74 and F75 upon binding to a cleft lined by residues N110, Y145, and Y152 in the central domain of ERCC1 (Figure 5A) [28]. Importantly, this 14 residue XPA peptide acts as a specific inhibitor of NER in cell-free extracts by blocking access of endogenous XPA to the ERCC1 binding pocket. This inhibitory effect was found to be highly specific as the mutant XPA-F75A peptide does not interfere with the NER reaction highlighting the importance of the F75 residue in mediating the XPA-ERCC1 interaction (Figure 5B) [28].

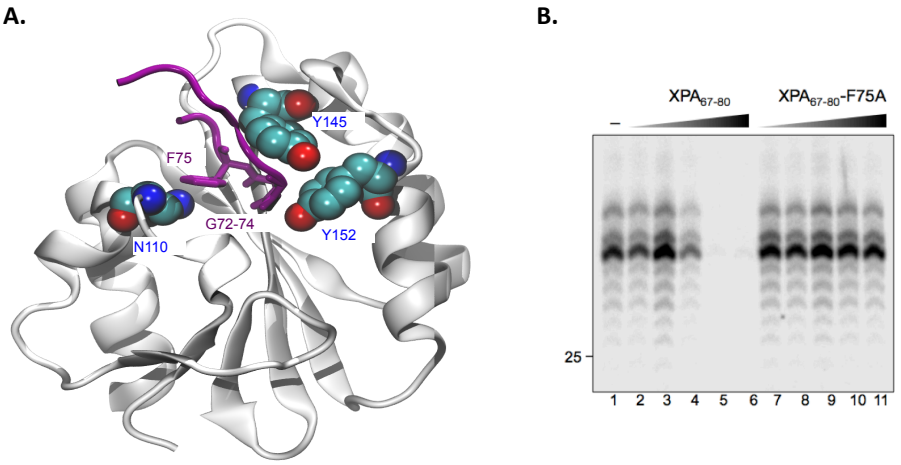

Figure 5. The ERCC1-XPA interaction is required for NER. (A) The structure of an XPA peptide (residues 67-80 shown in purple) bound to the central domain of ERCC1 (gray). The peptide undergoes a disorder to order transition upon binding ERCC1. The residues in XPA and ERCC1 that are important for this interaction are highlighted in pink and atom color, respectively. Figure adapted from [28, 35] (B) The XPA_{67-80} peptide inhibits the *in vitro* NER reaction. Addition of increasing concentration of the XPA_{67-80} peptide to a cell-free extract inhibits the excision of the cisplatin-contining oligonucleotide from a plasmid by NER. The XPA peptide containing the F75A mutation has no effects on the NER reaction, indicating the importance of the F75 residue in the ERCC1-XPA interaction. The NER reaction is visualized by radiolabeling excised DNA fragments, resulting in a characteristic pattern of 28-34mer oligonucleotides, which are separated on a DNA sequencing gel and visualized by autoradiography. Figures adapted from [28] and [35].

Conversely, mutation of the ERCC1 residues N110, Y145, and Y152, located within the XPA-binding pocket in the central domain of ERCC1, resulted in reduced NER activity of the ERCC1-XPF complex *in vitro* [35]. The most severely affected mutant ERCC1-N110A/Y145A, failed to colocalize with other NER proteins at sites of UV damage in cells, indicating a deficiency in the interaction with XPA under physiological conditions. Significantly, ERCC1-deficient UV20 CHO cells transduced with ERCC1-N110A/Y145A displayed significant sensitivity to UV, consistent with a defect in NER [35]. An intriguing question was whether these mutations would also affect other roles of ERCC1-XPF in ICL repair and homologous recombination, which are believed to be at least partially responsible for the severe phenotypes observed in some ERCC1- and XPF-deficient mice and patients that are not just due to defects in NER (Table 2) [89, 90]. ERCC1-N110A/Y145A expressing cells were not sensitive to other types of damage including mitomycin C- and cisplatin-induced interstrand crosslinks (ICLs) and ionizing radiation-induced double-strand breaks (DSBs), indicating that the XPA interaction and the XPA binding pocket are not required for these functions of ERCC1-XPF. Therefore, NER-specific functions of ERCC1-XPF can be separated by mutation of the XPA binding pocket.

Pathway	Function	Interacting Partners	References
NER	5’ incision	XPA	[27, 28, 70]
Interstrand Crosslink Repair	ICL unhooking, homologous recombination (?)	SLX4	[91-94]
DSB Repair/ ssDNA annealing	Flap cutting (?)	SAW1, SLX4	[95-97]
Telomere maintenance	?, Nuclease-independent	TRF2	[98-100]

Table 2. Known functions of the ERCC1-XPF protein

Is there a defined order of the two incisions in NER?

Once all the preincision factors are in place in the NER complex, the question arises whether the two incisions occur simultaneously or consecutively in a defined order. There are two main arguments why it would be advantageous for the two incisions to occur in an ordered fashion; first, simultaneous incisions could result in the instantaneous release of the damaged oligonucleotide, exposing a ssDNA gap in the non-damaged strand. ssDNA patches may activate DNA damage responses [101] and are considered to be more unstable than bulky DNA lesions in intact DNA. Secondly, consecutive incisions would allow a tighter regulation of the transition from the incision to repair synthesis steps. If the 5’ incision occurred first, this would provide a free 3’-OH tail

for the polymerase to initiate replication, while still protecting the DNA from exposure until the 3' incision is made. By contrast, making the cut 3' to the lesion would not set the system up for repair synthesis.

As will be described in Chapter 2 of this thesis, a recent study by our group using catalytically inactive forms of XPG and XPF showed that the incisions may indeed be ordered. In agreement with earlier studies [42, 43], using nuclease-dead XPG-E791A in an *in vitro* repair assay resulted in the formation of 5' uncoupled incision products (Figure 7). By contrast, the use of nuclease-dead XPF-D676A resulted in negligible 3' incision products [21, 33]. Interestingly, when XPG-E791A was used in an *in vitro* repair synthesis assay, formation of products of partial repair synthesis were observed, suggesting that after 5' incision the replication machinery had extended the incised DNA strand by 18-20 nucleotides from the 5' incision, even in the absence of the 3' incision (Figure 8). Furthermore, *in vivo* immunofluorescence experiments utilizing XP-F and XP-G cell-lines stably expressing the respective mutants, revealed that XPG-E791A supported recruitment of replication factors PCNA and Polδ and chromatin remodeler CAF-1 to sites of local UV lesions, whereas neither were visible in XPF-D676A cells (Figures 10 and 11) [33]. These findings suggest a model for the late steps in NER in which the 5' cut by XPF is made first, repair synthesis is initiated, followed by the 3' cut by XPG, and completion of repair synthesis, with ligase finally sealing the nick.

In contrast with these findings, 3' uncoupled incisions by XPG in the absence of ERCC1-XPF have been observed using a variety of substrates [20, 34, 102, 103]. While this discrepancy has not yet been resolved, one possible explanation could be that ERCC1-XPF and XPG can incise NER intermediates *in vitro* under conditions that do not reflect the *in vivo* situation. It is known that both endonucleases are able to cleave model NER intermediates *in vitro* in the absence of additional proteins [51, 69, 70]. It is therefore possible that excess amounts of XPG, or the absence of ERCC1-XPF in a cell extract could allow the 3' incision by XPG in the absence of a properly assembled complex.

Factors contributing to the coordination of dual incision and repair synthesis

The fact that repair synthesis can occur prior to XPG incision, suggests that there might be a trigger that renders XPG catalytically active. Consistent with this notion, XPG has distinct requirements for binding and cleaving its substrates, which are mediated by its spacer region [48, 51] and it has also been shown that the XPG family member FEN1 undergoes a conformational change to become catalytically active [49]. What are potential factors that could facilitate this transition? In principle the transition could be due to a simple structural rearrangement caused by the approaching DNA polymerase, but there is increasing evidence that additional protein factors may contribute to and facilitate the transition.

One of the factors that has a key role in dual incision and repair synthesis is RPA. Studies have shown that RPA remains bound to sites of NER after dual incision and that it can stimulate the XPG incision on model substrates and is therefore a strong candidate for the regulation of XPG incision activity [104, 105]. Perhaps an even more attractive candidate is PCNA. PCNA has been shown to interact with XPG [55], and has been established to play a key role in triggering incision of DNA by FEN1 during the resolution of Okazaki fragments during replication [106, 107]. In NER, such an interaction between XPG and PCNA could be involved in triggering 3' incision by XPG. Our data showing that repair synthesis proceeds a little more than half way through the repair patch in the presence of catalytically inactive XPG [33] suggests that there is an obstacle that blocks the DNA polymerase. This situation is reminiscent of replicative polymerases stalling at DNA lesions. This is known to result in PCNA ubiquitylation, which results in the recruitment of translesion synthesis (TLS) polymerases [108]. Two observations suggest that a related mechanism could apply during NER. First, a recent study by Ogi *et al.* showed that three different polymerases, polδ, polκ, and polε are responsible for repair synthesis in NER, and that selective recruitment of at least polκ is dependent on functional Rad18 and PCNA ubiquitylation [36]. Second, sequence analysis of XPG shows that, like TLS polymerases [109], it contains a ubiquitin-binding motif (UBM) [110] in addition to its PIP domain for PCNA interaction [55], raising the possibility

that an interaction between ubiquitylated PCNA and XPG could be involved in triggering 3' incision in NER. To what extent such protein-protein interactions among PCNA, XPG, Polκ and other factors contribute to the completion of repair synthesis and possibly to the triggering of UV induced DNA damage signaling are intriguing areas for future investigations [111]. The focus of this thesis is the relevance of the interaction of XPG with ubiquitin (via its UBM domain) [110], and with PCNA via the previously identified C-terminal PIP domain (PIP-C) [55] and a new putative N-terminal PIP domain (PIP-N) that we have identified.

Conclusions

Structure-specific endonucleases are important for maintaining the integrity of DNA in many DNA repair pathways. ERCC1-XPF and XPG are two such endonucleases and make the incisions 5' and 3' to a lesion in the NER pathway, respectively. The work discussed in this chapter has helped to understand how the function of these two proteins is tightly regulated such that the inadvertent incision of DNA is avoided and that nicks or gaps formed as reaction intermediates are promptly subjected to an appropriate repair synthesis process to avoid the formation of deleterious intermediates. Much remains to be done to understand exactly how the dual incision and repair synthesis processes are coordinated to complete the NER process.

Preview

This thesis addresses the role of the structure-specific endonuclease XPG, which performs the 3' incision in NER in coordinating the late steps in NER, the transition from dual incision to repair synthesis. Chapter 2 describes studies showing that the presence of XPG, but not its catalytic function, is necessary for XPF to perform the 5' incision, and importantly that the replication machinery is recruited prior to the 3' incision by XPG. These findings strongly support the hypothesis that XPG somehow mediates the transition from dual incision to repair synthesis. Chapter 3 describes studies investigating the role of different domains in XPG that may mediate this function of the protein. Analysis of three different domains of XPG, two PCNA-interacting domains, and one ubiquitin-binding motif, reveals that the interaction between XPG and PCNA, and between XPG and ubiquitin is indeed important for the transition from dual incision to repair synthesis, possibly in the context of ubiquitylated PCNA. Conclusions and future directions are discussed at the end of the thesis.

CHAPTER 2

Coordination of Dual Incision and Repair Synthesis in Human Nucleotide Excision Repair

Adapted from the manuscript by Lidija Staresincic, Adebanke F. Fagbemi, Jacqueline H Enzlin, Audrey M. Gourdin, Nils Wijgers, Isabelle Dunand-Sauthier, Giuseppina Giglia-Mari, Stuart G. Clarkson, Wim Vermeulen, and Orlando D. Schärer, published in The EMBO Journal in 2009, Vol 28, pages 1111-1120

Abstract

Nucleotide excision repair (NER) requires the coordinated sequential assembly and actions of the involved proteins at sites of DNA damage. Following damage recognition, dual incision 5' to the lesion by ERCC1-XPF and 3' to the lesion by XPG leads to the removal of a lesion-containing oligonucleotide of about 30 nucleotides. The resulting single-stranded DNA gap on the undamaged strand is filled in by DNA repair synthesis. Here, we have asked how dual incision and repair synthesis are coordinated in human cells to avoid the exposure of potentially harmful single-stranded DNA intermediates. Using catalytically inactive mutants of ERCC1-XPF and XPG, we show that the 5' incision by ERCC1-XPF precedes the 3' incision by XPG and that the initiation of repair synthesis does not require the catalytic activity of XPG. We propose that a defined order of dual incision and repair synthesis exists in human cells in the form of a "cut-patch-cut-patch" mechanism. This mechanism may aid the smooth progression through the NER pathway and contribute to genome integrity.

Introduction

Nucleotide excision repair (NER) is a versatile DNA repair pathway that enables cells to eliminate a plethora of helix-distorting lesions caused by different environmental agents. Versatility and specificity in NER are achieved through the sequential and highly coordinated actions of at least 30 polypeptides that detect the lesion and excise a damage-containing oligonucleotide followed by repair synthesis and ligation events to restore the DNA sequence to its original state [6, 7, 12]. A subpathway of NER, transcription-coupled NER (TC-NER), preferentially removes damage from the transcribed strand of active genes and is initiated through stalling of an elongating RNA polymerase at DNA lesions [112, 113]. In bulk DNA, XPC-RAD23B appears to be the initial sensor of DNA damage and is essential for the assembly of all subsequent NER factors in the process known as global genome NER (GG-NER) [14, 57, 114, 115]. TFIIH, the next factor to be recruited, is responsible for strand separation around the lesion [20, 30, 116], enabling XPA, RPA and XPG to join the complex. ERCC1-XPF is then engaged [21, 28, 117] to perform the incision 5' to the damage [70, 83] while XPG cleaves 3' to the lesion [69]. An oligonucleotide of 24-32 nucleotides in length containing the lesion is then released and the resulting gap is filled by DNA polymerase δ/ε (and/or possibly κ) [118], replication factor C (RFC), PCNA, RPA and the nick is sealed by DNA ligase I or DNA ligase III/XRCC1 [37, 119] to restore the original DNA sequence. At a higher level of organization, chromatin assembly factor 1

(CAF-1) has been implicated in the restoration of chromatin following the repair reaction [120].

While many recent studies have been concerned with the mechanisms of damage recognition [15], less is known about the coordination of the two incision and the repair synthesis steps. For repair synthesis to occur, the 5' incision by ERCC1-XPF is required to generate a free 3'-OH group, the substrate for the DNA polymerase. By contrast, the 3' incision by XPG may not necessarily be needed to initiate polymerization. If both incisions occurred without any DNA repair synthesis, simple release of the oligonucleotide containing the damaged residue could result in the formation of a single-stranded DNA (ssDNA) gap, another deleterious DNA lesion with a key role in activating DNA damage signaling pathways [121]. Furthermore, the occurrence of aberrant DNA breaks is associated with inadvertent NER activity at non-damaged sites in XP/CS cells, underscoring the importance of avoiding the formation of unwanted NER incision reactions [122, 123]. It therefore appears likely that a mechanism ensuring the smooth transition between the dual incision and repair synthesis steps would have evolved.

The similar kinetics of the damage removal and repair synthesis indeed suggests a coordination of these two events [32]. Analysis of the literature, however, reveals that there is no consensus concerning the order of the two incisions. Although there is agreement that the 5' and 3' incisions are made in a near-synchronous manner [124], both 5'

uncoupled [103, 124] and 3' uncoupled [20, 34, 102] incisions have been observed in different experimental contexts *in vitro*. Using catalytically inactive forms of XPG it has been shown that the presence of XPG, but not its catalytic activity, is required for the generation of the 5' incision by ERCC1-XPF [42, 43]. Another study showed that the efficient 3' incision by XPG required the presence and catalytic activity of ERCC1-XPF [21].

Here we report the use of catalytically inactive mutants of XPF and XPG to establish the relative temporal order of the two incision reactions and DNA repair synthesis in human cell-free extracts and cells. The results suggest a novel "cut-patch-cut-patch" mechanism whereby the dual incision and repair synthesis events of NER are highly coordinated. In turn, this mechanism can explain how the potentially dangerous effects of ssDNA intermediates are minimized or even prevented.

Results

Active site mutants of XPG and XPF do not support dual incision

To try to determine if there is a strict temporal order to the 5' and 3' incisions in human NER, we made use of mutants of ERCC1-XPF and XPG that are catalytically inactive, but retain full DNA binding ability, with the hope of trapping NER intermediates. Three useful active site mutants of XPG have previously been reported. These D77A, E791A and D812A proteins do not display 3' nuclease activity and prevent dual incision in NER *in vitro*, but allow 5' incision by ERCC1-XPF to occur [42, 43]. We

have recently characterized the active site of human XPF and constructed several mutants with severely impaired endonuclease activity while retaining full DNA binding activity [86]. Here, we further characterized these mutants to select a catalytically inactive mutant devoid of NER dual incision activity analogous to the previously characterized XPG mutants. Hence, we tested the ability of these purified recombinant proteins, expressed as heterodimers with ERCC1, to restore NER activity in XP-F deficient cell extracts [124].

The wild-type, R678A, R681A, E701A and R715A XPF proteins fully restored the excision of a lesion-oligonucleotide from a plasmid containing a site-specific 1,3-intrastrand d(GpTpG) cisplatin DNA crosslink (Figure 6, lanes 1, 3, 5, 6 and 9, respectively), whereas E679A displayed residual activity (Figure 6, lane 4). By contrast, the D676A, D704A, E714A, K716A and D720A XPF mutants (Figure 6, lanes 2, 7, 8, 10 and 11, respectively) failed to restore any NER activity. An earlier study using a fully reconstituted system showed that XPF-D676A was devoid of any NER activity, whereas D720A had some residual activity [21].

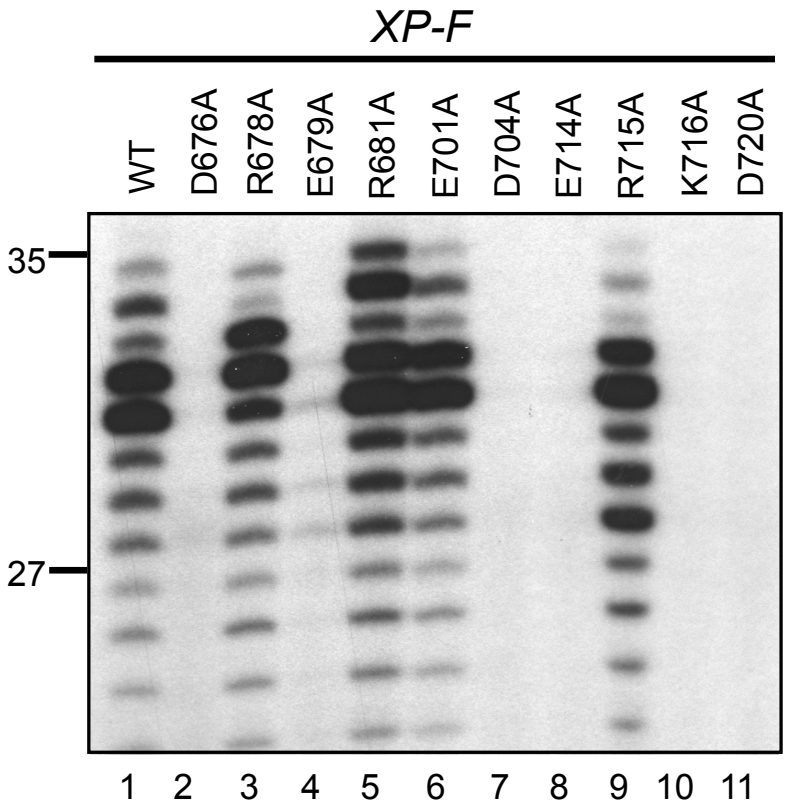

Figure 6. XPF Active Site Mutants Deficient in NER *in vitro.* Cell extracts prepared from XPF-deficient XP2YO cells were incubated with a plasmid containing a single 1,3 cisplatin intrastrand crosslink in presence of 200 fmol purified recombinant wild-type ERCC1-XPF (lane 1) or ERCC1-XPF proteins with different point mutations in XPF (lanes 2-11). The excision products containing the cisplatin adduct were labeled by the annealing of an oligonucleotide complementary to the excised oligonucleotides with a G_4 overhang and filling in with Sequenase 2.0 and [α-^{32}P] dCTP. The products were separated on a 14% denaturing polyacrylamide gel and visualized by autoradiography. The positions of size markers are indicated on the left.

Efficient 3' incision by XPG is dependent on prior 5' incision by ERCC1-XPF

We used ERCC1-XPF-D676A and XPG-E791A for further studies to discern any possible interdependency of the 5' and 3' incision steps. Covalently closed circular DNA containing a single 1,3-intrastrand cisplatin

DNA crosslink was incubated with an XPF- or XPG-deficient cell-free extract complemented with wild-type or nuclease-deficient ERCC1-XPF and XPG proteins. The reaction products were purified and cleaved with *Bss*HII to excise a 190 bp fragment from the plasmid DNA. Incision products were detected by annealing an oligonucleotide complementary to the *Bss*HII incision site 3' to the lesion followed by a fill-in reaction to generate a fragment of approximately 130 nt for the 5' uncoupled incision by XPF and a 100 nt fragment for incision by XPG, in the presence or absence of the 5' incision (Figure 7A).

Incubation of the plasmid with XP-G or XP-F cell extract did not yield any incision products (Figure 7B, lanes 2 and 5, respectively). When the extracts were complemented with wild-type XPG or XPF proteins (lanes 3 and 6, respectively), products specific for 3' incision by XPG (3 bands around 100 nt) were visible. Addition of XPF-D676A to the XP-F cell extract only yielded marginal amounts of 3' incision products (lane 7), suggesting that 3' uncoupled incision by XPG does not occur efficiently in the presence of catalytically inactive XPF. By contrast, addition of XPG-E791A to the XP-G cell extract resulted in the appearance of two intense specific bands (of around 130 nt in length) corresponding to the product of XPF 5'-uncoupled incision (lane 4). These results indicate that efficient 3' incision by XPG is dependent on the presence and catalytic activity of ERCC1-XPF, while the presence, but not the catalytic activity of XPG is

required for the 5' incision by ERCC1-XPF. Hence, the 5' incision might precede the 3' incision.

DNA Repair Synthesis can be initiated *in vitro* without the 3' incision by XPG

To determine if both 5' and 3' incisions need to occur before DNA repair synthesis can be initiated, we investigated the nature of the repair synthesis products in XP-G cell extracts complemented with wild-type XPG and XPG-E791A, by incubating a plasmid containing a single defined cisplatin lesion in the extracts together with [α-^{32}P]-dCTP and [α-^{32}P]-TTP. After the reaction, DNA was purified and digested with *Kpn*I and/or *Sac*I or *Xho*I and/or *Bsr*BI (Figure 8A).

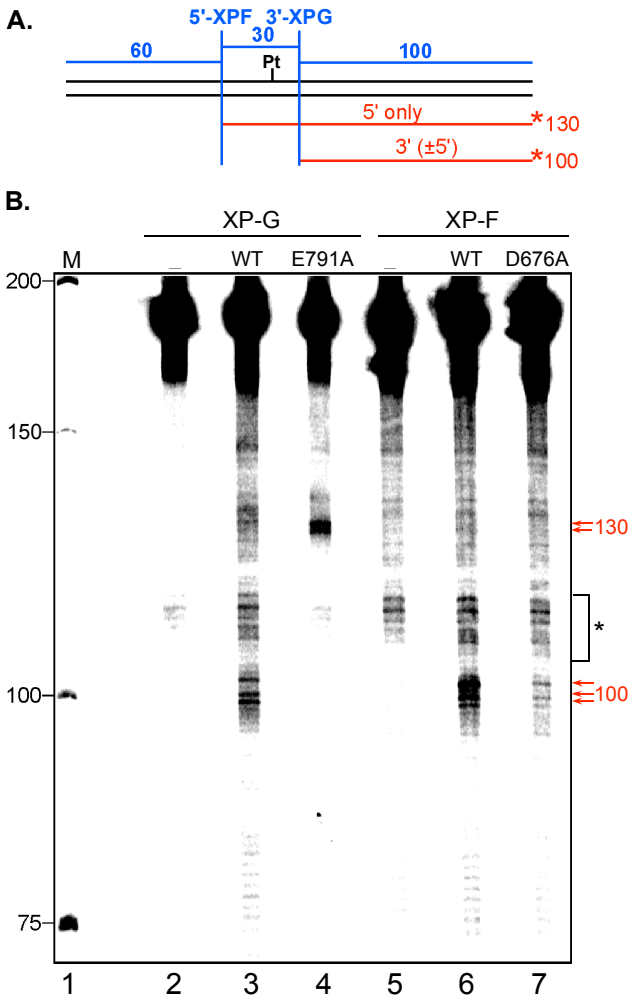

Figure 7. Efficient XPG cleavage is dependent on the catalytic activity of XPF. (A) Schematic representation of a 190bp *Bss*HII fragment with a single defined cisplatin lesion. Incision sites by ERCC1-XPF and XPG are indicated. Incisions were detected using fill-in reactions with Sequenase 2.0 and [α-^{32}P] dCTP by annealing an oligonucleotide complementary to a *Bss*HII cleavage site containing a G_4 overhang allowing for the visualization of the 130 and 100 mer products for 5' and 3' incision respectively. Possible excision products are indicated in red and the position of the [α-^{32}P] label is indicated by an asterisk. **(B)** cccDNA with a single defined cisplatin lesion was incubated with cell extracts lacking XPG- (XPCS1RO, lanes 2-4) or XPF-deficient (XP2YO, lanes 5-7), either alone (lanes 2 and 5) or complemented with wild-type XPG (lane 3), XPG E791A (lane 4), wild-type XPF (lane 6), XPF D676A (lane 7), purified, digested with *Bss*HII, radioactively labeled and analyzed on a denaturing PAGE gel. The positions of size markers are indicated on the left, and the position of the reaction products on the right of the gel. Unspecific bands present in all the lanes are marked by asterisk.

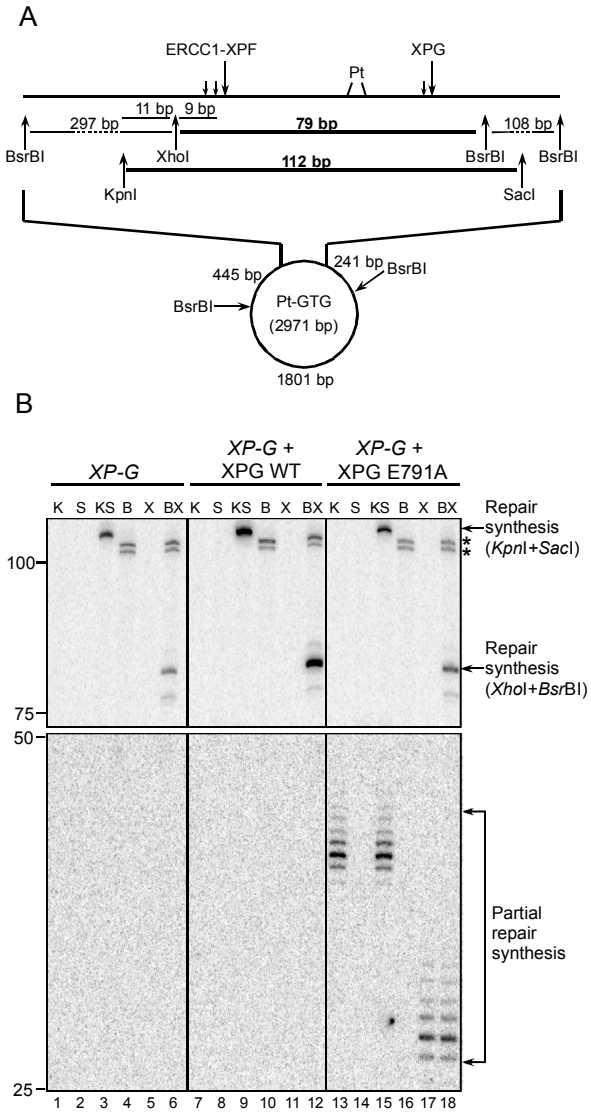

Figure 8. XPG E791A Supports Partial DNA Repair Synthesis *in vitro*. (A) Schematic representation of the lesion-containing plasmid used in the assay. Incision sites by ERCC1-XPF and XPG and the sizes of restriction fragments are indicated. The fragments containing the repair synthesis products are shown in bold (79 bp and 112 bp). **(B)** cccDNA with a single defined cisplatin lesion was incubated with a XP-G cell extract, either alone (lanes 1-6) or in the presence of 600 fmol of wild-type XPG (lanes 7-12) or XPG E791A (lanes 13-18) as well as 10 µCi of [α-^{32}P]dCTP and 10µCi [α-^{32}P]TTP. DNA was further purified, digested with *Kpn*I (lanes 1, 7, 13), *Sac*I (lanes 2, 8, 14), *Kpn*I+*Sac*I (lanes 3, 9, 15), *Bsr*BI (lanes 4, 10, 16), *Xho*I (lanes 5, 11, 17), *Bsr*BI+*Xho*I (lanes 6, 12, 18) and analyzed on a denaturing PAGE gel. The positions of size markers are indicated on the left, the nature of the observed products on the right of the gel. Two unspecific bands are marked by asterisk. Abbreviations: K= *Kpn*I, S= *Sac*I, KS= *Kpn*I+*Sac*I, B= *Bsr*BI, X= *Xho*I, BX= *Bsr*BI+*Xho*I.

When DNA was incubated with the XP-G deficient cell extract alone, products of nonspecific DNA synthesis were observed with signal intensities roughly proportional to the length of the DNA fragments (Figure 8B, lanes 1-6). These signals are likely due to random nicks produced by topoisomerases and/or nucleases present in the cell-free extracts [125]. Addition of wild-type XPG to the mixture led to a significant increase in the intensity of the bands of 112 nt (*Kpn*I and *Sac*I) and 79 nt (*Bsr*BI and *Xho*I), corresponding to newly synthesized and ligated DNA at the site of the cisplatin lesion (lanes 9 and 12, respectively). Note that the 79 nt signal of the *Bsr*BI/*Xho*I digestion in lane 12 is much more intense than the non-specific signal at 108 nt, strongly suggesting that the 79 bp band results from XPG-induced repair synthesis. The specificity of the signal for repair synthesis was further supported by the observation that no increase of the specific band at 79bp was seen if the reaction was carried out with the parental non-damaged plasmid (data not shown). Addition of nuclease deficient XPG-E791A, which permits incision by ERCC1-XPF, to the XP-G cell extracts, did not result in a change in the intensity of the full-length repair synthesis products of 112 bp and 79 bp. However, two new products with the most intensive bands of 39 nt (*Kpn*I and *Sac*I) and 28 nt (*Bsr*BI and *Xho*I) were visible after a longer exposure of the gel (lanes 15 and 18, respectively). These bands were also present after the cleavage with only one restriction enzyme 5' to the damaged site (lanes 13 and 17), while no products were visible after cutting with restriction enzymes 3' to

the incision sites (lanes 14 and 16). The appearance of these bands is therefore consistent with their being partial repair synthesis products, in which the polymerase extended the 3'-OH group generated by ERCC1-XPF about 18-20 nt in the absence of XPG incision. These observations indicate that initiation of repair synthesis is dependent on cleavage by ERCC1-XPF and that it can occur prior to 3' cleavage by XPG. No partial repair synthesis products were observed when an extract made from XP-F cells was complemented with wild-type XPF or XPF-D676A (data not shown). These results demonstrate that repair synthesis can be initiated *in vitro* prior to the 3' incision by XPG.

Catalytically inactive XPF persists at sites of UV damage

Having observed partial repair synthesis without XPG cleavage *in vitro*, we wished to test whether repair synthesis could also be initiated *in vivo* prior to 3' incision by XPG. For repair synthesis to take place, the DNA replication machinery has to be recruited to the sites of DNA damage. Therefore, we examined the localization of PCNA, a component of the DNA replication machinery, after local UV irradiation of a XP-G cell line expressing wild-type XPG or XPG-E791A and a XP-F cell line expressing wild-type XPF or XPF-D676A. We have previously described the generation and characterization of XP-G cell lines expressing wild-type XPG and XPG-E791A using lentiviral transduction. Both XPG proteins were localized to UV-damaged spots in cell nuclei shortly after damage infliction, but only XPG-E791A persisted in these spots, suggesting that

completion of NER is required for the dissociation of XPG [46]. XP-F cell lines expressing wild-type XPF and XPF-D676A were generated in an analogous fashion by transducing XPF-deficient XP2YO cells with lentiviral recombinants encoding HA-tagged wild-type XPF and XPF-D676A. The resulting cells were subjected to local UV irradiation and the recruitment of XPC, the initial damage recognition factor, and XPF to sites of UV damage was analyzed [57].

At 0.5 hrs after irradiation, we observed co-localization of XPC with both HA-tagged wild-type XPF and XPF-D676A (Figure 9A). 3 hours after local UV irradiation, XPC and XPF were no longer present at the damaged sites in the cells expressing wild-type XPF. However, in the mutant XPF transductants XPC remained co-localized with the catalytically deficient XPF-D676A (Figure 9B). Hence, cleavage by ERCC1-XPF and XPG is needed for both nucleases to dissociate from the damaged site and for the completion of NER.

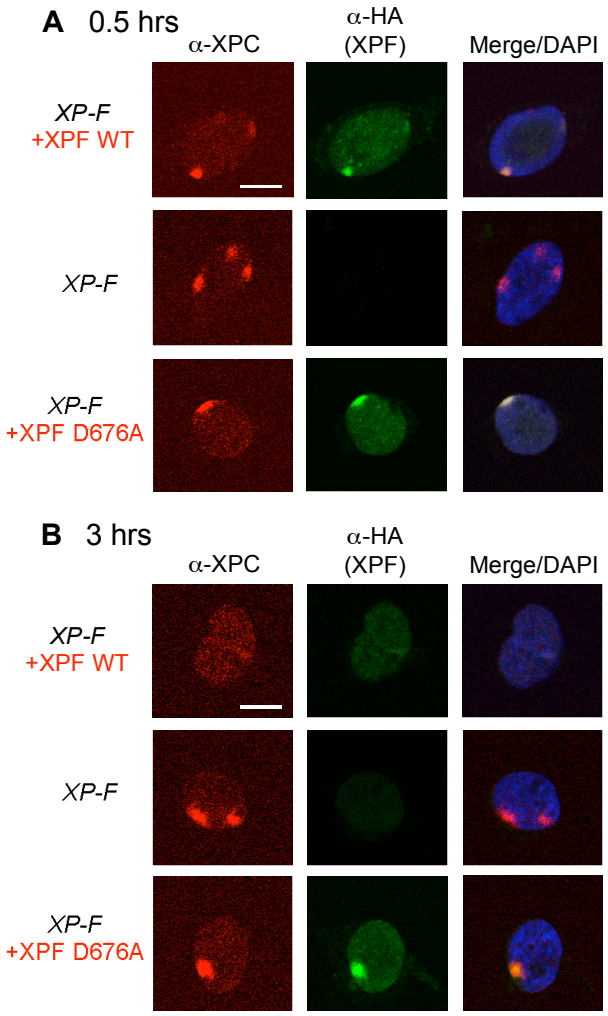

Figure 9. Recruitment of XPF to Sites of Local UV Damage in Different XP-F Cell Lines. XP2YO (XP-F) cells, untransduced or transduced with XPF-WT or XPF-D676A, were grown on coverslips and locally irradiated with a UV dose of 150 J/m^2 through filters with 5 μm pores and fixed 0.5 hours **(A)** or 3 hours **(B)** after irradiation. The cells were immunolabeled with antibodies against XPC (red) or the HA tag present on the C-terminus of XPF (green). Merged images indicate the overlay of XPC, XPF and DAPI staining. Scale bars, 10 μm.

Recruitment of PCNA and CAF-1 to sites of local UV damage depends on the presence, but not the catalytic activity of XPG

Using the transduced XP-G and XP-F cell lines, we studied how the catalytic activity of the two nucleases correlates with the recruitment of repair synthesis factors to NER sites. It has been shown before that recruitment of PCNA to the sites of local UV damage is severely affected in XPG-deficient cells [126]. In agreement with this report, we did not observe any co-localization of PCNA with XPC in XP-G cells 0.5 hrs after UV irradiation (Figure 10A, middle row). However, as expected, XPC and PCNA co-localized at sites of UV damage in XP-G cells expressing wild-type XPG (Figure 10A, top row). PCNA also co-localized with XPC in the XPG-E791A transductants (Figure 10A, bottom row). In principle, the recruitment of PCNA to sites of UV damage in the presence of catalytically inactive XPG could reflect partial DNA repair synthesis or be due to the recruitment of PCNA prior to incision, possibly by direct interaction with XPG [55]. To distinguish between these possibilities, we investigated the recruitment of PCNA in various XP-F cells. While PCNA was found at sites of UV damage in XP-F cells expressing wild-type XPF, PCNA did not colocalize with XPC in untransduced XP-F cells or in the XPF-D676A transductants (Figure 10B). These observations demonstrate that the recruitment of PCNA to sites of UV damage is dependent on the catalytic activity of XPF and therefore likely on active DNA repair synthesis. Consistent with this notion, the presence of Pol δ at sites of UV damage

also required catalytically active XPF (Figure 11).

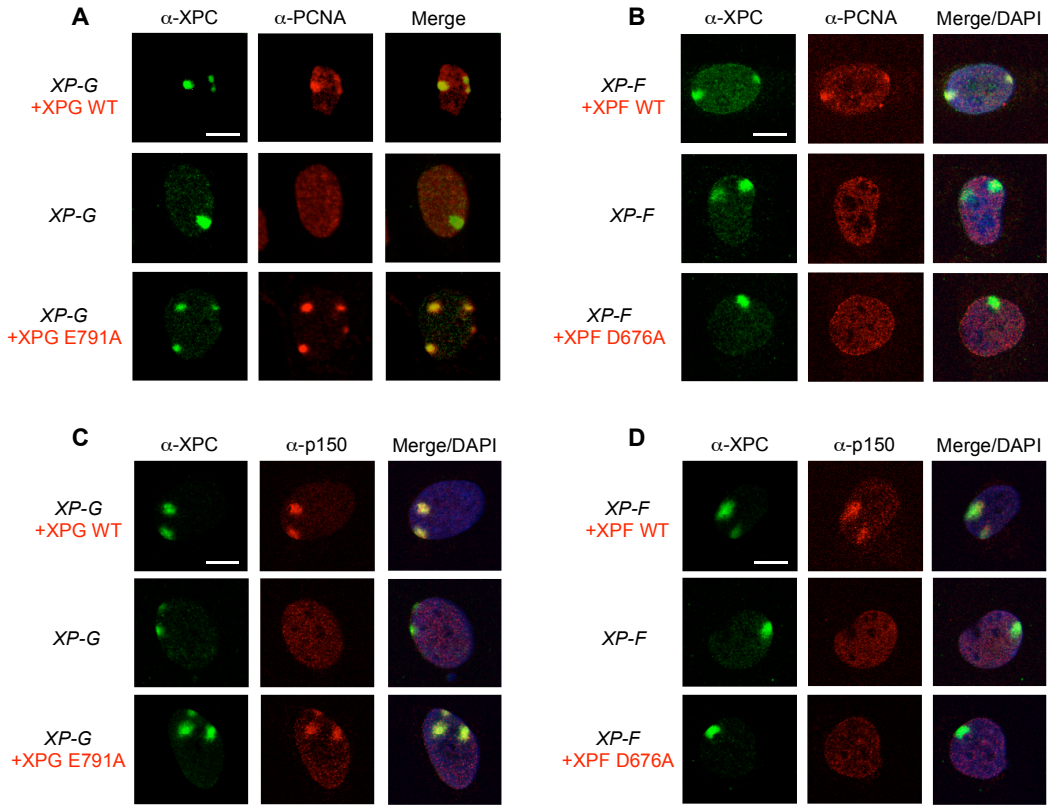

Figure 10. XPF- and XPG-Dependent Co-localization of PCNA and CAF-1 with XPC. XPCS1RO (XP-G) cells, untransduced or transduced with XPG-WT or XPG-E791A (**A** and **C**) and XP2YO (XP-F) cells, untransduced or transduced with XPF-WT or XPF-D676A (**B** and **D**), were grown on coverslips and locally irradiated with a UV dose of 150 J/m^2 through filters with 5 μm pores and fixed 0.5 hours after irradiation. The cells were immunolabeled with antibodies against XPC (green), PCNA (red) and CHAF150, the largest subunit of CAF-1 (red). Merged images indicate the overlay of XPC, PCNA or CHAF150 and DAPI staining. Scale bars, 10 μm.

Having established that the recruitment of the replication machinery required the catalytic activity of XPF, but not that of XPG, we asked if factors acting even later in NER could be recruited to sites of UV damage in the absence of XPG incision. We examined the recruitment of

CHAF150, a subunit of the chromatin assembly factor 1 (CAF-1) that is involved in the restoration of chromatin after a NER reaction [120]. CHAF150 behaved like PCNA in that its recruitment was dependent on the catalytic activity of XPF, but not on that of XPG (Figure 10D and 10C). These results are consistent with an earlier observation that CAF-1 is recruited to the sites of DNA damage in a PCNA-dependent manner [120]. The important novel conclusion is that even factors acting downstream of DNA repair synthesis can be recruited to sites of UV damage before the second incision 3' to the lesion has occurred.

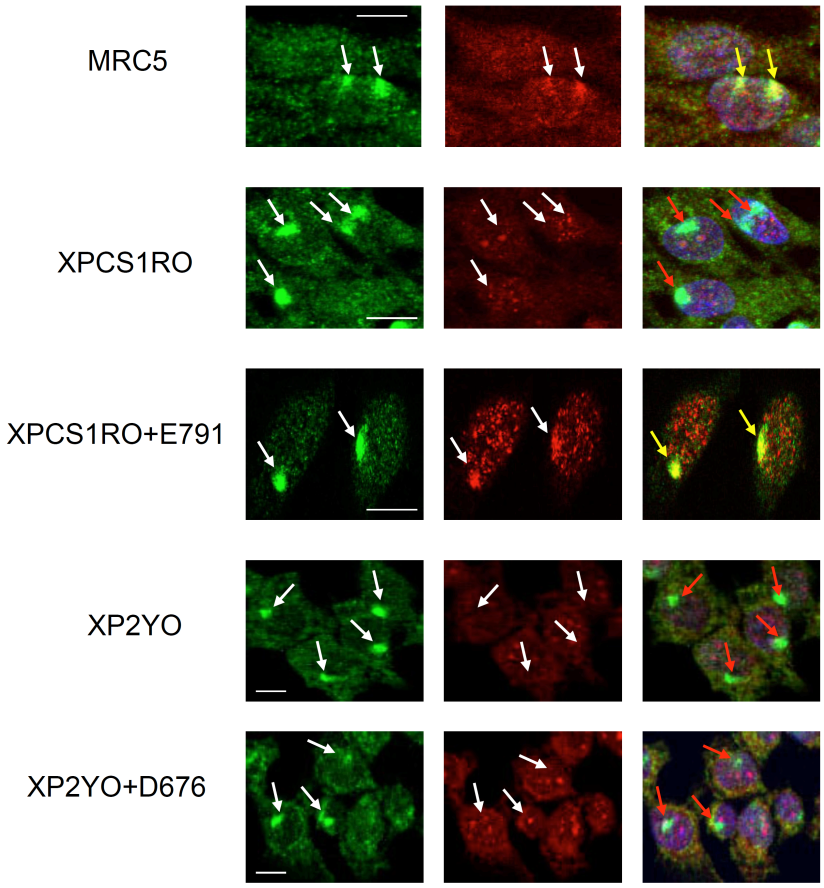

Figure 11: **Catalytically active XPF is required for pol δ recruitment to damage regions.** MRC5 cells, XPCS1RO (XP-G) cells untransduced or transduced with XPG-E791A, and XP2YO (XP-F) cells, untransduced or transduced with XPF-D676A, were grown on coverslips, cultured in the presence of HU-AraC for 30 mins, locally irradiated with a UV dose of 150 J/m^2 through filters with 5um pores and fixed 1 hour after irradiation. The cells were immunolabeled with antibodies against XPB (green) used as a marker to locate UV-damaged spot (white arrows) and polymerase δ (red).Merge indicates the overlay of XPB, polymerase δ and DAPI stainings. Scale bars, 10 um, yellow arrows indicate co-localization between XPB and polymerase δ, whereas red arrows indicate absence of this co-localization.

Partial unscheduled DNA synthesis (UDS) occurs in the absence of 3' incision by XPG

To test whether loading of PCNA to NER sites is able to stimulate DNA repair synthesis in the absence of 3' incision *in vivo*, we examined DNA repair by unscheduled DNA synthesis (UDS) after UV-irradiation of XP-G transductants expressing wild-type XPG or XPG-E791A. Wild-type XPG complemented the severe UDS defect of untransduced XP-G cells (102 % versus 7%, with NER-proficient cells assayed in parallel set at 100%, Figure 12A). In line with the observed partial DNA repair synthesis *in vitro* and recruitment of PCNA, the very low UDS level of untransduced XP-G cells was significantly increased upon expression of the catalytically inactive XPG (from 7 to 49 % UDS, Figure 12A). To try to ensure that this UV-induced DNA synthesis occurs at sites of NER rather than being a non-specific artifact, we monitored repair synthesis at locally UV-damaged areas (Figure 12B). Although quantification is difficult, significant numbers of autoradiographic grains were found clustered together over non-S phase nuclei in NER-proficient cells and XP-G transductants expressing wild-type XPG (Figure 12B, upper panels). Very few grains were found over comparable nuclear areas of untransduced XP-G cells but the nuclei of the XPG-E791A transductants exhibited a significant amount of grain clustering, roughly mid-way between the wild-type and XP-G levels (Figure 12B, lower panels). In contrast, the levels of UDS in XP-F cells expressing XPF-D676A (8% of wild-type, Figure 12A) was at the same

background level as untransduced XP-F cells, whereas UDS was restored to normal levels in cells expressing wild-type XPF (104%, Figure 12A). Local UDS experiments confirmed these findings; while UDS sites were clearly observed for XP-F transductants expressing wild-type XPF, they were not evident in XPF-D676A transductants (Figure 12C). Together, these results strongly suggest that the observed UDS is linked to sites of local UV damage, and that partial repair synthesis can occur in living cells in the absence of the catalytic activity of XPG, whereas it does require the catalytic activity of XPF.

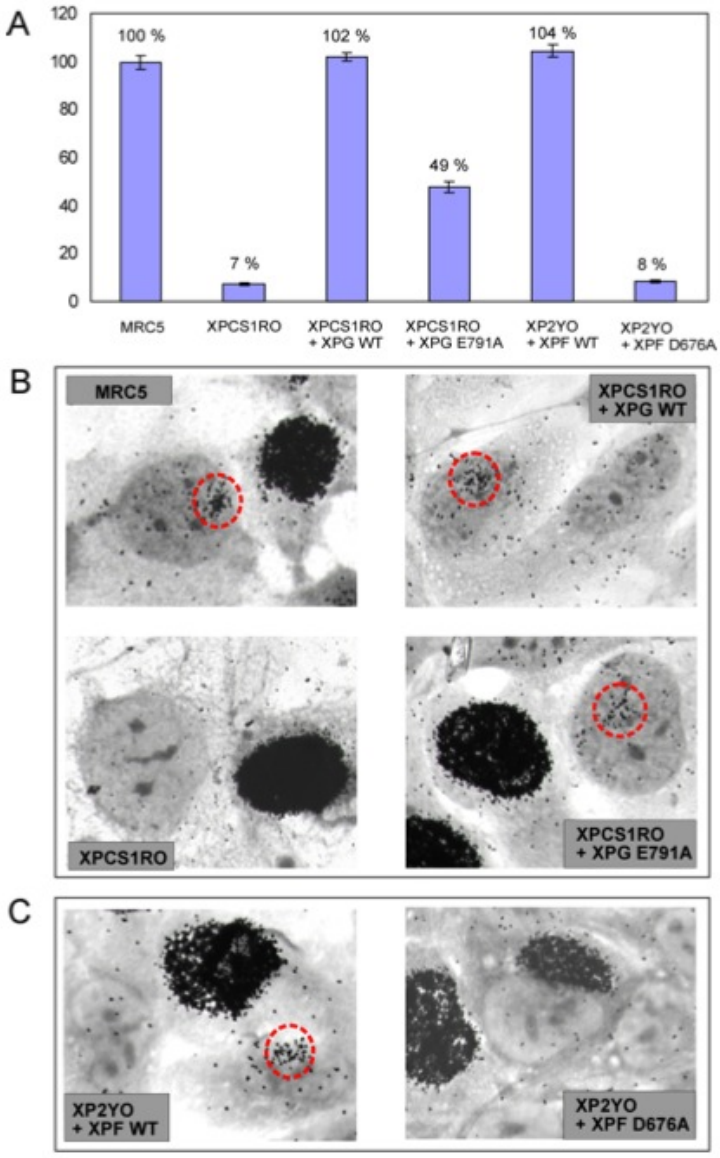

Figure 12. UDS in XP-F and XP-G Cells transduced with wild-type and mutant *XPF* and *XPG*, respectively. (A) DNA repair synthesis or UV-induced UDS levels of different, as indicated, XP-F and XP-G cells, expressed as percentage of the UDS of a NER-proficient cell line (MRC5) assayed in parallel. (B and C) UDS in cells locally irradiated through a 5 μm microporous filter (60 J/m^2), NER-proficient MRC, XPCS1RO, XPCS1RO transduced with wild type XPG and XPG-E791A (B) and XP2YO transduced with wild-type XPF or XPF-D676A (C). Heavily labeled cells were cells in S-phase, incorporating large amounts of tritiated thymidine by replicative DNA synthesis. Dotted circles indicate the position of pores in which UV damage has been induced and UDS has been observed.

Discussion

An unresolved longstanding issue for human NER is whether the 5' and 3' incisions, by ERCC1-XPF and XPG, respectively, occur in a strict temporal order and, if so, which one occurs first. A related issue is how these incisions are coordinated with DNA repair synthesis to prevent the exposure of potentially extremely damaging ssDNA gaps. We believe that the following observations described here provide novel and important insight into these issues.

First, the 5' incision by ERCC1-XPF depends on the presence, but not catalytic activity, of XPG whereas efficient 3' incision by XPG requires the catalytic activity of ERCC1-XPF (Figure 7), extending previous findings [21, 42, 43]. Second, and most important, partial DNA synthesis is detectable *in vitro* in the presence of catalytically inactive XPG (Figure 8), thereby demonstrating that the incision 5' to the lesion by ERCC1-XPF is both necessary and sufficient for the initiation of repair synthesis, whereas 3' incision by XPG is needed for the completion, but not the initiation of repair synthesis. Third, some late NER factors, including the replication factors PCNA and Polymerase δ as well as the chromatin assembly factor CAF-1, are recruited to sites of local UV damage in cells expressing catalytically inactive XPG, but not in cells expressing catalytically inactive XPF (Figure 10). Fourth, cells expressing catalytically inactive XPG, but not those expressing catalytically inactive XPF, are capable of undergoing intermediate levels of unscheduled DNA repair synthesis (Figure 12).

A defined temporal order for human NER incision and DNA repair synthesis events

Based on these observations we suggest that the human NER pathway does indeed have a defined temporal order for the 5' and 3' incisions and for DNA repair synthesis. Specifically, we propose the following model for the coordination of the dual incision and repair synthesis steps (Figure 13). After assembly of all the factors of the preincision complex, 5' cleavage by ERCC1-XPF takes place, generating a free 3'-OH group. The repair synthesis machinery consisting minimally of polymerase δ, the clamp loader RFC and the processivity factor PCNA are recruited and repair synthesis is initiated. Which factors and interactions may facilitate this recruitment remains to be established, but it is possible that RPA has an important role in this transition [32]. DNA synthesis is then initiated and proceeds about half way through the repair patch. The stalling of the polymerase at this point might trigger the XPG endonuclease activity, allowing the repair synthesis to be completed. We have previously shown that XPG has distinct requirements for binding and cleaving DNA [51] raising the possibility that a conformational change in the NER complex brought about by the polymerase activity triggers the catalytic activity of XPG.

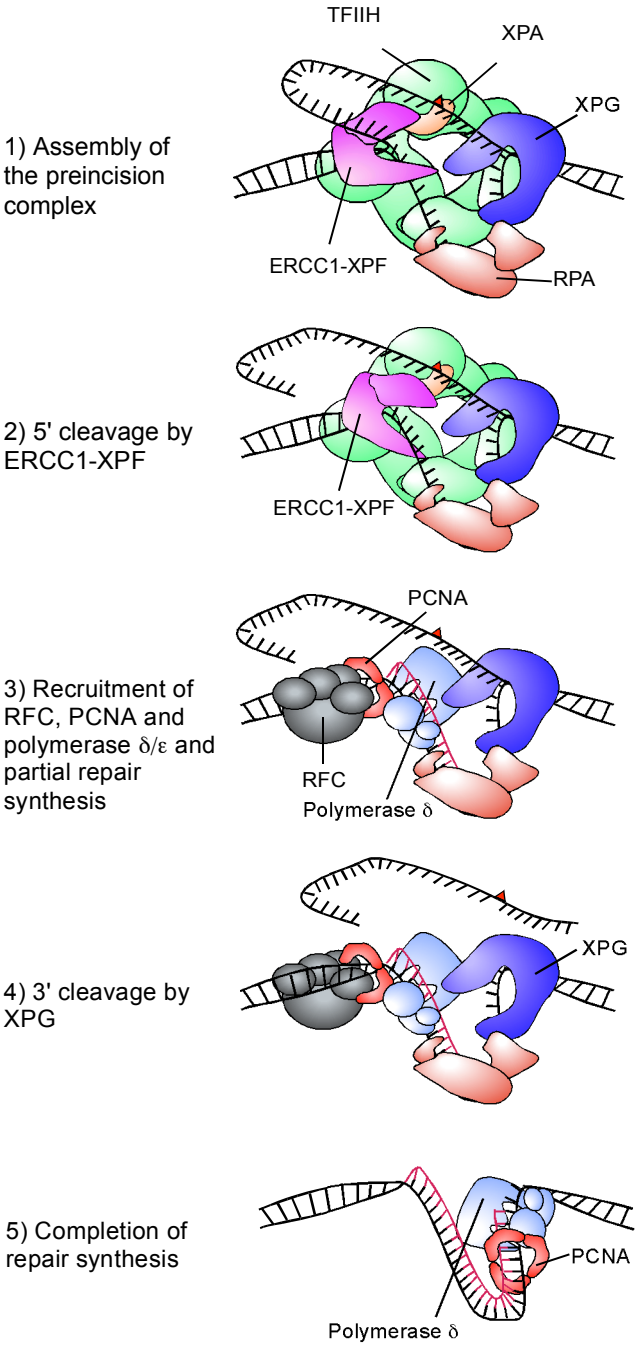

Figure 13. Model for the Coordination of Dual Incision and Repair Synthesis Steps in NER. Schematic representation of the proposed sequence of events following the assembly of the preincision complex. The red triangle stands for the DNA lesion. Individual proteins involved in each step are indicated.

Due to the near simultaneous occurrence of the two incision reactions, we have not yet been able to prove that this reaction sequence is also favored in a situation where wild-type XPF and XPG proteins are present. However, indirect support for our model comes from a recent study that considered the sequence of arrival and release of various NER factors during the dual incision and repair synthesis steps in NER using a fully reconstituted system [104]. This study showed that ERCC1-XPF is released from repair complexes with the arrival of RFC, while XPG (and RPA) are only fully displaced once the entire repair synthesis machinery (RFC, PCNA and Pol δ) has been recruited.

One prediction of our model is that the addition of DNA polymerase inhibitors might inhibit 3' incision by XPG. We observed that addition of the Pol δ inhibitor aphidicolin to an *in vitro* NER reaction did not show a significant inhibition of the 5' or 3' incision (data not shown), consistent with an earlier study [124]. While further studies will be necessary to fully delineate the biochemical relationship of repair synthesis and the 3' incision by XPG, it is interesting to note that a recent study found that treatment of cells with HU and AraC inhibited the removal of 6-4PPs [37]. Although the molecular basis for this observation is currently unknown, it is consistent with our model and the notion that inhibition of repair synthesis blocks at least one of the incisions (presumably by XPG).

The model proposed here does not exclude an involvement of additional protein-protein interactions or protein modifications in the

various steps. For example, it is known that XPG has a PIP box and that it can interact with PCNA [55]. Although it has not yet been shown convincingly that the interaction between PCNA and XPG is required for NER, an interaction between the two proteins may contribute to the activation of the XPG incision. A recent study has implicated polκ in the repair synthesis step of NER [118]. It is possible that two polymerases, polδ and polκ, act at different steps of repair synthesis, for example before and after incision by XPG has taken place. Further studies will be required to determine how the various phases of repair synthesis in NER are regulated by protein-protein interactions and possibly post-translational modifications.

Apparent contradictions

One set of experimental observations appears to be in contrast with our results. Under very similar experimental conditions and using a variety of substrates either 5' uncoupled [103, 124] or 3' uncoupled [20, 34, 102, 103] incisions have been observed. Our model would predict that 3' uncoupled incision by XPG should not occur at any appreciable frequency. One possible explanation is that ERCC1-XPF and XPG, can incise NER intermediates under certain conditions *in vitro* that would be disfavored *in vivo*. It is known, for example, that the intrinsic structure-specific endonuclease activity of both ERCC1-XPF and XPG can be readily detected *in vitro* without the need for additional proteins [69, 70]. Similarly, high relative concentration of XPG or the absence of ERCC1-XPF in a cell

extract or reconstituted system may allow incision by XPG 3' to the lesion in the absence of a properly assembled NER complex. We have previously shown that XPG has distinct requirements for binding and cleaving DNA substrates and that the XPG spacer region has a critical role in mediating this substrate preference [48, 51, 52]. We suggest that XPG is present in a catalytically inactive conformation prior to 5' incision and partial repair synthesis and that its catalytic activity is revealed by a change in the complex brought about by partial repair synthesis. The barrier for XPG to cleave certain substrates does not occur at the level of substrate binding, but likely involves a subsequent rearrangement of an XPG-substrate complex [51]. We propose that a lowering of this activation barrier under certain experimental conditions leads to 3' uncoupled incisions.

A "Cut-patch-cut-patch" mechanism

Early investigations of excision repair in bacteria focused on the discrimination between two models: "patch and cut", involving a first incision close to the damage, followed by repair synthesis and second incision, and "cut and patch", invoking excision of the damaged base/s prior to repair synthesis [127, 128]. Subsequently, in particular with the ability to reconstitute NER *in vitro*, and the ability to observe dual incision on NER substrates in the absence of repair synthesis, the "cut and patch" model became accepted as the way by which NER operates [124, 129, 130].

The present work suggests that the human NER machinery operates via a “cut-patch-cut-patch” mechanism that includes features of both previous models. Interestingly, long-patch base excision repair (BER) has been shown to proceed in a similar way. In long-patch BER, polymerase δ/ε, supported by the replication accessory factors RFC and PCNA or polymerase β, carries out repair synthesis past the abasic site and introduces 2-6 nucleotides. The short oligonucleotide overhang generated in this way is excised by the Flap endonuclease FEN1, and the nick is sealed by DNA ligase I [131, 132]. In line with this model, it was demonstrated that PCNA facilitates excision in long-patch BER [133]. Stimulation of the dual incision by PCNA has also been observed in NER, leading to the proposal that PCNA may promote the turnover of the early NER factors [134] linking the excision and repair synthesis steps. Although many aspects of these important DNA repair processes remain to be discovered, it is interesting and unexpected that both NER and long-patch BER have operational similarities.

Materials and Methods

Protein Purification

Wild type XPF, XPF D676A, XPF R678A, XPF E679A, XPF 681A, XPF E701A, XPF D704A, XPF E714A, XPF R715A, XPF K716A, XPF D720A, wild type XPG and XPG E791A proteins were expressed in Sf9 insect cells and purified as described previously [51, 86]. The purity of the enzyme preparations was very similar to the ones previously reported and 0.2-0.5 mg of proteins were obtained at concentrations of 0.2-0.3 mg/ml.

In vitro NER Dual Incision Assay

Covalently closed circular DNA (pBluescript) containing a single 1,3-intrastrand d(GpTpG) cisplatin-DNA crosslink was prepared as described previously [124] and additionally purified over two consecutive sucrose gradients. Reactions were carried out in a buffer containing 40 mM HEPES-KOH (pH 7.8), 70 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP, 0.36 mg/ml BSA, 22 mM phosphocreatine (di-Tris salt) and 50 ng/μl creatine phosphokinase. Each reaction contained 200 ng DNA and 30 μg of cell-free extract prepared from XPG- or XPF-deficient fibroblast cells (XPCS1RO and XP2YO, respectively). Complementation was assayed upon addition of 730 fmol wild-type or mutant protein (XPG or XPF). Reactions were incubated at 30°C for 45 minutes. 50 nM of an oligonucleotide complementary to the excision product with a G_4 5'-overhang (5'-GGGGGAAGAGTGCACAGAAGAAGACCTGGTCGACC)

was added, followed by heat inactivation at 95°C for 5 minutes. Alternatively, for detection of individual incisions, the incision reaction was inactivated by addition of 1M EDTA, pH 8.0, 3% SDS and 12μg proteinase K. DNA was extracted with phenol:chloroform and ethanol precipitated as described previously [135], then 50nM of an oligonucleotide complementary to the BssHII restriction site with a G_5 5'-overhang was added (5'–GGGGGCAATTAACCCTCACTAAAGGGAACAAAAGCTGG) followed by heat inactivation at 95°C for 5 minutes. After cooling down the reactions for 15 minutes at room temperature, 0.5 units of Sequenase and 3.5 μCi of [α-^{32}P]-dCTP (both from Amersham-Pharmacia, diluted in Sequenase dilution buffer) were added. Reactions were incubated for 3 minutes at 37°C prior the addition of 1.2 μl dNTP mix (100 μM of each dATP, dGTP, TTP and 50 μM dCTP) and incubation for another 12 minutes at 37°C. This fill-in reaction labeled the product using the G_4 overhang provided by the respective complementary oligonucleotides as a template. Reactions were stopped by addition of formamide loading buffer, heated at 95°C for 5 minutes and analyzed on a 12% or 8% denaturing polyacrylamide sequencing gel. The gel was exposed on a phosphor screen and scanned on a PhosphorImager.

In vitro NER Repair Synthesis Assay

The assay was performed using the same substrate, cell extracts and proteins as described for the excision assay. The reaction mixtures

additionally contained 10 μM dATP, 10 μM dGTP, 5 μM dCTP, 5 μM TTP, 10 μCi of [α-^{32}P]-dCTP and 10μCi [α-^{32}P]-TTP. Complementation was assayed upon addition of 600 fmol of wild-type or mutant protein (XPG or XPF). Reactions were incubated at 30°C for 3 hours. DNA was purified using MinElute PCR Purification Kit (Qiagen), cleaved with *Kpn*I and *Sac*I or *Bsr*BI and *Xho*I and analyzed on a 10% denaturing polyacrylamide sequencing gel.

Cell Culture Conditions and Preparation of Whole Cell Extracts

For the generation of whole cell extracts, SV40-transformed fibroblast cells XPCS1RO (XPG-deficient, [136] and XP2YO (XPF-deficient, GM08437) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in the presence of 5% CO2. Cells were grown to near confluency, and whole cell extract was prepared accordingly to a published procedure [137].

Cell Transduction with Lentiviral Recombinants

XPG wild type cDNA, XPG-E791A cDNA were cloned into the pLOX/EWGFP lentiviral vector and XPF wild type (with a C-terminal HA tag) and XPF-D676A (with a C-terminal HA tag) cDNAs were cloned into the pWPXL lentiviral vector by replacing the GFP cDNA. Lentiviruses containing the different constructs under the control of the EF1α promoter were produced by co-transfecting 293T cells with the following 3 plasmids:

the packaging plasmid psPAX2, the envelope plasmid pMD2G and the lentiviral vector containing the different XPG and XPF cDNAs. Details of the vectors and protocols are described on the following web site http://www.lentiweb.com. XP-G/CS (94RD27, patient XPCS1RO) and XP-F (XP2YO) SV40 immortalized fibroblasts at 50% confluency were infected with viral particles containing the different XPG and XPF recombinants. Transduced cells were then cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a 5% CO_2 humidified incubator. The transduction efficiency was further assessed by immunofluorescence.

Local UV Irradiation and Immunofluorescence

Local DNA damage infliction within cultured cells was performed as described previously [138]. Briefly, cells cultured on coverslips were rinsed with PBS and covered with a micro-porous polycarbonate filter containing 5μm pores (Millipore). Cells were irradiated through the filter with a Philips TUV lamp (254 nm) with a dose of 150 J/m^2. After UV-irradiation cells were cultured for 0.5 hrs, washed first with PBS and then with PBS containing 0.05% Triton X-100 for 30 seconds prior to fixation with 3% paraformaldehyde for 15 minutes at room temperature or with ice-cold methanol for 20 min (for PCNA staining). Subsequently, cells were permeabilized by a 2 times 10 min. incubation in PBS containing 0.1% Triton X-100, and washed with PBS$^+$ (PBS containing 0.15% glycine and 0.5% bovine serum albumin). For the experiments with Polδ, cells were

incubated with Hu-AraC (10mM HU, 0.1mM AraC) from 30 minutes prior to irradiation until fixation and cells were fixed with MeOH rather than formaldehyde. Cells were incubated at room temperature with the primary antibody (diluted in PBS^+) for 2 hours in a moist chamber. Subsequently cells were washed 5 times for 10 min. with PBS Triton X-100, washed with PBS^+, and incubated at room temperature with the secondary antibody (diluted in PBS^+) for 1 hour in a moist chamber. Cells were washed 5 times for 10 mins in PBS Triton X-100, washed in PBS, and embedded in Vectashield mounting medium (Vector) containing 0.1 mg of DAPI (4′-6′-diamidino-2- phenylindole)/ml.

Primary antibodies were as follows: mouse monoclonal anti-PCNA (Dako, clone PC10), 1:1000, rabbit polyclonal affinity purified anti-XPC [139], 1:300, rabbit polyclonal anti-XPB (TFIIH p89, S-19m Santa Cruz), 1:1000, mouse monoclonal anti-Polδ (A-9, Santa Cruz), 1:25, mouse monoclonal anti-CHAF150 (CAF-1) (abcam, ab7655), 1:2000, and mouse monoclonal FITC-conjugated anti-HA (Roche, clone 3F10) Secondary antibodies were as follows: Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories), 1:1000, Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories), 1:1000, and Alexa-488 conjugated goat anti-rabbit (Molecular Probes), 1:800.

Confocal Microscopy

Confocal images of the cells were obtained using a Zeiss LSM 510 microscope equipped with a 25mW Ar laser at 488 nm, a He/Ne 543 nm

laser, and a 40 X 1.3 NA oil immersion lens. Alexa-488 was detected using a dichroic beam splitter (HFT 488), and an additional 505- to 530-nm bandpass emission filter. Cy3 was detected using a dichroic beam splitter (HFT 488/543) and a 560- to 615-nm bandpass emission filter.

Unscheduled DNA Synthesis (UDS)

To determine global genome NER activity in cultured cells, UV-induced DNA repair synthesis or unscheduled DNA synthesis (UDS) was measured. Coverslip cultures were rinsed with PBS, UV-irradiated (16 J/m^2, Philips 254 nm TUV lamp) and subsequently incubated for 2 hours in culture medium supplemented with 20μCi/ml [^{3}H-1',2']-thymidine (120 Ci/mmol, Amersham TRK565). After fixation coverslips were dipped in Ilford K2 photographic emulsion, exposed for three days an after development stained with Giemsa. Autoradiographic grains above the nuclei of 50 cells were counted and compared to the number of grains above nuclei of NER-proficient fibroblasts (MRC5, set at 100% UDS), assayed in parallel. UDS in locally damaged cells (local UDS) with 60 J/m^2 was performed in a similar fashion with the exception of an extended exposure time to six days.

Acknowledgements

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CHAPTER 3

A UBM and new PIP domain in XPG Regulate the Late Steps in Nucleotide Excision Repair

Introduction

Nucleotide excision repair (NER) is a highly versatile DNA repair pathway capable of eliminating a variety of lesions within DNA [6]. The pathway involves the concerted action of more than 30 proteins that sequentially recognize a lesion, excise it in the form of a 24-32-nucleotide long stretch of DNA containing the damage, fill in the ensuing gap by repair synthesis and finally ligate the DNA to seal the nick, restoring it to its original context [6, 12]. Two subpathways of NER exist that differ primarily in their mode of damage recognition. Transcription-coupled NER removes damage in the actively transcribed regions of the genome, and is triggered by stalling of the transcribing RNA polymerase at a lesion [112, 113]. Alternatively, lesions within the bulk DNA are recognized by XPC-RAD23B, which is essential for recruitment of all other NER factors [57, 114]. XPC-RAD23B recruits the multi-subunit transcription and repair factor TFIIH, which is responsible for opening up the DNA around the lesion [17, 18, 34], allowing recruitment of XPA, RPA and XPG to the complex, with simultaneous release of XPC [32]. ERCC1-XPF joins the complex [28, 32] and positions itself via its interactions with XPA [28, 35] and RPA to perform the 5' incision [140]. ERCC1-XPF performs the incision 5' to the damage while XPG makes the 3' incision [33, 69, 70] releasing a 24-32 nucleotide stretch of ssDNA containing the lesion. The resulting gap is filled in by polymerase δ, ε, or κ [36] and associated

factors and the nick sealed by ligase I or IIIα [37] restoring the DNA to its original state.

While the early steps of NER, starting with damage recognition by XPC-RAD23B [15, 18], and leading up to the dual incisions by ERCC1-XPF and XPG [32, 33, 57] have been studied extensively, less is known about how dual incision and repair synthesis are linked [6, 33]. We have recently shown that the 5’ incision by ERCC1-XPF and partial repair synthesis can occur in the absence of 3’ incision by XPG (Chapter 2) [33]. This observation suggests that the dual incision events may be ordered, with the 5’ incision occurring first, releasing a free 3’-OH group which serves as a substrate for initiation of repair synthesis. This order of events likely represents a regulatory mechanism, preventing unwanted cuts within the DNA and avoiding deleterious ssDNA regions that could occur if the dual incisions are uncoupled from repair synthesis. Furthermore, XPG has been shown to interact with PCNA [55] and more recent evidence suggests that XPG has a role in the transition from dual incision to repair synthesis via its interaction with PCNA [104].

In light of these observations, we were interested in exploring whether an interaction between XPG and PCNA (and possibly other factors) has a role triggering the 3’ incision by XPG, providing an additional layer of regulation. Precedent for such a mechanism exits in resolution of Okazaki fragments by the flap endonuclease-1 (FEN1), a member of the same nuclease family as XPG [141]. FEN1 remains bound

to the DNA in an inactive conformation until it interacts with PCNA from the approaching replication fork [106, 107], upon which it undergoes a conformational change to become catalytically active [49].

We considered whether the XPG incision might be similarly regulated. We were intrigued by a possible analogy between a polymerase carrying out repair synthesis blocked by the presence of XPG and a replicative polymerase blocked at a DNA lesion (Figure 14). Stalling of a polymerase at a lesion results in the ubiquitylation of PCNA, which serves as a signal for recruitment of TLS polymerases that have the ability to bypass DNA lesions [108]. In addition to their PIP domain, TLS polymerases contain a ubiquitin-binding motif, and the PIP and UBM domains act synergistically in recruiting TLS polymerases [109]. Intriguingly, sequence analysis reveals that XPG, like the translesion synthesis (TLS) polymerases, also possesses a ubiquitin-binding motif (UBM) in addition to its PIP domain for PCNA interaction [110], raising the possibility that XPG activity might also be regulated by interaction with ubiquitylated PCNA. Indeed, it has recently been shown that ubiquitylation of PCNA occurs during NER, and that this ubiquitylation was required for the recruitment of polκ, one of three polymerases involved in NER repair synthesis (the other ones being polδ and polε) [36]. A possible scenario during NER could therefore be that XPG initially binds DNA in an inactive conformation, and the approaching replication machinery collides with XPG allowing interaction with PCNA and stimulation activity of XPG. It is

conceivable that such collision could lead to ubiquitylation of PCNA, which could further stimulate XPG activity (Figure 14).

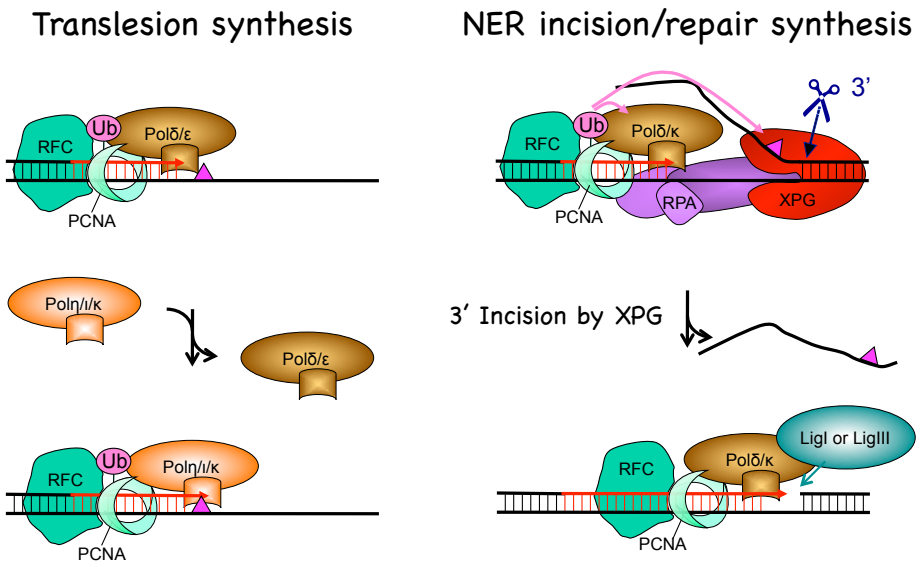


Figure 14: Schematic of proposed similarities between TLS synthesis and NER dual incision and repair synthesis. Left panel shows a stalled replication fork at a DNA lesion that leads to ubiquitylation of PCNA, which serves as a signal to switch from replicative polymerase to TLS polymerase allowing bypass of the lesion. The right panel is our proposed model whereby replication machinery stalls during repair synthesis, triggering PCNA ubiquitylation which could in turn trigger 3' incision by XPG.

To test these hypotheses we made mutations in the PIP (PIP-C) and UBM domains of XPG. We report here an additional putative PIP domain (PIP-N) in the protein, and observed the effects of these mutations on recruitment and departure of NER factors from regions of damage. We observed that mutations in the UBM and PIP-N domains impair damage repair, and the late steps of NER, but see no effects of PIP-C on XPG activity. Intriguingly, the UBM mutants exhibit a phenotype similar to the E791A mutant with regards to damage repair, and PCNA localization. Our

findings suggest that the role of XPG in coordinating dual incision with repair synthesis is multifaceted, and that it maintains several subtle interactions with PCNA to carry out this role.

Results

XPG contains a UBM that interacts specifically with ubiquitin.

An alignment of the XPG sequence with those of the TLS polymerases revealed the presence of a conserved ubiquitin-binding motif (UBM) in XPG [110] which, like the UBMs in the TLS polymerases [109] spans ~30 residues and is comprised of two predicted helical segments separated by a “Leucine-Proline” motif (Figures 15 and 16A).

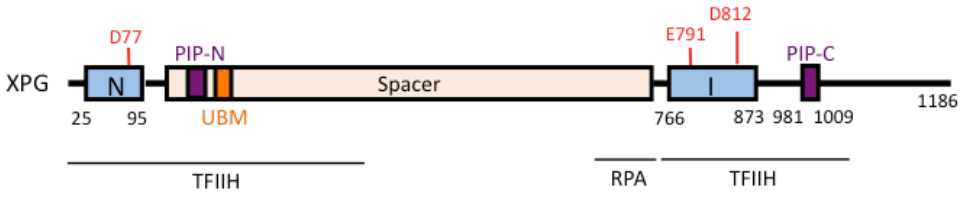
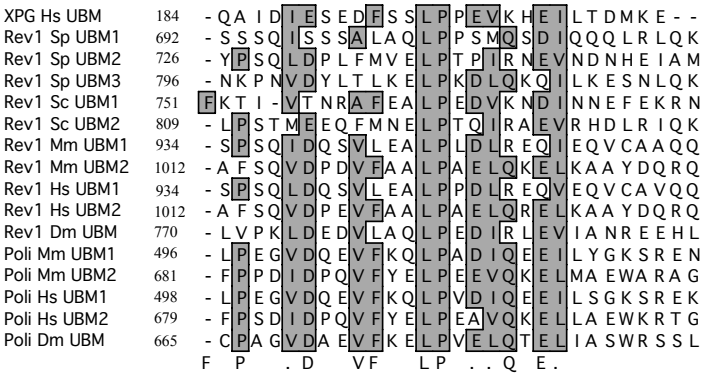


Figure 15: XPG structure and domains: The primary structure of XPG shows the 1186 amino acid protein with its active site made up of the N and I domains (light blue) separated by the 600 amino acid spacer region. The active site residues within the N and I domains are shown in red. Regions for interaction with TFIIH, and RPA are shown, and the PCNA-interaction domains (PIP-C and PIP-N) are indicated in purple, while the ubiquitin-binding motif (UBM) is shown in orange. Mutants used in this study include E791 (mutated to A) shown in red.

A.



B.

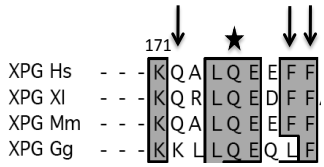


Figure 16: UBM and PIP-N domain alignments. (A) Alignment of the XPG UBM domain with UBM domains of the TLS polymerases is shown with conserved residues highlighted. The numbers indicate the position of the first amino acid of each UBM in the sequence. For the ΔUBM mutant the conserved LP residues were mutated to AA. (B) Alignment of the PIP-N domain of XPG in different species. The canonical PIP residues conserved in humans are indicated by the arrows, while the non-canonical residue is indicated by a star. The conserved FF residues were mutated to AA to generate the ΔPIP-N mutant. Species abbreviations used: Hs, *Homo sapiens*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Xl, *Xenopus laevis*; Gg, *Gallus gallus*.

We generated XPG mutants by site-directed mutagenesis of the critical Leu-Pro residues thought to be important for ubiquitin-binding, L196A, P197A (ΔUBM mutant) or deletion of the entire UBM domain (ΔUBM ^{domain} mutant) [48]. Purified recombinant XPG^{WT}, XPG$^{\Delta UBM}$ and XPG$^{\Delta UBM\text{-}domain}$ were incubated with GST-Ub_4, pulled down with GST beads. Analysis of the GST-bound and supernatant fractions revealed that XPG^{WT} bound

specifically to ubiquitin, whereas XPG$^{\Delta UBM}$ and XPG$^{\Delta UBM\text{-}domain}$ lost the ability to bind ubiquitin (Figure 17, lanes 5-7). *In vitro* nuclease and NER assays revealed that the ΔUBM mutant retained normal nuclease activity (data not shown). These data show that XPG binds ubiquitin via its UBM domain but that the UBM domain is not essential for the activity of XPG *in vitro*.

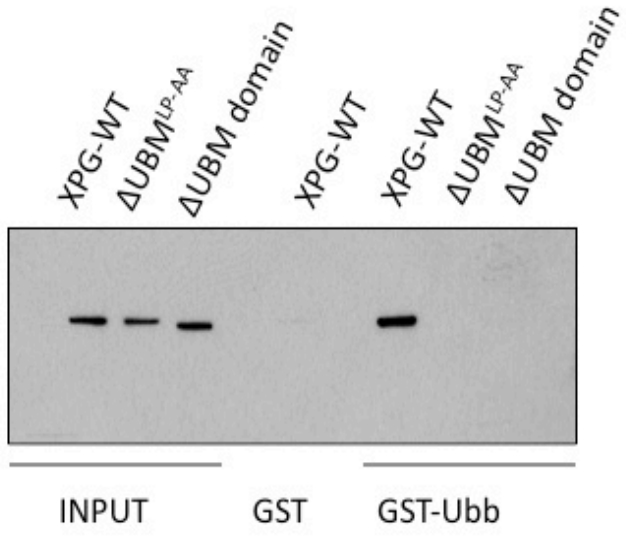


Figure 17: The UBM domain in XPG mediates its binding to ubiquitin. *In vitro* binding of GST-Ubb (GST fused to four ubiquitin residues) to the indicated XPG constructs, XPG-WT, XPGΔUBM^{LP-AA}, and XPGΔUBM domain. XPG-WT bound to GST-Ubb but not to GST alone (compare lanes 4 and 5), whereas mutation of the LP residues to AA as well as deletion of the UBM domain in XPG inhibited binding of the protein (compare lane 5 to lanes 6 and 7).

A putative PIP in the spacer region of XPG.

It has previously been shown that XPG contains a C-terminal PIP domain by which it specifically interacts with PCNA (PIP-C) (Figure 15) [55]. Mutation of the R992 residue in the PIP-C domain had minimal effect

on NER activity of XPG, although the PCNA interaction was specifically abolished [55]. Interestingly, the related endonuclease FEN1 also interacts specifically with PCNA via a PIP domain, and requires this interaction to activate nuclease activity of FEN1 for resolution of Okazaki fragments during replication [106, 107]. Although the XPG nuclease activity clearly does not require an interaction with PCNA, we were interested in determining whether the C-terminal PIP domain of XPG might contribute to NER in conjunction with other protein interaction domains. It has been reported that a number of TLS polymerases contain both PIP and UBM domains, and that mutations in both domains were required for TLS activity in cells [109]. These findings suggest a similar mechanism of action in XPG whereby it interacts with PCNA via both of these domains, possibly in a manner whereby it maintains subtle interactions with PCNA via these domains. It has also been reported that several PCNA-interacting proteins such as CAF-1 [142] contain more than one PIP. We searched for additional PIPs in XPG, and found another possible PIP domain in its spacer region (PIP-N), located immediately N-terminal to the UBM domain (Figures 15 and 16B). The newly found PIP-N domain lacks a critical residue that is conserved amongst most PIP domains, as canonical PIPS conform to the sequence QXXhXXaa, where Q is a glutamine, h is a hydrophobic residue (valine, methionine, leucine, or isoleucine), a is an aromatic residue (phenylalanine, tyrosine, tryptophan, or occasionally histidine), and X is any amino acid (see Figure 16B –

Q175 in XPG Hs instead of “h” indicated by a star). Yet alignment of XPG sequences in various organisms revealed conservation of this putative PIP in the higher order vertebrates *M. musculus*, and *X. laevis* (Figure 16B).

To better understand whether the PIPs and UBM domains in XPG have a role in NER, we generated mutations in the PIP-C domain, F996A, F997A ($XPG^{\Delta PIP\text{-}C}$), the PIP-N domain, F179A, F180A ($XPG^{\Delta PIP\text{-}N}$), and the UBM ($XPG^{\Delta UBM}$), separately and in combination ($XPG^{\Delta UBM\Delta PIP\text{-}C}$, and $XPG^{\Delta UBM\Delta PIP\text{-}N}$). We used the lentiviral vector transduction system to stably transduce the XPG-deficient cell-line XPCS1RO with either of these mutants as well as the previously generated active site mutant XPG^{E791A} [33] and analyzed how these mutations affected the repair of DNA lesions and the recruitment of NER factors to the sites of local UV damage [57].

Damage removal is impaired in ΔUBM, and ΔPIP-N mutants but not in ΔPIP-C mutants.

We analyzed *in vivo* damage repair kinetics of the cell-lines by UV-irradiating them with 150J/m^2 of UV through a micro-pore filter generating sites of local UV damage (LUD). Following irradiation cells were fixed either immediately, or following incubation for various time-periods, and then washed and probed with antibodies to (6-4)PPs, and the NER factor ERCC1 (for images of cells with co-localized (6-4)PP and ERCC1 foci at 0.5h see Figure 26 in the Appendix). Cells containing damage foci were counted and graphed as illustrated in Figure 18. As expected, damage foci

levels persisted in the untransduced XP-G cells, with greater than 50% damage foci persisting to 24h, while in XPG^{WT} cells (6-4)PPs disappeared completely between 3h and 6h. The catalytically inactive XPG^{E791A} mutant, which we have previously shown to be severely impaired in NER (Chapter 2 and [33]), exhibited much reduced damage-removal ability with ~30% damage foci persisting up to 24h (Figures 18 and 19). Intriguingly, the ΔUBM mutant showed levels of damage foci very similar to those in XPG^{E791A} despite having normal nuclease and NER activity *in vitro* (Figure 18 and data not shown). Of the two PIP domains, PIP-N appeared to play a more important role in NER, as $XPG^{\Delta PIP\text{-}N}$ cells, but not $XPG^{\Delta PIP\text{-}C}$ cells exhibited a moderately reduced rate of damage removal (Figures 18 and 19). Importantly, incorporation of the UBM mutation into any other mutant resulted in phenotypes similar to that of $XPG^{\Delta UBM}$ alone, suggesting that the UBM domain is of particular importance for damage removal *in vivo*.

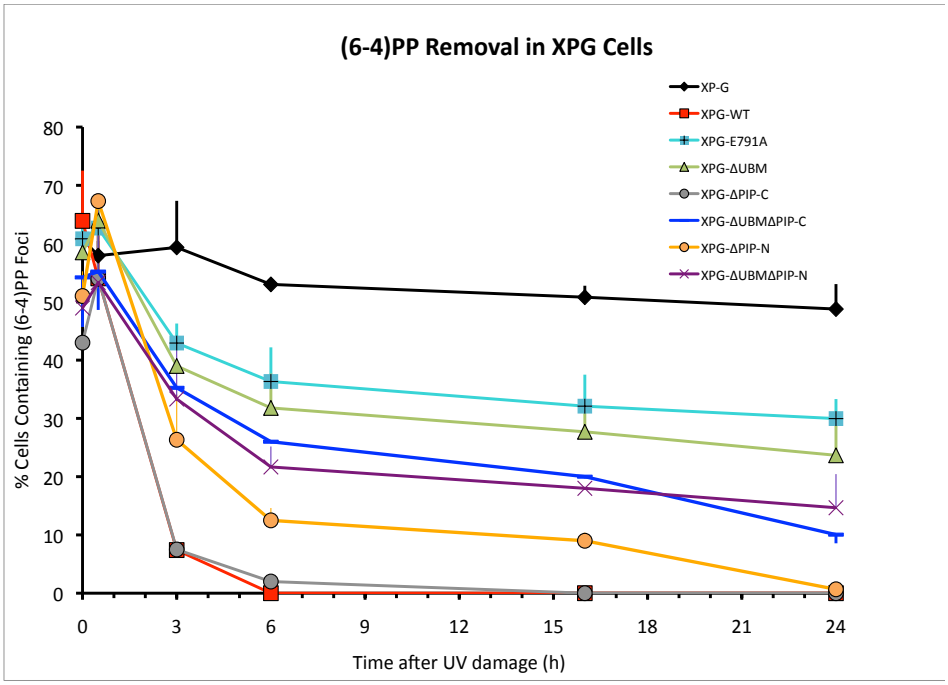


Figure 18: Removal of (6-4)PPs is impaired in the E791A, UBM and PIP-N mutants. XP-G deficient cells (XPCS1RO) were transduced with wild-type or mutant XPG and irradiated with UV light (150 J/m^2) through a polycarbonate filter with 5μm pores and fixed and stained for (6-4)PP (red) and the nuclear marker DAPI (4'-6'-diamino-2-phenylindole) in blue at the indicated time-points after irradiation. This is a graphical representation of the percentage of cells with persistent (6-4)PP foci at various time-points. Data represent the average of three independent experiments ± S.D. (error bars).

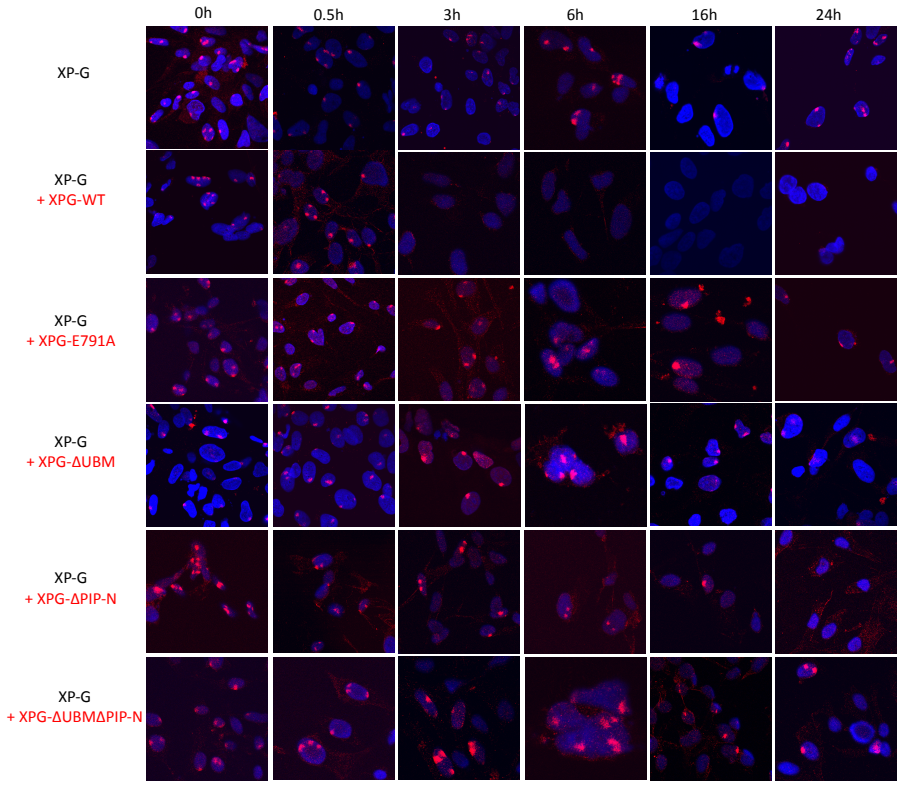


Figure 19: IF images of (6-4)PP foci in mutants at all time-points analyzed. Images of the UV irradiated cells at different time-points following irradiation, (see Figure 18) with (6-4)PP foci shown in red, and DAPI staining in blue.

The PIP-N but not PIP-C domain is required for the association of PCNA with (6-4)PPs.

Next we analyzed the localization of PCNA to damage regions by probing for PCNA along with the NER factor ERCC1 at various time points following UV-irradiation. We have previously shown that PCNA is recruited to damage regions in the XPG-E791A mutant, despite its inability to carry out incision, suggesting that the presence, but not catalytic activity of XPG

is required for the recruitment of PCNA to LUDs (Chapter 2 and [33]). We observed the same results in a quantitative manner, with PCNA foci visible in XPG^{E791A} cells as early as 0.5h following UV-irradiation, the same as is seen in XPG^{WT} cells. The $XPG^{\Delta UBM}$ and $XPG^{\Delta PIP\text{-}C}$ mutant cell-lines also supported normal recruitment of PCNA at the earliest time-point (Figures 20 and 21), demonstrating that the PIP-C domain was not required for the recruitment of PCNA. Interestingly however, mutations in putative PIP domain, ΔPIP-N, led to abolished PCNA recruitment to LUDs at 0.5h (Figures 20 and 21), and only 5-10% colocalization PCNA of foci with (6-4)PPs was observed at 1h following UV irradiation (Figure 21). It is important to note that XP-G deficient cell-lines did not show any recruitment of PCNA at the early time-points tested. These results suggest that the PIP-N domain plays a role in PCNA recruitment to damage regions, or at least in maintaining its stable interaction with the DNA at damage regions.

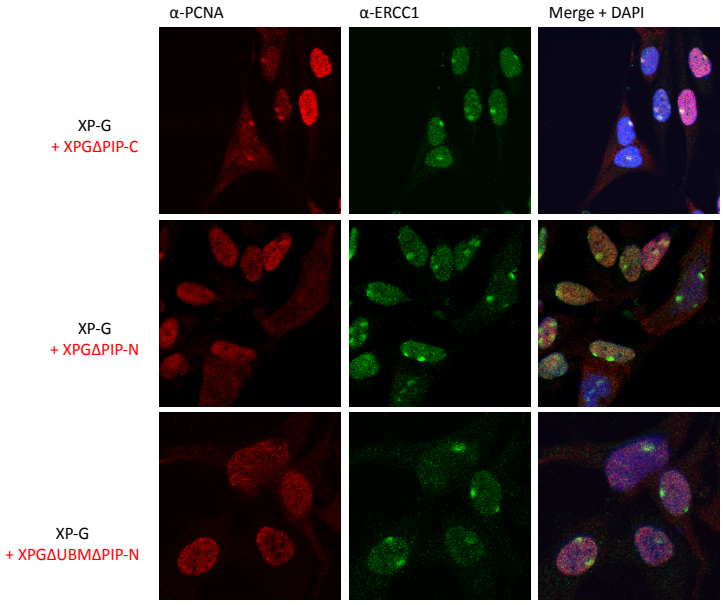


Figure 20: PCNA recruitment in the PIP mutants. XP-G cells transduced with either wild-type or mutant XPG were UV-irradiated, returned to culture, and fixed and stained for PCNA at the indicated times following irradiation. Images of the PIP mutants $XPG^{\Delta PIP-C}$, $XPG^{\Delta PIP-N}$ and $XPG^{\Delta UBM\Delta PIP-N}$ at 0.5h after UV irradiation showing PCNA foci (red) and ERCC1 foci (green). No PCNA foci are visible in $XPG^{\Delta PIP-N}$, and $XPG^{\Delta UBM\Delta PIP-N}$ mutants while PCNA foci are clearly visible in the $XPG^{\Delta PIP-C}$ mutant.

PCNA persists at sites of damage in active site and ΔUBM mutants

At the later time-points we observed some interesting results. In XPG^{WT} cells, PCNA disappears from damage regions by 16h with only a very small fraction (~5%) still visible at 6h following UV damage. The ΔPIP-C mutant once again behaved similar to XPG^{WT} with respect to PCNA kinetics further suggesting that this mutation does not affect NER. However in the XPG^{E791A}, $XPG^{\Delta UBM}$, and $XPG^{\Delta UBM\Delta PIP-C}$ expressing cells, PCNA foci persisted, with ~15-25% PCNA foci remaining in these cells at 24h after UV-irradiation (Figure 21). These results suggest that, as would

be expected in XPG-E791A, PCNA is recruited normally and stably to the DNA, where it initiates, but is unable to complete its function in repair synthesis, possibly because it is blocked by inactive XPG. Persistence of PCNA foci in $XPG^{\Delta UBM}$, and $XPG^{\Delta UBM\Delta PIP\text{-}C}$ also supports such an explanation. However PCNA behaved distinctly in $XPG^{\Delta PIP\text{-}N}$ and $XPG^{\Delta UBM\Delta PIP\text{-}N}$ cells. In those mutants, PCNA levels remained low (<10%) at all time points examined (Figure 21). At 16h, and 24h following UV-irradiation, PCNA foci dropped to 0-5% in both mutants, although the $XPG^{\Delta PIP\text{-}N}$ mutant contained ~10-15% of the other NER factor ERCC1 at the same time-points (Figure 22) suggesting that this mutant specifically affects the association of PCNA with LUDs (for images of PCNA and ERCC1 foci colocalized in cells at all time-points examined see Figures 27-31 in the Appendix).

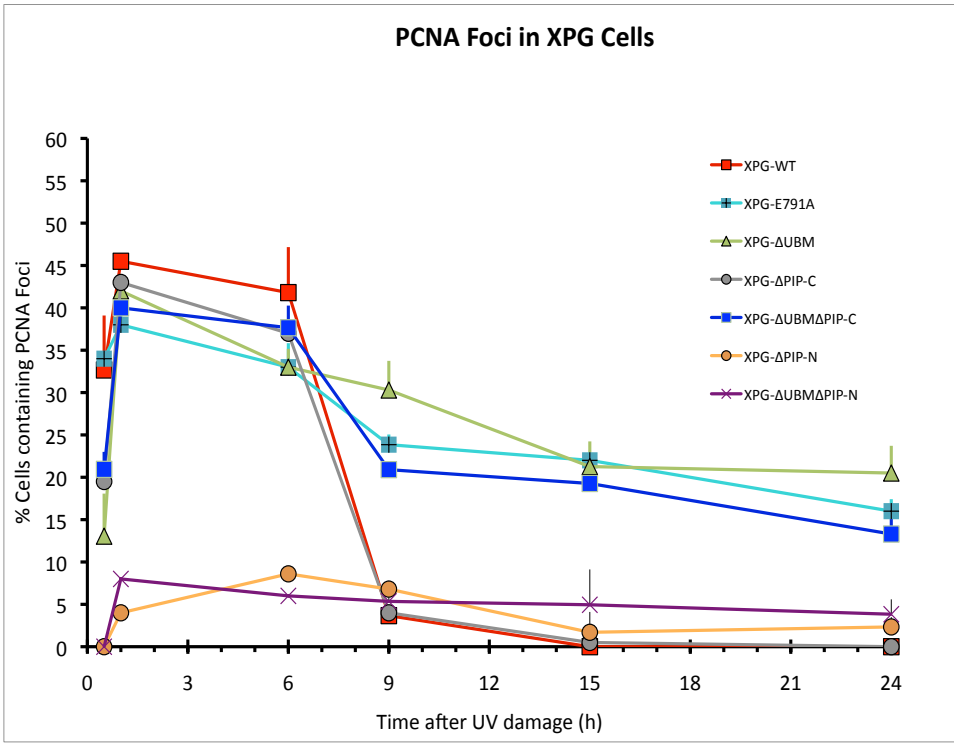


Figure 21: PCNA association with damaged regions is impaired in PIP-N mutants, while its dissociation is impaired in UBM and E791A mutants. A graphical representation of the percentage of cells containing PCNA foci at various time-points after UV. PCNA foci accumulate within 0.5h in XPG-WT, XPG-E791A, XPG$^{\Delta UBM}$, XPG$^{\Delta PIP-C}$, and XPG$^{\Delta UBM\Delta PIP-C}$, but not in XPG$^{\Delta PIP-N}$, or XPG$^{\Delta UBM\Delta PIP-N}$ indicating impairment of PCNA recruitment as a result of the PIP-N mutation. PCNA foci persist up to 24h in XPG$^{\Delta UBM}$, XPG$^{\Delta UBM\Delta PIP-C}$, and XPG-E791A mutants indicating inability of the replication factor to dissociate from repair factories in these mutants.

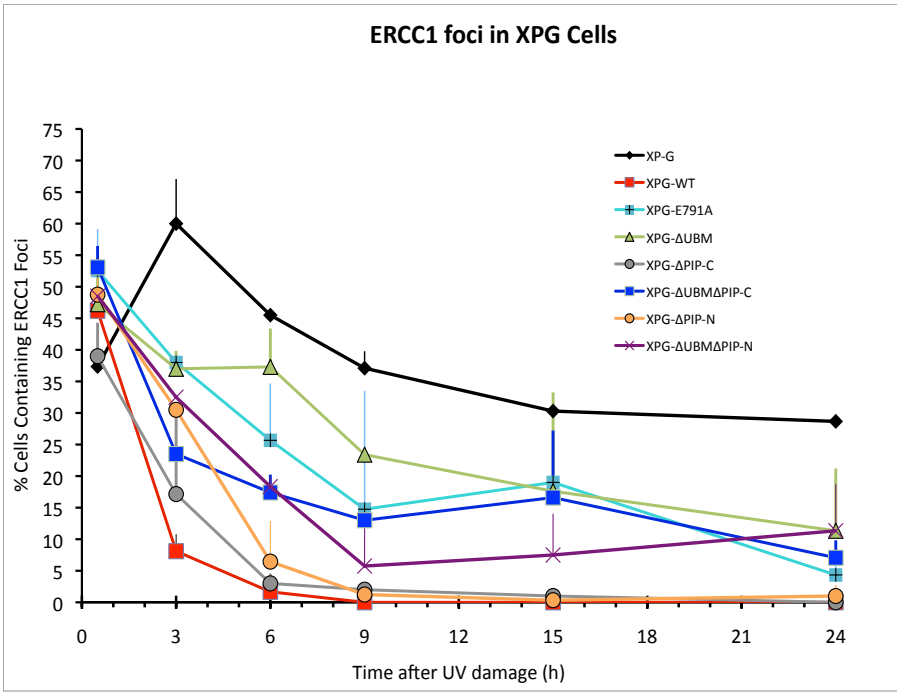


Figure 22: ERCC1 remains associated with damaged regions in E791A and UBM mutants up to 24h. A graphical representation of the percentage of cells containing ERCC1 foci at various time-points after UV.

XPG foci persist in XPG-E791A, ΔUBM, and ΔPIP-N mutants to different extents.

We analyzed the levels of association of XPG with (6-4)PPs in our XPG-mutant cell-lines. XPG was recruited to LUDs with similar dynamics in all of our cell lines, with significant foci levels seen at 0.5h following UV irradiation. In XPG^{WT} and $XPG^{\Delta PIP\text{-}C}$, XPG levels at LUDs dropped dramatically by 3h, with only <5% XPG foci present (Figures 23 and 24). However, the XPG-E791A mutant retained ~30-35% XPG foci at 3h, while the $XPG^{\Delta UBM}$, $XPG^{\Delta PIP\text{-}N}$, and $XPG^{\Delta UBM\Delta PIP\text{-}N}$ mutants retained 10-15% XPG foci levels at the same time point (Figures 23 and 24).

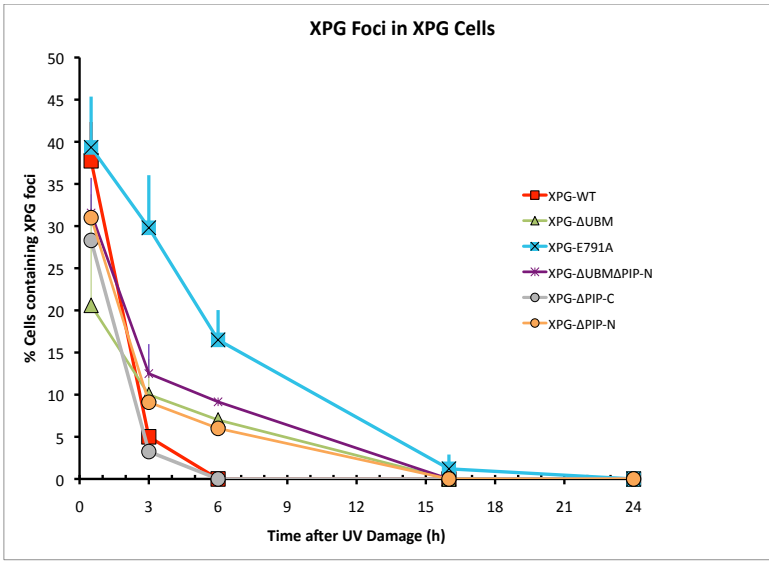


Figure 23: XPG remains associated with damaged regions for longer periods in the E791A, UBM and PIP-N mutants than the wildtype. XP-G deficient cells and those transduced with either wild-type or mutant XPG were UV-irradiated, returned to culture, and fixed and stained for the HA-tag on XPG constructs and (6-4)PP at the indicated times following irradiation. A graphical representation of the cells with persistent XPG foci at different time-points after irradiation. Compare XPG-WT to the E791A, ΔUBM, and ΔPIP-N mutants at 3h and 6h after UV irradiation. XPG does not appear to persist until 16h and 24h in any of the mutants in the conditions tested.

Likewise, at 6h following UV, whereas the XPG^{WT} and $XPG^{\Delta PIP\text{-}C}$ were no longer visible at LUDs, XPG^{E791A}, $XPG^{\Delta PIP\text{-}N}$, $XPG^{\Delta UBM}$, and $XPG^{\Delta UBM\Delta PIP\text{-}N}$ were found at LUDs in 20, 10, 6, ~6% of the cells (Figure 23). At the later time-points of 16h and 24h, no XPG was visible at LUDs in any of the cell-lines used in our experiments, which was surprising as XPG-E791A mutants have previously been shown to exhibit the maintenance of XPG foci even up until 24h after UV irradiation [46]. Figure 24 shows images of XPG foci co-localized with (6-4)PP in the different cell-lines at 0.5h and 3h following UV irradiation.

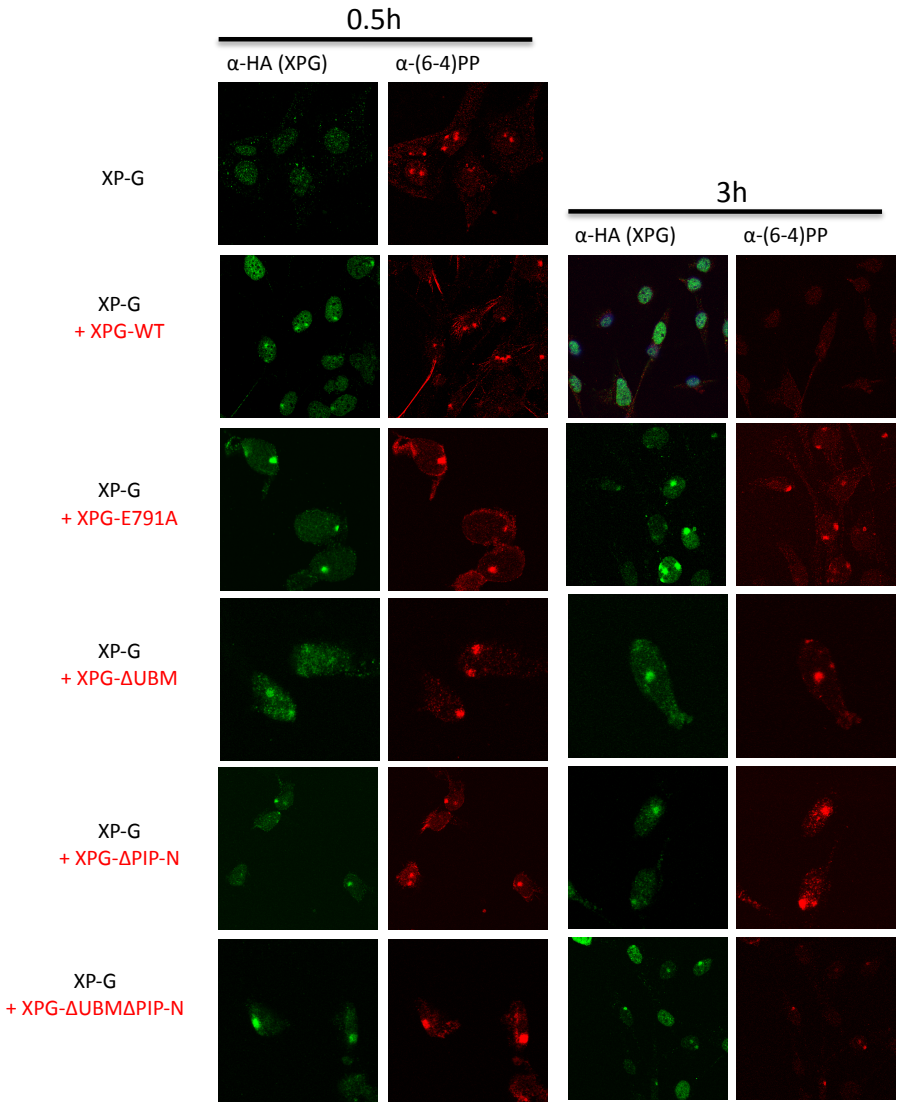


Figure 24: IF images of cells showing co-localization of XPG with (6-4)PP foci at 0.5h and 3h after UV irradiation. XP-G deficient cells and those transduced with either wild-type or mutant XPG were UV-irradiated as described, returned to culture, and fixed and stained for the HA-tag on XPG constructs (green) and (6-4)PP (red) at the indicated times following irradiation. These are images of the cells at 0.5h and 3h after UV showing XPG foci (green) and (6-4)PP foci (red).

The association and dissociation of the damage recognition factor XPC-RAD23B with (6-4)PPs is not affected by mutations in XPG.

The damage recognition factor XPC-RAD23B is ubiquitylated in NER by the UVDDB/CUL4A/RBX1 complex [143-146], and has been shown to depart from NER complexes upon, or possibly as a consequence of XPG arrival (Figures 2B-2C) [30-32]. This raised the possibility that the UBM domain in XPG is actually important for interacting wtih ubiquitylated XPC and facilitating the exchange of XPG with XPC in NER complexes. To address this issue, we probed for XPC-RAD23B in our assays using an antibody specific to XPC at various time-points following UV irradiation (Figure 25). We observed rapid accumulation of XPC at 0.5h, with >35%-50% XPC foci present in all cell-lines. While XPC persisted in XPG-deficient cells, it quickly disengaged in XPG^{WT} and all the mutants tested to ~20% and 2-10% at 1h and 3h, respectively (Figure 25). At the later time-points of 16h and 24h there were no XPC foci visible in any of the transduced cells, whereas XP-G deficient cells maintained 20% and >10% foci respectively. These results strongly indicate that the UBM in XPG does not interact with ubiquitylated XPC, nor does it facilitate the exchange of XPG with XPC on the DNA (for images of XPC and ERCC1 colocalized in cells at all time-points analyzed see Figures 32-36 in the Appendix).

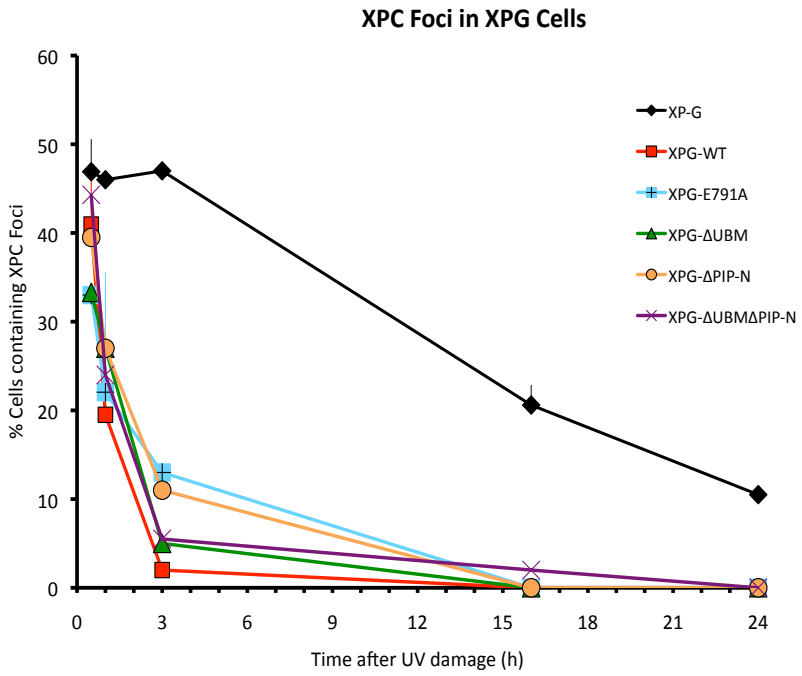


Figure 25: The damage recognition factor XPC-RAD23B behaves with similar kinetics in XPG-WT and all mutants tested. XP-G deficient cells (XPCS1RO) and those transduced with either wild-type or mutant XPG were UV-irradiated, returned to culture, and fixed and stained for XPC (red) and ERCC1 (green) at the indicated times following irradiation. The graph represents the percentage of cells containing XPC foci at the different time-points following UV irradiation. XPC foci are mostly gone from damage regions by 3h in XPG-WT and all mutants, whereas they persist in XP-G cells until 24h.

Discussion

An important unresolved issue in NER has been how the dual incision and repair synthesis steps are coordinated. XPG has long been suggested to play a role in this coordination, particularly because of known interaction with PCNA via its C-terminal PIP domain. To address this question further we have used local UV irradiation coupled with immunofluoresence to analyze XPG mutants impaired in nuclease activity, PCNA-binding via the PIP-C and PIP-N domains, and in ubiquitin-binding. The present study provides insights into how these XPG domains may regulate the transition from dual incision to repair synthesis, and suggest a mechanism by which a few subtle interactions between XPG, PCNA and ubiquitin could stabilize the NER complex and allow tight regulation of incisions.

Damage Removal

We examined the removal of (6-4)PP lesions in all the mutants and observed that the E791A, UBM, and PIP-N mutants are all impaired in damage removal although to different degrees (Figure 18). As expected, and as observed previously, the E791A mutant was significantly impaired in damage removal, however we were surprised to observe that the UBM mutant showed similar levels of impairment, although it appeared to function normally in *in vitro* repair assays. We also observed a weaker yet significant impairment in damage repair by the PIP-N mutant. The

difference in the *in vitro* versus *in vivo* repair ability of the UBM mutant can be attributed to a tendency for reactions to unnaturally go to completion *in vitro*, whereas *in vivo* certain conditions need to be met for the same reactions to occur. We have previously shown that the dual incisions in NER could be ordered with XPF first making the 5' incision, allowing initiation of repair synthesis, prior to the 3' incision by XPG [33]. If the ordered incisions were to take place *in vivo* as a mode of tighter regulation of the transition for incision to repair synthesis, then one could hypothesize that the interaction between XPG and PCNA could serve as a signal for XPG to make the 3' incision. These findings allow us to speculate that in certain instances, if the normal NER reaction is impaired from reaching completion, either due to structural DNA perturbations, or some other form of obstruction, the 5' cut could take place allowing initiation of repair synthesis, but leading to stalling of PCNA and the replication machinery. During replication, stalling of the replication machinery leads to ubiquitylation of PCNA which serves as a signal for the switch from replicative polymerases to TLS polymerases which are able to synthesize past the lesion, and allow replication to continue (Figure 14, left panel) [109, 147]. These take on added significance when considering the recent work of Ogi *et al.* involving the role of ubiquitylated PCNA and Polκ in NER [36]. They showed that ubiquitylated PCNA along with Polκ is involved in NER, and is involved in the completion of repair synthesis along with Polδ in about 50% of the NER reactions. Therefore in NER, it is

possible that stalled PCNA becomes ubiquitylated and then interacts with XPG via its UBM, as an additional trigger to stimulate the nuclease function of the protein. One unresolved discrepancy exists between the model proposed here and the findings of Ogi *et al.* They show the ubiquitylation of PCNA regardless of whether NER reaches completion or not, observing ubiquitylated PCNA in XP cells, and suggest that ubiquitylated PCNA is loaded onto the DNA independently of NER. Regardless, it is still possible that an interaction between such ubiquitylated-PCNA and XPG serves as an additional trigger for the 3' incision. Clearly, the temporal and spatial kinetics of ubiquitylation of PCNA during NER warrant further investigation.

The PIP-N mutant shows a lower level of impaired damage removal that cannot be explained by the above phenomenon, however it is possible that the PCNA-XPG interaction, which also appears to be disrupted in this mutant is responsible for this impairment. Taken together it appears that XPG nuclease activity in NER may be regulated by its interaction with PCNA and ubiquitin.

Recruitment and Disassembly of NER Factors

We were also interested in observing the behavior of other NER factors in our different mutants following UV irradiation, and towards that end we observed the recruitment of the early damage recognition factor XPC, the dual incision factors ERCC1-XPF and XPG, and the repair

synthesis factor PCNA. Recruitment of all above factors proceeded normally in all our mutants, with presence detected at 0.5h. We were particularly interested in departure of XPC from damage regions, as XPC is also ubiquitylated during NER and it was a necessary question to consider whether the UBM in XPG actually interacts with ubiquitylated XPC rather than PCNA. We observed that unlike in the XP-G cells, XPC had mostly departed from LUDs within 3h in all our mutants, similar to its behavior in wildtype cells. The XPG-UBM mutant did not alter the XPC kinetics within the damage regions, showing that displacement of XPC by XPG was normal in these mutants (Figure 25). Importantly XPC departure was the same in XPG-E791A mutant, showing that the incision by XPG is not necessary for the departure of XPC from the damaged DNA, consistent with earlier *in vitro* experiments [32].

The dual incision factors behaved differently, and in some ways unexpectedly in our mutants. ERCC1-XPF appeared to remain bound to damage regions in XPG-E791A, and all mutants containing the ΔUBM mutation as well as in XP-G cells (Figure 22). This was unexpected, especially in XPG-E791A, as we have clearly previously demonstrated that this mutant supports 5' uncoupled incision and it seems counterintuitive that ERCC1-XPF would remain at damaged sites once PCNA has been recruited and repair synthesis initiated. Based on the expected loading of PCNA onto the free 3' OH made available following XPF incision, it is as yet unclear how both factors could remain stably

associated with the DNA for extended periods of time. It is worth noting that there were much greater margins of error when measuring ERCC1-XPF foci at the later time-points as compared with most other factors (Figure 22). XPG also gave some unexpected results. Based on the observation that damage repair was impaired in the E791A, ΔUBM and ΔPIP mutants, we expected a delay in XPG departure from damaged regions in these cell-lines. A previous report by Thorel *et al*. using XPG-E791A cells in a similar assay showed the clear presence of XPG foci in these cells along with the TFIIH factor XPB at 24h following UV irradiation [46]. In our hands, XPG^{E791A} persisted at damage sites at 3h and 6h when compared to XPG^{WT} cells, however by the later time-points of 16h and 24h, XPG foci were no longer visible in these cells even though damage foci were clearly still present (Figures 23 and 24). Likewise, $XPG^{\Delta UBM}$ and $XPG^{\Delta PIP\text{-}N}$ were present at LUDs at 3h and 6h although at lower levels than XPG^{E791A}. This discrepancy in XPG behavior in our mutants, especially as relates to the XPG^{E791A} mutant, could possibly be ascribed to the method used for detecting XPG in our assays. We detected XPG using an antibody specific to the C-terminal HA tag, rather than an XPG-specific antibody, as the HA antibody produced better, reproducible results. It is possible that the signal visible from the HA antibody is lost over time. Alternatively, a study by Essers *et al.* observing the behavior of a ubiquitylation mutant of PCNA, revealed that the residence time of this mutant (which is unable to participate in TLS) at UV damaged regions was

significantly shorter than that of wildtype PCNA [126]. Interestingly the GFP-hPCNA^{K164R} mutant had kinetics similar to wildtype PCNA in DNA replication foci. It is therefore possible that we are observing a similar phenomenon in our mutants, in that the XPG protein, although impaired in its function in NER, does not remain attached to the damaged regions. Although PCNA remains attached to the DNA, it is possible that the interaction between such ubiquitylated PCNA and XPG is necessary for the completion of dual incision, and PCNA is delayed at the DNA while XPG eventually dissociates after a slight delay (Figure 23). Furthermore, recent studies have reported a connection between NER and damage signaling. Studies by Marteijn *et al.* showed activation of ATR signaling following NER, with later recruitment of DSB repair factors 53BP1 and BRCA1 to LUD regions [148]. Subsequently it was shown that ssDNA intermediates generated by NER are processed by Exo1 endonuclease, generating much larger single-stranded regions, which then trigger the damage response [111]. The absence of XPG at damage regions at later time points could be due to its displacement by other factors resulting from damage signaling.

The transition from dual incision to repair synthesis

XPG has long been suggested to play additional roles in NER other than just performing the 3' incision [6]. The observation that XPG binds PCNA via a PIP domain in XPG [55], along with the delayed residence time of XPG at sites of incised DNA [32] encouraged the idea of XPG

involvement in coordinating the transition from dual incision to repair synthesis. This hypothesis is corroborated by recent observations by Mocquet *et al.* that the presence of XPG along with RPA at the damaged DNA is necessary for initiation of repair synthesis [104], as well as our findings that the XPG-E791A mutant supports uncoupled 5' incision, recruitment of replication factors, and partial repair synthesis (Chapter 2 and [33]). The observed function of ubiquitylated PCNA in NER [36] and the presence of a UBM in XPG [110] necessitated the next question of whether an interaction of XPG with ubiquitylated PCNA during NER is important for its role for the transition from dual incision to repair synthesis. This work shows that the UBM domain of XPG is indeed important for its role in NER, and suggests that this function is mediated by an interaction between XPG and ubiquitylated PCNA. The ΔUBM mutant, although impaired in repair, supports recruitment of all NER factors tested for, and like XPG-E791A, maintains the delay of PCNA at damaged regions within the cell. This impairment of PCNA signifies an inability of the complex to complete NER, and likely indicates that the replication machinery is blocked by the presence of XPG. Alternatively, PCNA ubiquitylation could be a mechanism that comes into play when repair synthesis is impaired, possibly due to irregularities in the DNA or other problems, and in this case, would be the alternative method for stimulating XPG activity.

The normal departure of the earlier factor XPC strongly suggests that the earlier steps in NER occur with normal dynamics. A new putative PIP domain in XPG (PIP-N) also impaired damage removal and XPG$^{\Delta PIP\text{-}N}$ mutant did not allow stable recruitment of PCNA to damage regions, either at early or late time-points following UV damage. Although the PIP-N is non-canonical (Figure 16B), it appears to be important for NER function of XPG, and other factors possess non-canonical PIPs, such as chromatin assembly factor 1 (CAF-1), which contains two distinct PIPs that differ in the strength of their interaction with PCNA, yet contribute to CAF-1 function in replication [142].

It appears that the relationship between XPG and PCNA is an important and dynamic one, possibly mediated by several, subtle interactions that have different roles with regards to recruitment of PCNA and its eventual departure from NER regions. Although a lot remains to be understood about the process, the work described here highlights the intricacy of the mechanism that drives NER from damage removal to repair synthesis.

Materials and Methods

Cell Culture Conditions

SV40-tranformed XPG deficient human fibroblast cells XPCS1RO [136] were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in the presence of 5% CO_2.

Lentiviral Cell Transduction

The cDNA of XPG wildtype, XPG-E791A, XPG-UBM, XPG-PIP-N, XPG-PIP-C, XPG-UBMPIP-N, and XPG-UBMPIP-C were cloned into the pWPXL lentiviral vector with a C-terminal his_6HA tag using *Xma*I and *Mlu*I restriction sites and thereby replacing the GFP cDNA. Each lentiviral construct was co-tranfected into 293T cells along with the packaging plasmid psPAX2, and the envelope plasmid pMD2G. Production of high titer viruses was achieved as described on the LentiWeb website http://lentiweb.com/. SV40 immortalized XP-G/CS (94RD27, patient XPCS1RO) fibroblasts [136] at 50% confluency were infected with the recombinant XPG viral particles to generate stably transduced XPG-mutant cell-lines. The transduced cells were maintained in cell culture as described above. Transduction efficiency was assessed by immunofluorescence.

Local UV Irradiation and Immunofluorescence

Cells were seeded and cultured on glass coverslips and processed as described previously [57]. Cells were briefly covered with a polycarbonate filter containing 5 μm pores (Millipore) and irradiated at 150 J/m^2 using a UV-C lamp (EL series, UVP, model UVLS-28). Following UV-irradiation cells were returned to culture for appropriate recovery times. Subsequently, cells were washed with PBS, then with PBS containing 0.05% Triton X-100 prior to fixation with 3% paraformaldehyde in PBS containing 0.1% Triton X-100 for 15 min. (for PCNA staining, upon recovery from UV irradiation cells were washed with PBS, and immediately fixed in ice-cold methanol at 4°C for 15 min). Then the cells were permeabilized by washing 5 times with PBS containing 0.1%-0.2% Triton X-100. To detect (6-4)PP, cells were treated with 0.07M NaOH for 5 min to denature the DNA, and the cells were again washed 5 times with PBS + 0.1% Triton X100. Cells were blocked in PBS+ (PBS containing 0.15% glycine and 0.5% bovine serum albumin) for a minimum of 30 min and then incubated with primary antibody diluted in PBS+ at room temperature in dark, humid conditions for 2 h. Subsequently the cells were washed 5 times with PBS + 0.1% Triton, washed briefly with PBS+, and incubated with secondary antibody diluted in PBS+ at room temperature in dark, humid conditions for 1 h. The cells were washed 5 times in PBS + 0.1% Triton X-100, and once in PBS, and finally the coverslips were mounted on vectashield mounting medium (Vector Laboratories)

containing 4'-6'diamino-2-phenylindole (DAPI) at a concentration of 0.1 mg/ml.

For quantification, at least 100 cells were counted in different fields per experiment, and three independent experiments were performed to generate standard error.

Primary antibodies used are as follows: to detect XPG, mouse monoclonal anti-XPG [8H7] (abcam, ab46), 1:2000, or rabbit polyclonal anti-HA (abcam, ab9110), 1:2000; to detect PCNA, mouse monoclonal anti-PCNA (DakoCytomation, clone PC10), 1:1000; to detect XPC, mouse monoclonal anti-XPC (abcam, ab6264), 1:1000; to detect ERCC1, rabbit polyclonal anti-ERCC1 (Santa Cruz, sc-10785), 1:100; and to detect (6-4)PPs, mouse monoclonal anti-(6-4)PPs (Cosmo Bio, clone 64M-2), 1:400.

Confocal Microscopy

Confocal images of the cells were captured using a Zeiss LSM 510 microscope equipped with a 25 mW Ar laser at 488 nm, a He/Ne 543 nm laser, and a 40 × 1.3 NA oil immersion lens. Alexa 488 was detected using a dichroic beam splitter (HFT 488), and an additional 505- to 530-nm bandpass emission filter. Cy3 was detected using a dichroic beam splitter (HFT 488/543) and a 560- to 615-nm bandpass emission filter.

Western Blotting

Western blotting was performed as described in [109].

Protein Expression and Purification

Wild-type XPG, XPGΔUBM^{LP-AA}, and XPGΔUBM^{Domain} proteins were expressed in Sf9 cells and purified as described previously [51]. Typically 0.2-0.5 mg of protein was obtained at concentrations of 0.2-0.3 mg/ml.

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Marzena Bienko in the laboratory of Dr. Ivan Dikic performed the experiment described in Figure 17 and Lidija Staresincic purified the proteins used in the same experiment.

Conclusions

And

Future Directions

The aim of this thesis was to explore the role of the 3' structure-specific endonuclease XPG in regulating the late steps of NER. To this end we first investigated the *in vitro* and *in vivo* function of the nuclease dead mutant XPG-E791A. We describe in Chapter 2 how this mutant is able to support uncoupled 5' incision and partial repair synthesis. XPG-E791A enables recruitment of the replication machinery, which is not recruited in the absence of XPG protein. The corresponding structure-specific endonuclease that makes the 5' cut in NER, ERCC1-XPF, was also tested in a similar manner in our assays. The nuclease dead ERCC1-XPF-D676A was unable to support any incision (5' or 3'), and did not allow any recruitment of downstream replicative factors [33]. These findings strongly suggest that the XPF incision occurs first and that XPG has a special role in coordinating the transition from dual incision to repair synthesis.

An interaction between XPG and the sliding clamp PCNA had previously been shown [55], and we were interested in testing whether the interaction of these two proteins was important for the late steps of NER. The discovery of a UBM (ubiquitin-binding domain) in XPG [110], coupled with the recent report indicating that ubiquitylated PCNA functions during NER [36] encouraged us to study the function of this UBM in NER. We also discovered a second putative PIP domain in XPG and considered whether there was some cooperation (or alternatively redundancy)

between two or more of these domains in mediating the XPG-PCNA interaction. Such synergistic relationships have been shown with relation to the PIPs and UBMs in the TLS polymerases [109], or the two PIPs in proteins such as CAF-1 [142]. To this end we generated mutant XPG cell-lines stably expressing mutations of either of these three domains alone or in combinations. We tested these cells along with XP-G deficient, XPG-WT, and the nuclease dead XPG-E791A cell-lines in immunofluoresence assays [57]. We generated local damage to the DNA in the cells by UV irradiation, and observed the kinetics of removal of damage, and of the recruitment and disassembly of several NER factors, to give us a better understanding of how these mutations affect various stages of the NER process. We observed that the E791A, UBM, and PIP-N mutants all exhibited impaired damage removal while the PIP-C mutant, like XPG-WT cells, did not. We also observed that the mutants behaved differently with regards to the kinetics of PCNA. The UBM and E791A mutant cells allowed normal recruitment of PCNA, but did not permit its normal disassembly and subsequent recycling, whereas the PIP-N mutants did not support the stable association of PCNA to damage regions.

The conclusions from our findings are that the XPG-PCNA interaction is indeed important for the smooth transition from dual incision to repair synthesis. It appears that XPG mediates the interaction by at least two different domains with differing affinities for PCNA, and possibly also interacts with the modified version of the protein by interacting with

ubiquitin on its surface. A closer look at these mutations by *in vitro* methods would give a good understanding of their role. The different mutants will be tested in pull-down assays utilizing PCNA and ubiquitylated PCNA to test how they affect the PCNA-XPG interaction directly. They will also be tested in *in vitro* repair synthesis assays to detect whether any repair synthesis is initiated as was seen with XPG-E791A. Such a finding would reiterate the idea that at least in certain conditions, the 5' incision occurs prior to the 3' incision, and, as we have observed in our immunofluorescence assays, that the UBM mutant behaves in a manner similar to XPG-E791A.

A closer examination of the role of ubiquitylated PCNA in the later steps of NER would be of importance. It would be relevant to detect NER-dependent ubiquitylation of PCNA possibly by UV irradiation followed by western blotting to detect modified and unmodified versions of PCNA. PCNA is typically ubiquitylated on its K164 residue by the Rad6-Rad18 E3 ubiquitin ligase [147, 149, 150].

Recent reports have linked NER to ATM and ATR signaling, an important finding as no such connection was previously known. One such study showed that single-stranded intermediates generated during NER (products of incision, particularly upon inhibition repair synthesis) led to recruitment of RPA, which in turn resulted in ATR activation [151]. Subsequently a different study showed that damage-signaling factors such as RNF8, MDC1, and 53BP1, which are typically active during DSB repair

were also activated in an NER-dependent fashion [148]. It has been shown, at least in *S. cerevisiae*, that the Exo1 nuclease, which has been shown to function in MMR and homologous recombination, processes NER intermediates to generate longer ssDNA regions that activate downstream signaling [111]. Some of the mutants described in this thesis would be relevant for testing this hitherto unresolved connection between the different damage pathways. The XPG-E791A mutant would be an interesting model for testing the possible up-regulation of Exo1, or RNF8, as 5' uncoupled incision would mimic inhibition of repair synthesis. The UBM and PIP-N mutants would also be of interest with this regard.

In conclusion, the findings described in this thesis add to the understanding of NER, particularly in how the late steps of the pathway proceed. It would be beneficial to further explore the dynamics of the interactions described here and possibly link the previously parallel damage repair responses, NER and damage signaling, more clearly.

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APPENDIX

The appendix contains supplemental figures for Chapter 3 as indicated within the text. These figures are listed in the preliminary pages under "List of Figures".

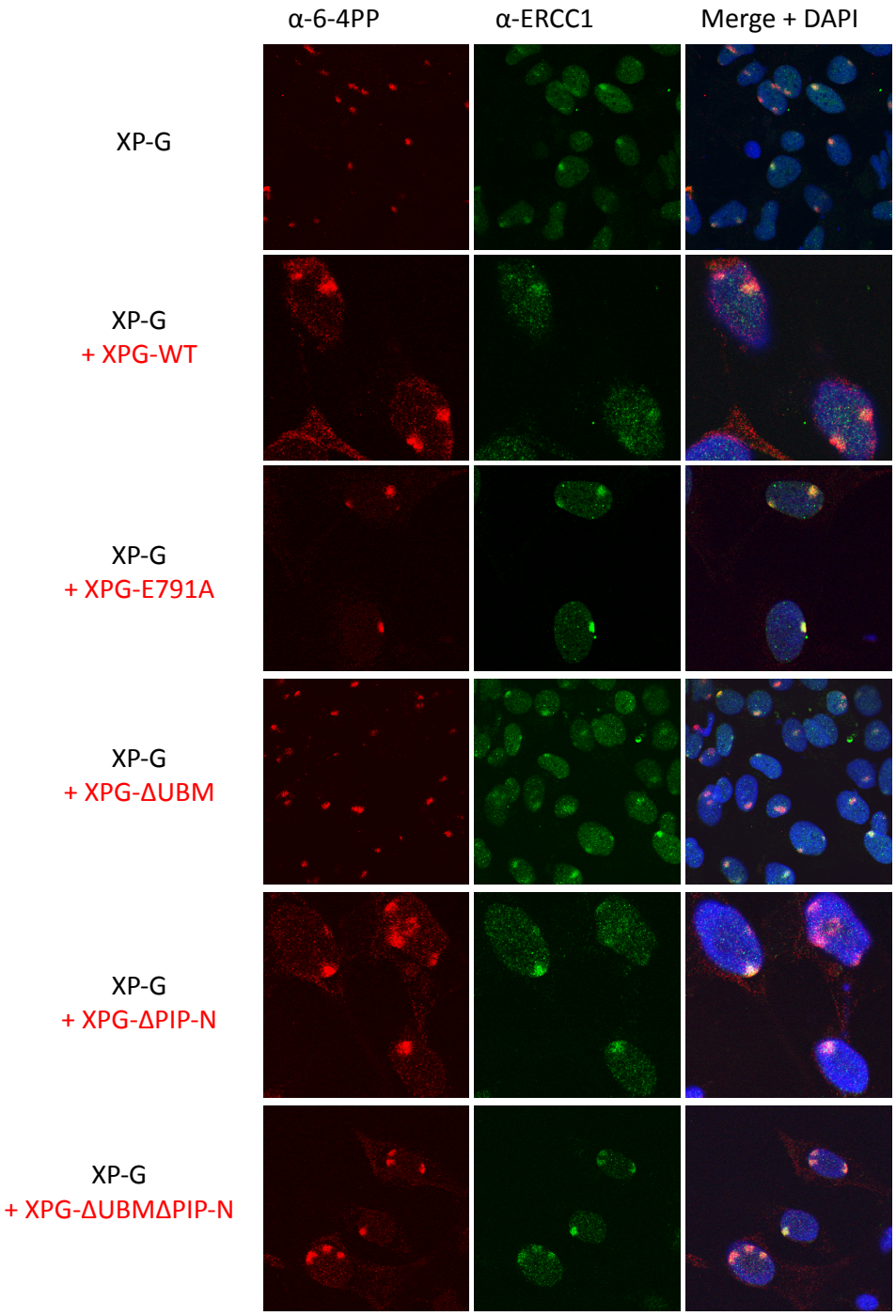

Figure 26: IF images of (6-4)PPs colocalized with ERCC1 foci in XPG cell-lines at 0.5h following UV irradiation. (6-4)PP foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

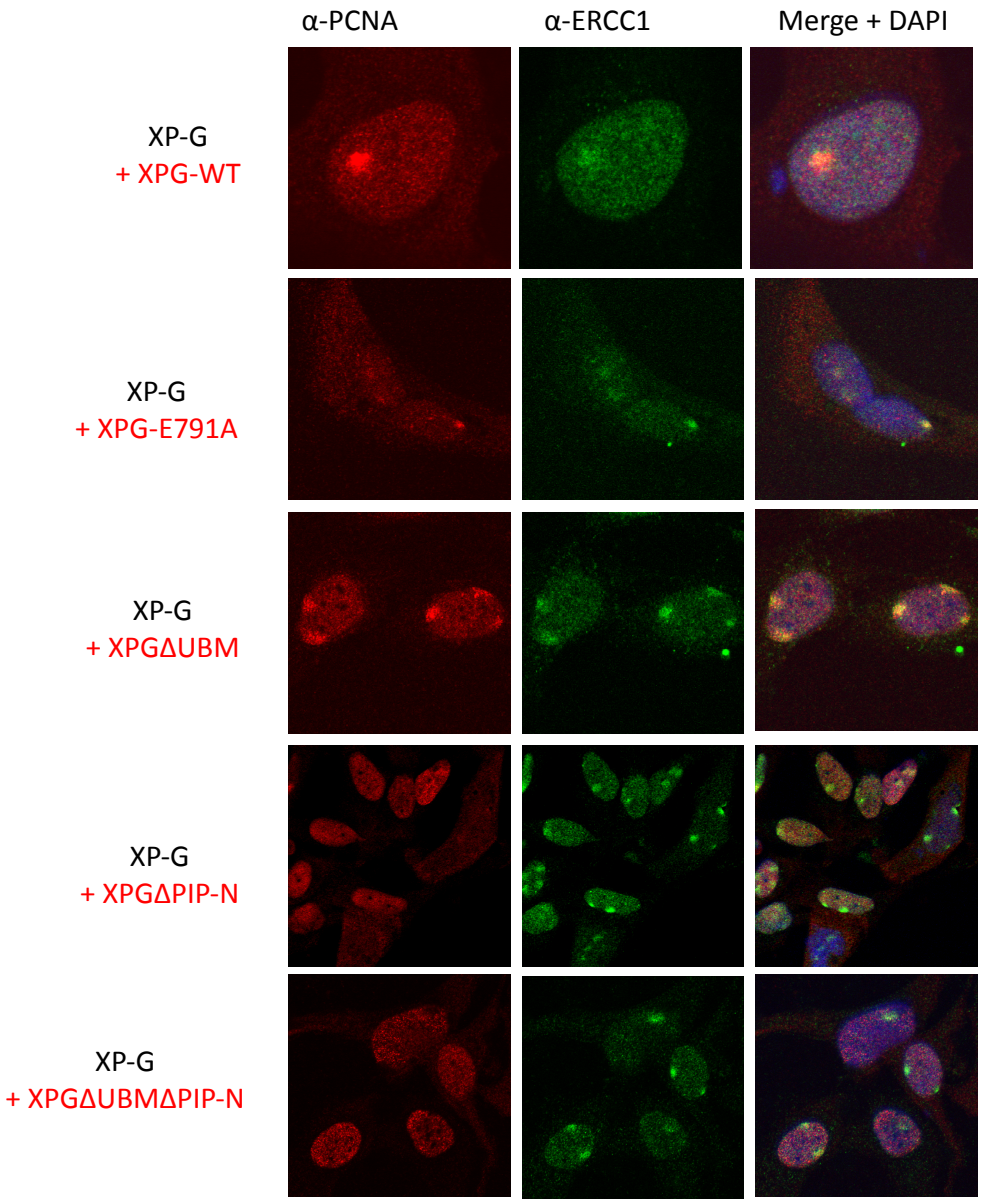

Figure 27: IF images of PCNA foci colocalized with ERCC1 foci in XPG cell-lines at 0.5h following UV irradiation. PCNA foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

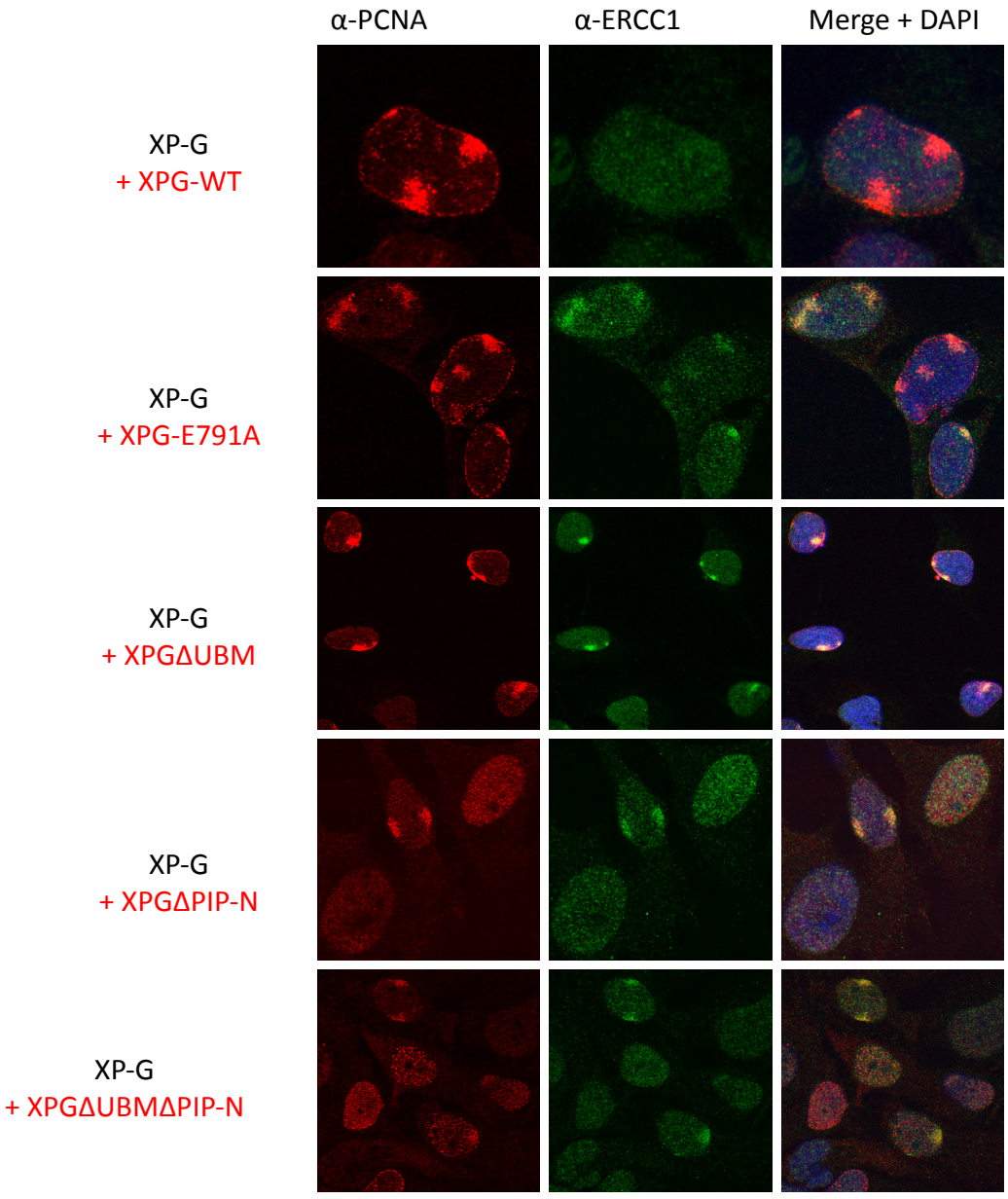

Figure 28: IF images of PCNA foci colocalized with ERCC1 foci in XPG cell-lines at 6h following UV irradiation. PCNA foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

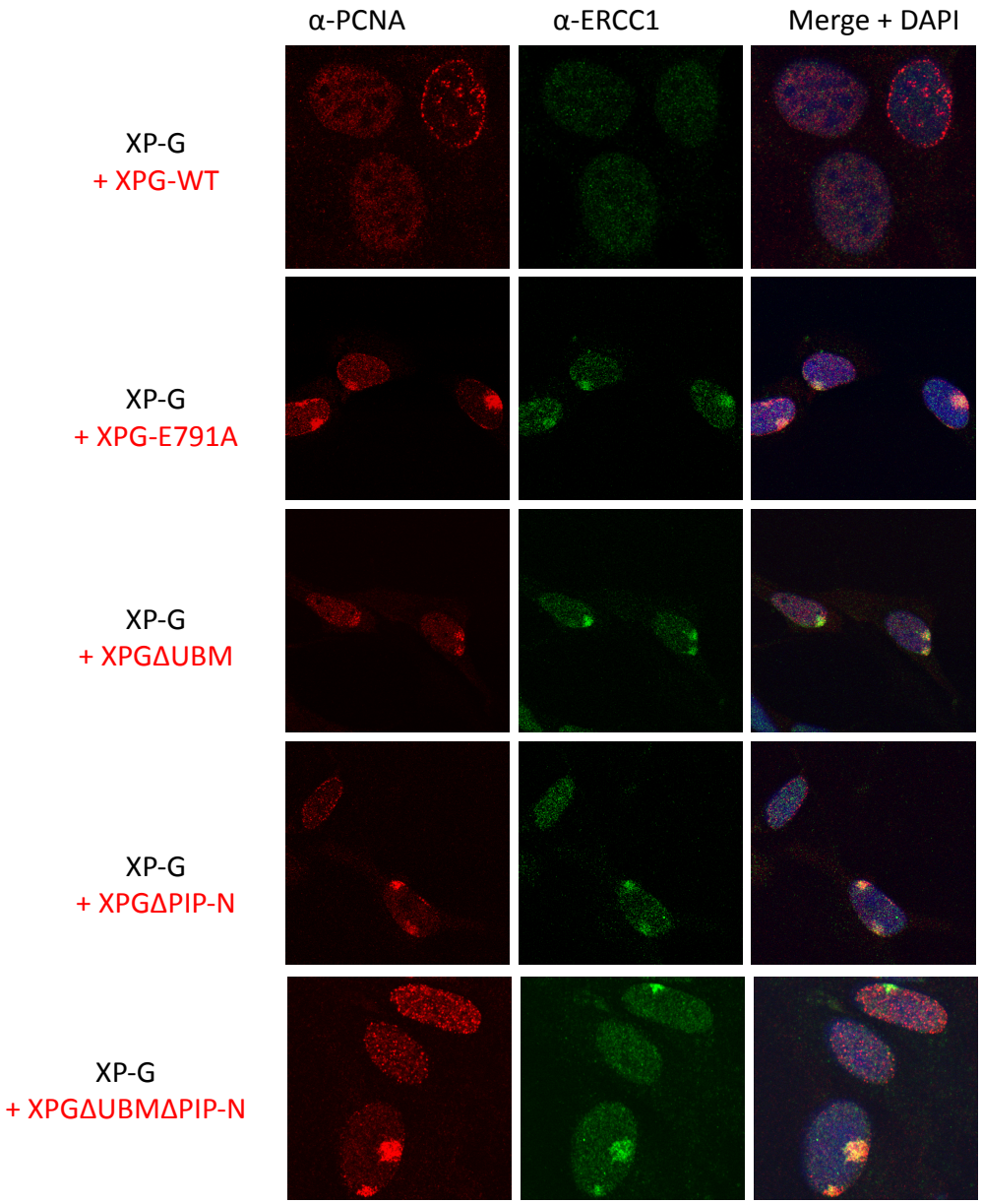

Figure 29: IF images of PCNA foci colocalized with ERCC1 foci in XPG cell-lines at 9h following UV irradiation. PCNA foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

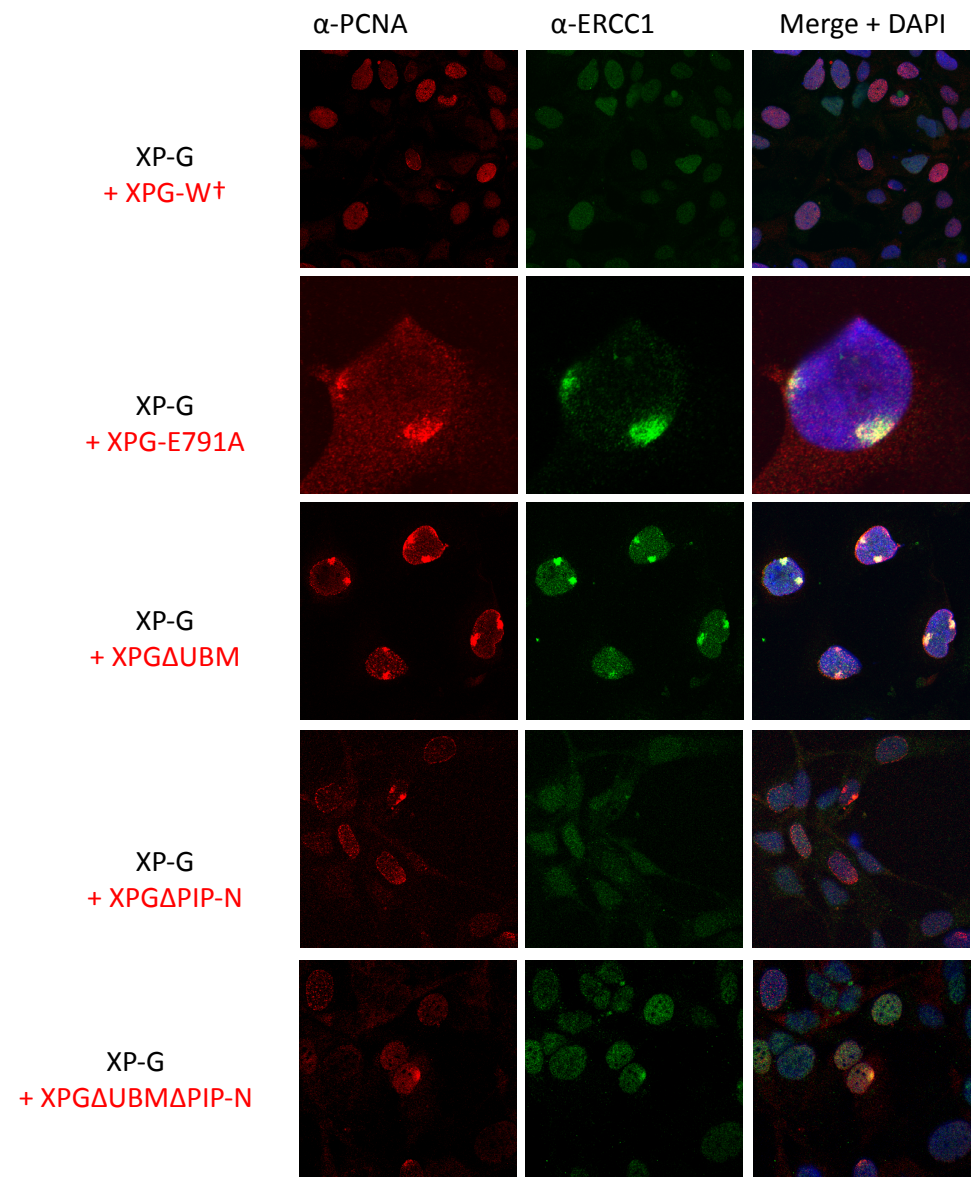

Figure 30: IF images of PCNA foci colocalized with ERCC1 foci in XPG cell-lines at 15h following UV irradiation. PCNA foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

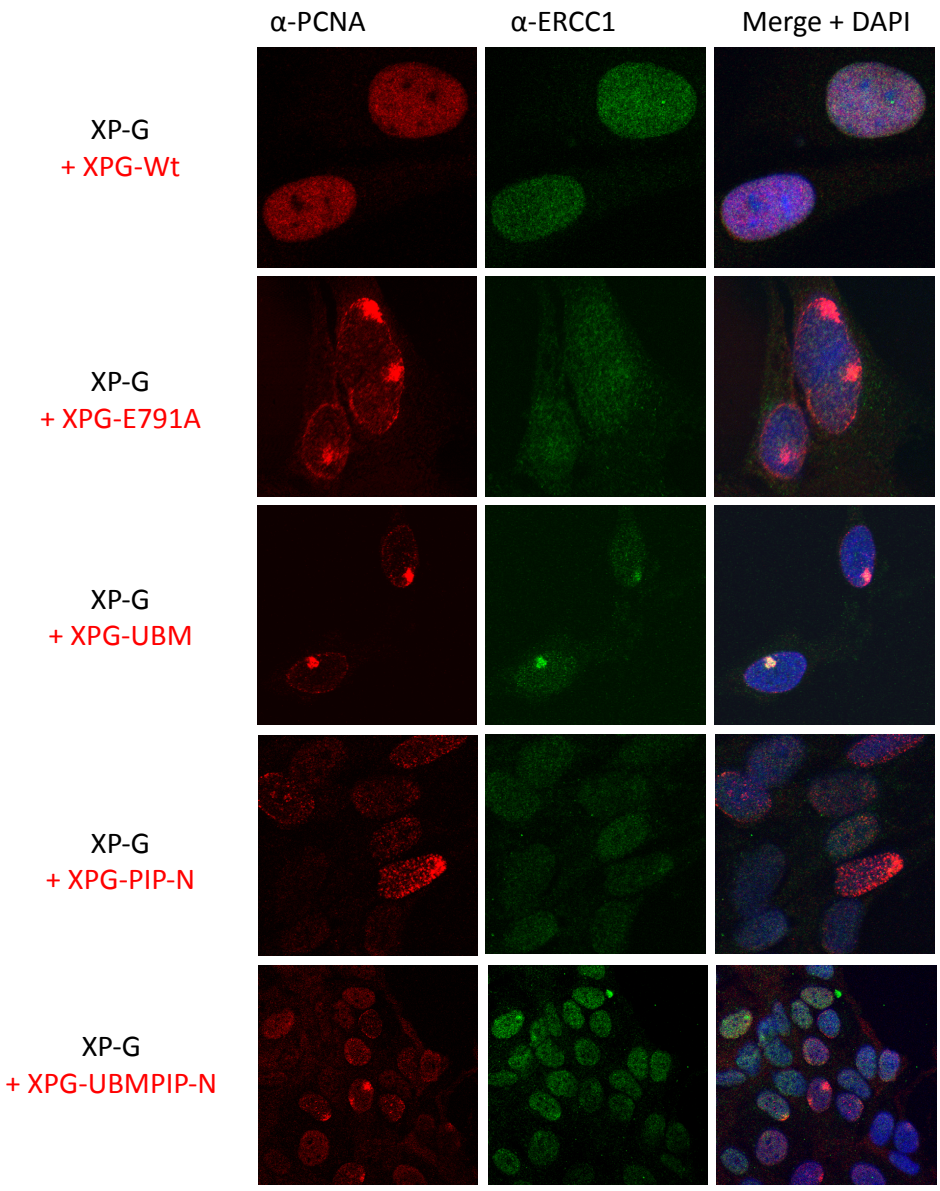

Figure 31: IF images of PCNA foci colocalized with ERCC1 foci in XPG cell-lines at 24h following UV irradiation. PCNA foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

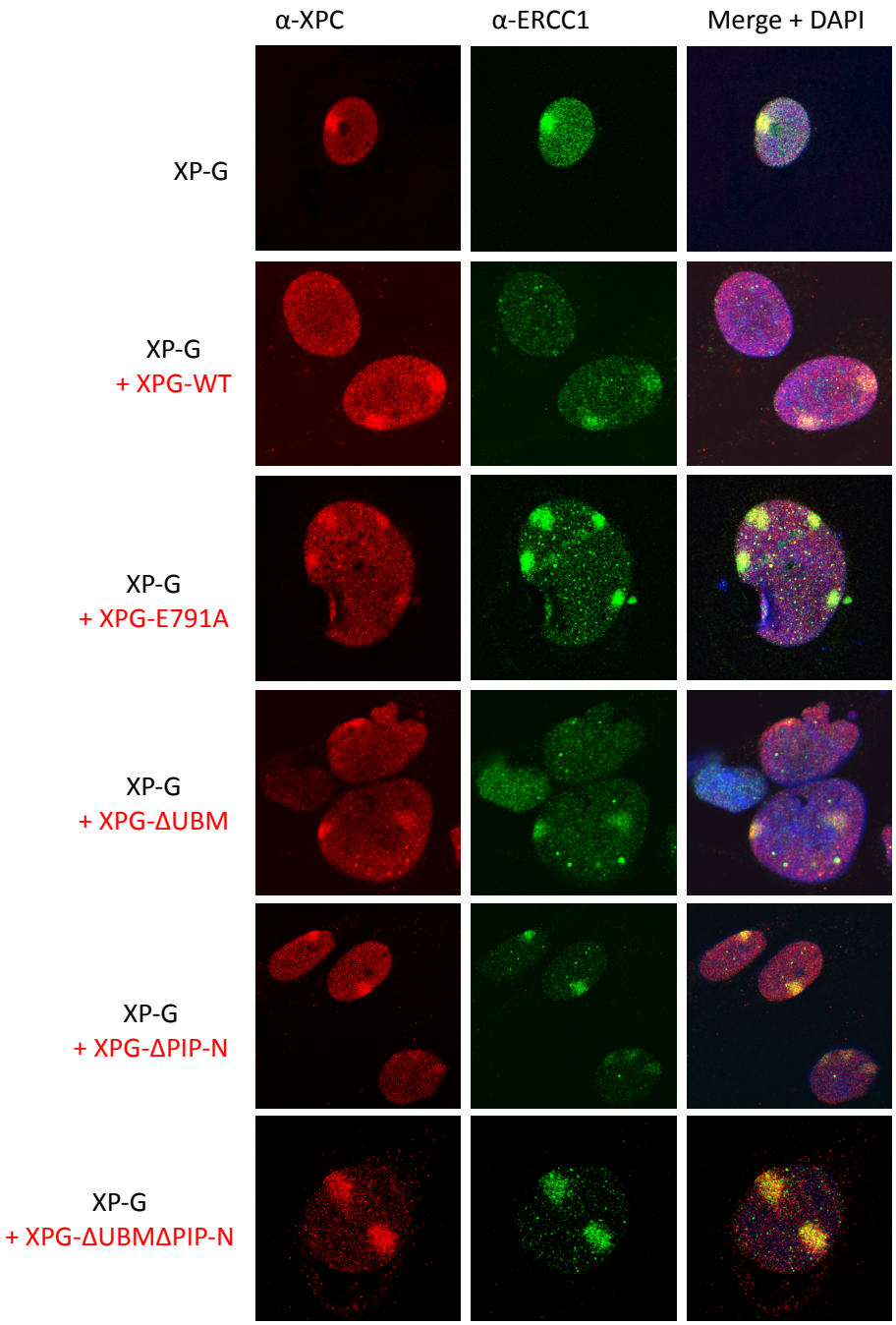

Figure 32: IF images of XPC foci colocalized with ERCC1 foci in XPG cell-lines at 0.5h following UV irradiation. XPC foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

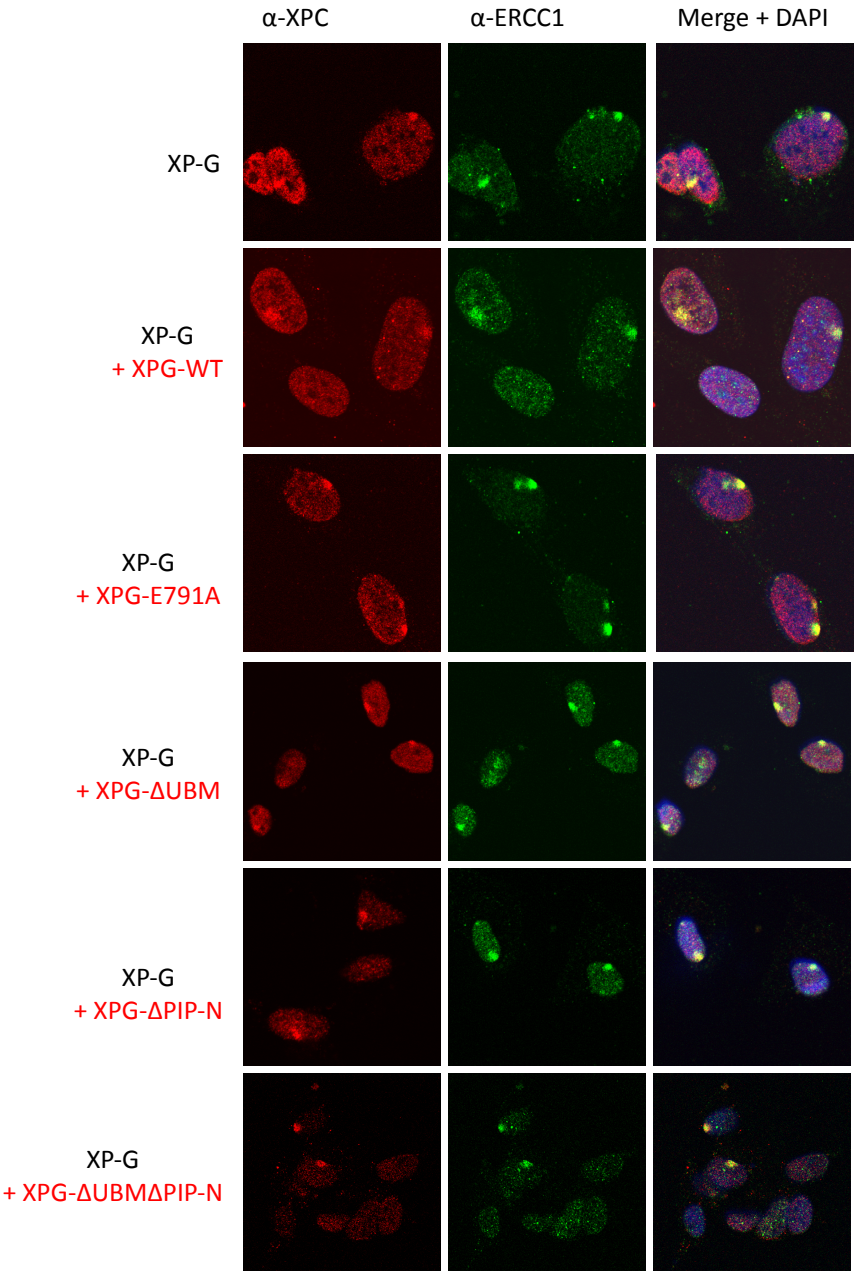

Figure 33: IF images of XPC foci colocalized with ERCC1 foci in XPG cell-lines at 1h following UV irradiation. XPC foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

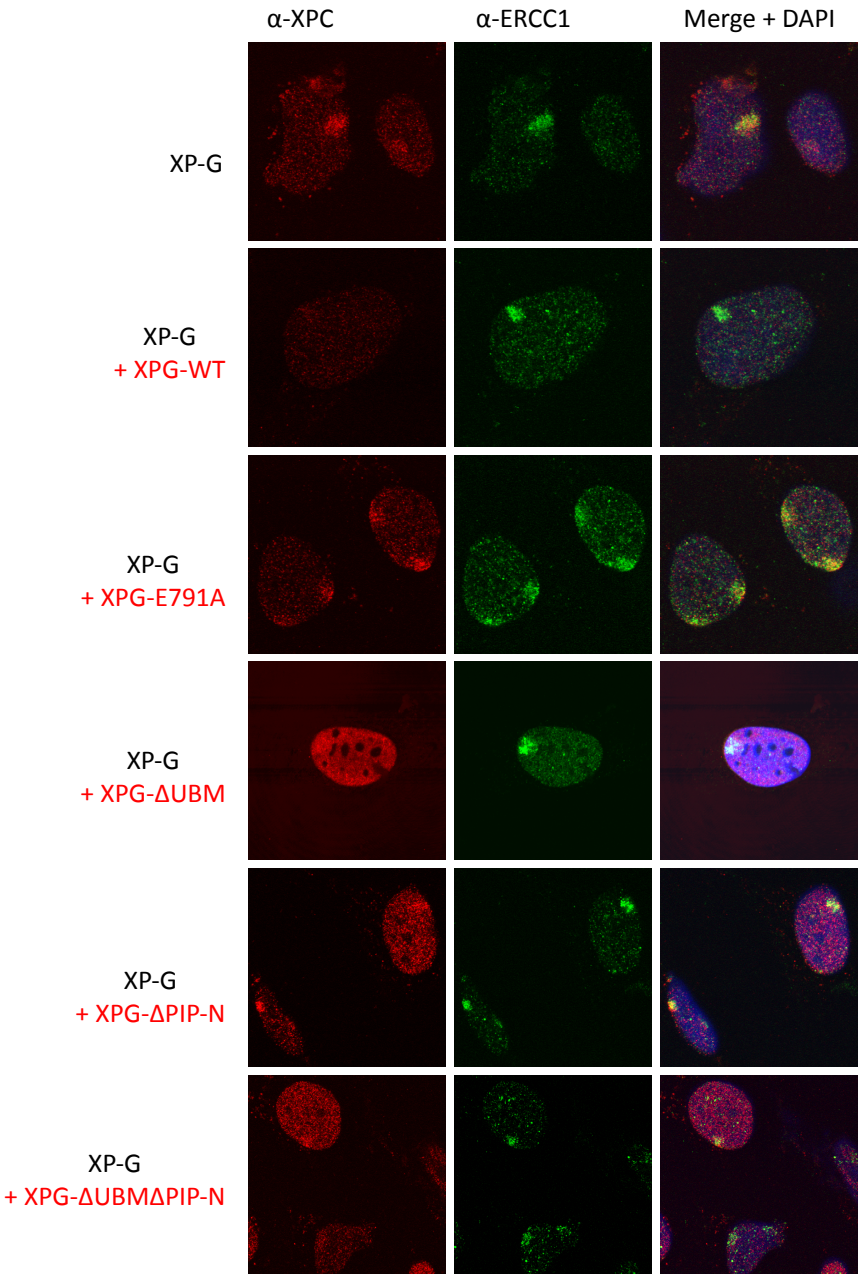

Figure 34: IF images of XPC foci colocalized with ERCC1 foci in XPG cell-lines at 3h following UV irradiation. XPC foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

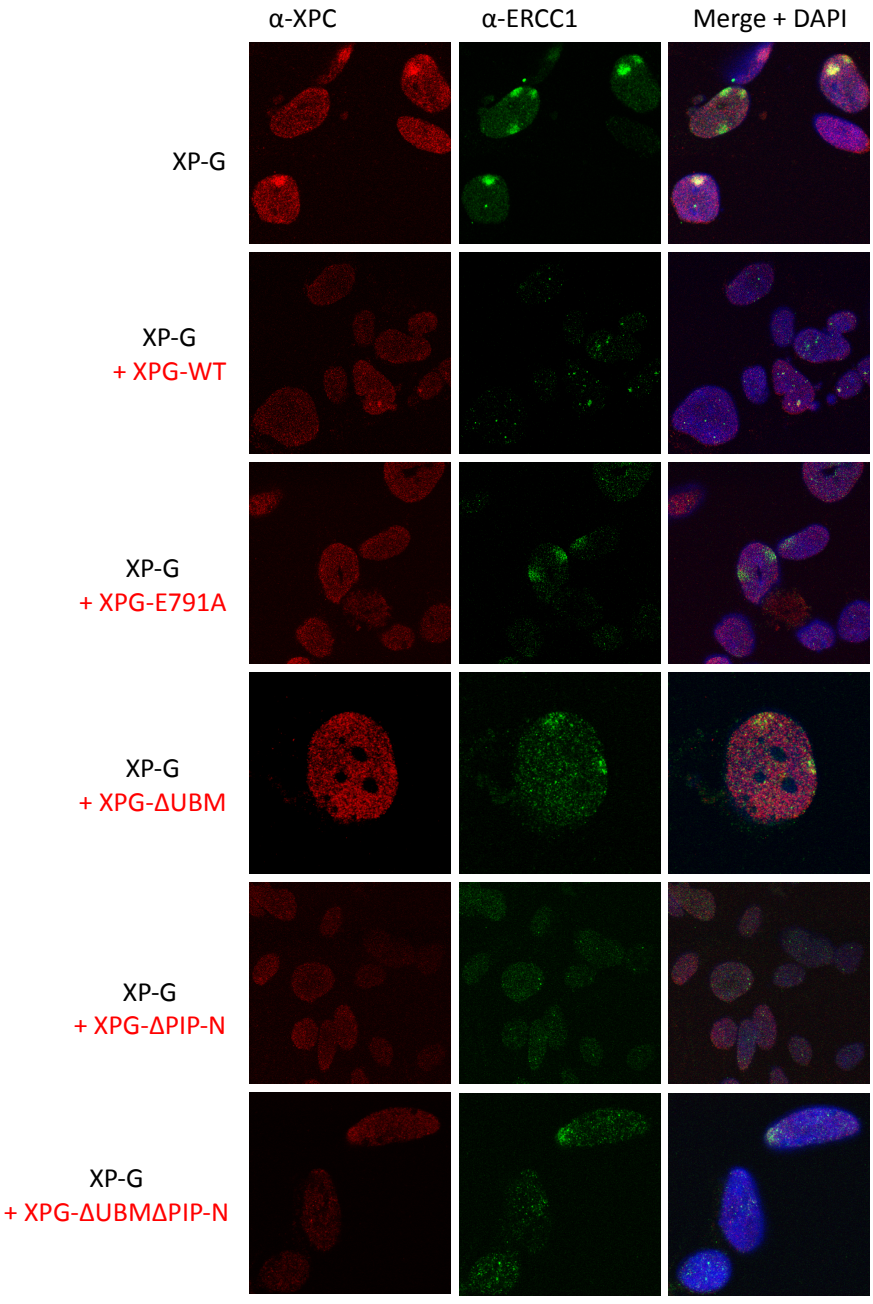

Figure 35: IF images of XPC foci colocalized with ERCC1 foci in XPG cell-lines at 16h following UV irradiation. XPC foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.

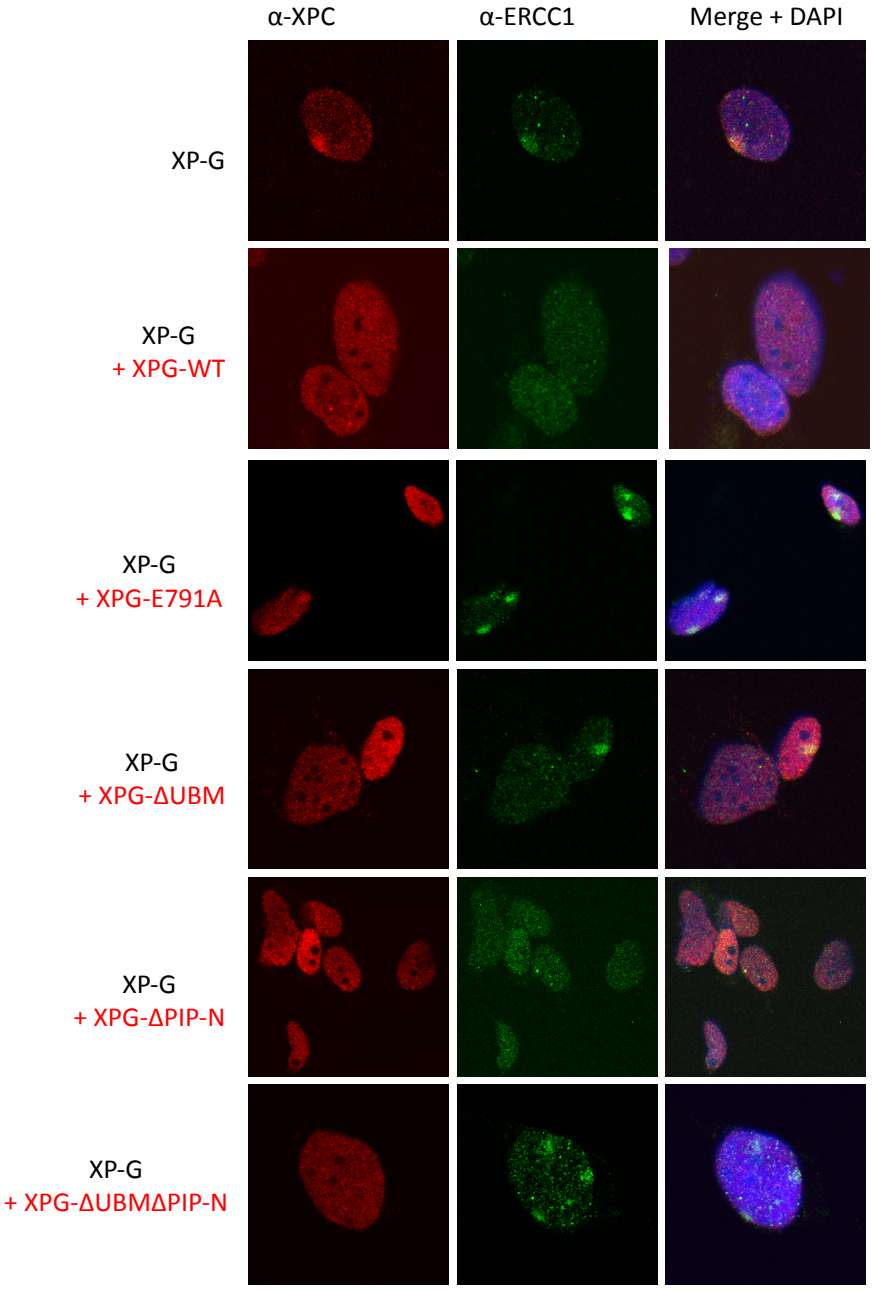

Figure 36: IF images of XPC foci colocalized with ERCC1 foci in XPG cell-lines at 24h following UV irradiation. XPC foci are shown in red, ERCC1 foci in green and merged images with DAPI staining in blue to highlight the nucleus.