Published in final edited form as:

DNA Repair (Amst). 2011 July 15; 10(7): 722–729. doi:10.1016/j.dnarep.2011.04.022.

Regulation of endonuclease activity in human nucleotide excision repair

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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. NER 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. This review focuses on 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.

1. Introduction

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) [1, 2]. 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. 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 [3, 4]. 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 [3], which are not caused by defects in NER, but are likely due to a mild impairment of transcription.

The NER pathway is highly versatile and complex and has the ability to process a myriad of different lesions [1]. 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 1) [1, 5].

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This review 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 be 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.

2. 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 [6]. 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 1A) [7-9]. XPC then recruits the ten-subunit complex TFIIH, which contains two helicase subunits. It is thought that XPB pries apart the two DNA strands [10], 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 1B) (for a more detailed discussion of damage recognition and verification and TFIIH function see contributions by Naegeli and Sugawara, Liu et al, Fuss and Tainer, and Egly and Coin in this issue of DNA Repair) [11, 12].

The result of TFIIH activity is an opened DNA structure [13, 14] that leads to the recruitment of the next three factors, XPA, RPA, and XPG, and formation of a stable preincision complex (Figure 1C). 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 [15, 16]. XPA additionally interacts with several NER factors, including RPA [17], TFIIH [18, 19] and ERCC1 [20, 21]. 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 [22]. 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 1C-E), where it helps to position the two endonucleases, while protecting the non-damaged strand following incision [5, 23]. 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 [24-26]. As we will discuss in more detail below, recent studies suggest that the catalytic activity of XPG is not revealed at this point [14, 27] and XPG fulfills a structural role in stabilizing the preincision complex generating an open-stable complex [28].

The last factor to join the preincision complex is the second endonuclease, ERCC1-XPF, which is recruited through interaction with XPA [21, 29]. Following its arrival, a number of catalytic steps are triggered (Figure 1D-F): dual incisions 5' and 3' to the lesion by XPF and

XPG, respectively [27]; repair synthesis filling in the gap by DNA polymerases δ , ϵ , or κ and associated factors [30] and finally, sealing of the nick by DNA ligase I or III α [31], 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.

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

3.1. 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 2A). Crystal structures of the family-members, T4 RNase H [32], T5 5'-exonuclease [33] and FEN1 [34, 35] 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 [36, 37]. 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 2A) [38]. 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 [25, 39, 40], and RPA [41] and is therefore required for the recruitment of XPG to sites of NER [42].

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 [34, 43, 44]. 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 [45]. 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 FEN-1, while also showing a preference for double-flap over single-flap substrates, typical of FEN-1 and not XPG [46]. 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 [47].

3.2. 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 [41, 48], PCNA [49] and TFIIH [25, 39, 40, 42, 50]. The strongest interaction is the one with TFIIH [25], and is required for the recruitment of XPG to NER complexes [51]. 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 [39]. 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 [40]. Nonetheless, the XPG Δ 225-231 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 [50].

3.3. 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 activity 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 [52, 53]. The other factors that are also associated with the XP/CS phenotype are the XPB and XPD subunits of TFIIH [3]. 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) [54, 55]. Furthermore, a role for the *S. cerevisiae* XPG homolog Rad2 in RNA polymerase II-mediated transcription has been demonstrated more directly [56]. 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 (see article by Lagerwerf *et al.* in this issue of DNA Repair) [47, 57-59]. 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) [60-62].

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

4.1. Properties, functional domains and structures of ERCC1-XPF

ERCC1-XPF forms an obligate heterodimer in mammalian cells [63] and *in vitro*, and the two proteins are unstable in the absence of each other [64, 65]. Both ERCC1 and XPF were originally cloned by complementation of UV sensitive rodent cell lines [66, 67] and by sequence homology to the yeast Rad1 gene [63]. ERCC1 and XPF dimerization occurs through two helix-hairpin-helix (HhH) motifs found at the C-termini of both proteins (Figure 3) [68, 69]. The HhH motifs are structurally similar to those found in other DNA binding proteins and also contribute to DNA binding in ERCC1-XPF [70-73]. The N-terminus of XPF is believed to be another important DNA binding region, which bears the same structure as the helicase domain of the archeal superfamily 2 (SF2) helicases, but lacks the residues necessary for ATP hydrolysis [74, 75].

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 [76]. An equivalent role was subsequently shown for the human ERCC1-XPF protein [63, 67, 77]. 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/IERKX₃D signature motif [78, 79]. 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 [68]. Indeed, the crystal structure of the ERCC1 central domain revealed a high structural homology with the nuclease domain found in archeal orthologs of XPF [70, 80]. 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.

4.2. 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 [20, 81], 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 [20]. 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 [81]. More recent studies revealed that a small peptide of XPA (residues 67-80) is necessary and sufficient for mediating the interaction with ERCC1 [21]. 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 4A) [21]. 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 4B) [21].

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* [29]. 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 [29]. 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) [82, 83]. 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.

5. 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 (see also article by Novarina *et al.* in this issue of DNA Repair) [84] 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.

A recent study by our group using catalytically inactive forms of XPG and XPF showed that the incisions might indeed be ordered. In agreement with earlier studies [36, 37], using nuclease-dead XPG-E791A in an *in vitro* repair assay resulted in the formation of 5'

uncoupled incision products. By contrast, the use of nuclease-dead XPF-D676A resulted in negligible 3' incision products [14, 27]. 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. 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 Pol δ and chromatin remodeler CAF-1 to sites of local UV lesions, whereas neither were visible in XPF-D676A cells [27]. 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 [13, 28, 85, 86]. 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 [45, 63, 87]. 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.

6. 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 [42, 45] and it has also been shown that the XPG family member FEN1 undergoes a conformational change to become catalytically active [43]. 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 [23, 88, 89]. Another attractive candidate is PCNA. PCNA has been shown to interact with XPG [49], and has been established to play a key role in triggering incision of DNA by FEN-1 during the resolution of Okazaki fragments during replication [90, 91]. 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 [27] 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 [92]. Two observations suggest that a related mechanism could apply during NER. First, a recent study by Ogi *et al.* showed that three different polymerases, pol δ , polk, and pol ϵ are responsible for repair synthesis in NER, and that selective recruitment of at least polk is dependent on functional Rad18 and PCNA ubiquitylation [30]. Second, sequence analysis of XPG shows that, like TLS polymerases [93], it contains a ubiquitin-binding motif (UBM) in addition to its PIP domain for PCNA interaction [94], raising the possibility that an interaction between ubiquitylated PCNA and XPG could be involved in

triggering 3' incision in NER. Preliminary data from our laboratory indeed indicates that mutations in the UBM domain in XPG impairs the late steps of NER, suggesting that this domain of XPG may mediate its interaction with ubiquitylated PCNA, and possibly regulates the transition from dual incision to repair synthesis. To what extent such protein-protein interactions among PCNA, XPG, Polk 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 (see article by Novarina *et al.* in this issue of DNA Repair) [95].

7. 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 review 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.

Acknowledgments

Work in the authors' laboratory on NER is supported by NIH grants GM080454, CA092584 and ES004068.

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Figure 1. 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 initiation of repair synthesis by the replication machinery, and in turn triggers 3' incision by XPG (E). Repair synthesis is completed and DNA ligase III α or ligase I seals the nick to complete the process (F).

Fig. 2. 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 domain (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.

Fig. 3. 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.

Figure 4. 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 [21, 29] (B) The XPA₆₇₋₈₀ peptide inhibits the *in vitro* NER reaction. Addition of increasing concentration of the XPA₆₇₋₈₀ peptide to a cell-free extract inhibits the excision of the cisplatin-containing 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. Figure adapted [21, 29].

Table 1

Known functions of the XPG protein

Pathway	Function(s)	Interacting Partners	References
GG-NER	Preincision complex formation; 3' incision	RPA, TFIIH, PCNA	[25, 39-41, 49, 87]
TC-NER	TC-NER complex assembly; 3' incision	TFIIH, PCNA, RNA Pol II, CSB	[47, 57, 58]
Transcription	?	RNA Pol II, TFIIH	[47, 54, 56]
BER	Stimulation of DNA glycosylase, other roles (?)	NTH1	[60, 61]

Table 2

Known functions of the ERCC1-XPF protein

Pathway	Function	Interacting Partners	References
NER	5' incision	XPA	[20, 21, 63]
Interstrand Crosslink Repair	ICL unhooking, homologous recombination (?)	SLX4	[96-99]
DSB Repair/ssDNA annealing	Flap cutting (?)	SAW1, SLX4	[100-102]
Telomere maintenance	?, Nuclease-independent	TRF2	[103-105]