Opinion

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# Interstrand crosslink repair: can XPF-ERCC1 be let off the hook?

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**The interstrand crosslink (ICL) presents a challenge to both the cell and the scientist. From a clinical standpoint, these lesions are particularly intriguing: ICL-inducing agents are powerful tools in cancer chemotherapy, and spontaneous ICLs have recently been linked with accelerated aging phenotypes. Nevertheless, the ICL repair process has proven difficult to elucidate. Here we discuss recent additions to the current model and argue that the endonuclease xeroderma pigmentosum complementation group F-excision repair cross-complementing rodent repair deficiency complementation group 1 (XPF-ERCC1) has been heretofore misplaced. During nucleotide excision repair, XPF-ERCC1 makes a single-strand nick adjacent to the lesion. XPF-ERCC1 has been thought to play an analogous role in ICL repair. However, recent data has implicated XPF-ERCC1 in homologous recombination. We suggest that this role, rather than its function in nucleotide excision repair, defines its importance to ICL repair.**

## Interstrand crosslink repair: linked to disease

The clinical implications of interstrand crosslink (ICL) repair make this process an exciting and important area of research. In contrast to mutagenic lesions, such as pyrimidine dimers and abasic sites, unrepaired DNA ICLs are highly cytotoxic [1]. This property has long been exploited in the clinic: ICL-inducing agents, including mitomycin C (MMC), nitrogen mustards (HN2s) and cisplatin, are powerful tools in cancer chemotherapy [1]. However, the utility of these agents is curtailed by the ability of cancerous cells to develop resistance. Importantly, cross-resistance to different ICL-inducing agents can develop after exposure to just one agent [2]. This observation shows that the cell has a mechanism for ICL repair and suggests that this mechanism is upregulated in resistant cells. Thus, a better understanding of ICL repair might help to identify new targets for chemotherapy enhancement. Intriguingly, recent evidence suggests that the clinical importance of ICL repair extends beyond chemotherapy. ICLs also occur during normal cell metabolism, and ongoing work suggests a critical link between deficient repair of such spontaneous ICLs and premature aging [3] (Box 1).

Although a great deal of work has been done to characterize the repair of ICLs in the yeast *Saccharomyces cerevisiae* [4], ICL repair in metazoans remains less well understood. Work in mammalian cells and *Drosophila melanogaster* has revealed contributions of proteins best known for their roles in other repair pathways, including homologous recombination repair (HRR), mismatch repair (MMR) and nucleotide excision repair (NER) (Figure 1). A general model for ICL repair has emerged: the replication machinery stalls at the site of the crosslink, where strand cleavage results in a one-ended double-strand break (DSB). Single-strand nicking of one strand on each side of the lesion enables one of the two crosslinked nucleotides to swing free of the helix, whereas the other remains connected to its phosphodiester backbone. This process is known as 'unhooking' (Figure 2). Unhooking is followed by homologous recombination, which enables the original replication fork to be restored. An unhooked crosslink could be removed by the NER machinery, which removes bulky mono-adducts from DNA. Intriguingly, two components of the NER pathway, xeroderma pigmentosum complementation group F, also called *ERCC4* (*XPF*) and excision repair cross-complementing rodent repair deficiency complementation group 1 (*ERCC1*), play a role in ICL repair outside of the NER pathway.

## Does XPF-ERCC1 mediate unhooking?

*XPF* belongs to the XP group of genes, mutations in which result in xeroderma pigmentosum (XP), a condition characterized by photosensitivity and susceptibility to skin cancer [5]. The proteins encoded by these genes make up the NER pathway, which is responsible for the excision of UV photoproducts (hence the photosensitivity) and other

### Glossary

**Interstrand crosslink (ICL)**: This term is used to describe a covalent bond between nucleotides on opposite DNA strands. These lesions are particularly toxic to the cell. The ability to induce ICLs underlies the value of several chemotherapeutic alkylating agents, such as melphalan and mitomycin C.
**Mismatch repair (MMR)**: The MMR pathway repairs base–base mismatches and insertion–deletion loops (stretches of unpaired DNA within a helix), which can result from errors during DNA replication. The MMR machinery recognizes the mismatch and excises the incorrectly paired nucleotide or loop. Defects in MMR underlie the majority of hereditary nonpolyposis colorectal cancers.
**Mono-adduct**: A chemical group covalently bound to a single nucleotide.
**Nucleotide excision repair (NER)**: The NER pathway is responsible for the repair of bulky mono-adducts and cyclobutane pyrimidine dimers. These lesions are the result of covalent binding between adjacent nucleotides and can occur after exposure to UV radiation. NER proteins recognize and excise the bound nucleotides. Patients suffering from xeroderma pigmentosum have defective NER and are thus hypersensitive to sunlight.
**Sister chromatid exchange (SCE)**: SCE is the exchange of DNA duplexes between paired chromatids during S phase. SCE can result from recombination after a double-strand break.

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**Box 1. A link between interstrand crosslinks and premature aging?**

Although interstrand crosslinks (ICLs) have been primarily addressed as a feature of cancer chemotherapy, these lesions can also arise during normal cell metabolism. It has been proposed that deficient repair of these spontaneous ICLs results in premature aging phenotypes [60]. Notably, the *XPF* mutations associated with xeroderma pigmentosum are relatively mild, resulting in defective nucleotide excision repair (NER) but not defective ICL repair [16,60]. A recent report described the characterization of a novel syndrome associated with an *XPF* mutation that causes defects in both pathways. The patient exhibited photosensitivity and progeroid symptoms, including increased apoptosis, liver defects and reduced growth factor signaling [3]. These symptoms are recapitulated in *ERCC1*$^{-/-}$ mice, but are not found in mice deficient for other NER pathway genes [3,61,62]. Together, these data suggest that deficient ICL repair is responsible for the progeroid symptoms.

Additional evidence to support this supposition is derived from molecular studies relating to the archetypical progeroid disease, Werner syndrome. This condition, characterized by both premature aging and cancer susceptibility, is associated with mutations in *WRN*. WRN, which has both helicase and exonuclease activity, is important for maintenance of genomic integrity, and several recent studies have implicated this protein in ICL repair [63–65]. More specifically, it has been shown that WRN's helicase activity is required for efficient ICL processing [63,66]. Very recent work using model substrates has shown that WRN can catalyze fork regression *in vitro* [67]. Such regression is a prerequisite for Mus81–Eme1 cleavage. Given this evidence, it is tempting to speculate that the failure to repair ICLs in patients with Werner syndrome underlies the progeroid symptoms of their condition, whereas inefficient repair of mutagenic lesions allows for tumorigenesis.

mono-adducts. XPF and ERCC1 make up a structure-specific nuclease that cleaves strands that transition from 5′ double-stranded to 3′ single-stranded DNA. During NER, this activity is required to create a nick 5′ to the lesion; another protein, XPG, creates a nick 3′ to the lesion [6].

The function of XPF-ERCC1 is not limited to NER. Evidence for the importance of XPF-ERCC1 to ICL repair has been accumulating for >20 years, beginning with work showing a 90-fold increase in sensitivity to MMC in XPF and ERCC1 mutant cell lines [7,8]. These findings suggested that the NER pathway is essential for cell survival in the presence of ICLs. However, it was subsequently determined that mutations in other NER genes failed to cause the same sensitivity. Mammalian cell lines with mutations in *ERCC1* or *XPF* are hypersensitive to crosslinking agents, but those mutant for *XPB*, *XPD* or *XPG* are not [9–12]. Likewise, fibroblast cells derived from XP-F patients show enhanced sensitivity to MMC and HN2, whereas those from XP-A patients do not, even though both are deficient in NER [3,13]. Moreover, ICL DNA is efficiently replicated *in vitro* in the absence of XPA [14]. These findings underline the difference between yeast and metazoan ICL repair, because all *S. cerevisiae* NER mutants are equally sensitive to crosslinking agents [15].

Traditionally, the function attributed to XPF-ERCC1 is the 'unhooking' step described above. Two lines of evidence suggest that XPF-ERCC1 mediates unhooking. The first is analogy: XPF-ERCC1 is required for single-strand nicking 5′ to a lesion during NER, which suggests that it might carry out the same function in ICL repair [5]. However, the lack of sensitivity of other NER deficiencies to ICL-inducing agents renders this analogy suspect. Notably, loss of XPA, which is required for recruitment of XPF-ERCC1 to an NER lesion, does not cause sensitivity to these agents. Another observation that calls the analogy into question is that cell lines from XP-F patients can show efficient processing of ICLs while failing to repair mono-adducts through NER [16]. This finding indicates that the function of XPF in ICL repair is separable from its function in NER.

The second line of evidence used to support a role for XPF-ERCC1 in unhooking derives from elegant *in vitro* studies, in which XPF-ERCC1 can mediate cleavage of an artificial substrate on both sides of a psoralen-induced crosslink, provided that the crosslink is adjacent to a single-stranded 3′ flap [17]. However, neither this structure nor this activity has been shown *in vivo*.

Based on these considerations, we argue that evidence for the involvement of XPF-ERCC1 in ICL unhooking might not be as strong as previously thought. An examination of the other steps in ICL repair might help shed light on an alternative role for XPF-ERCC1.

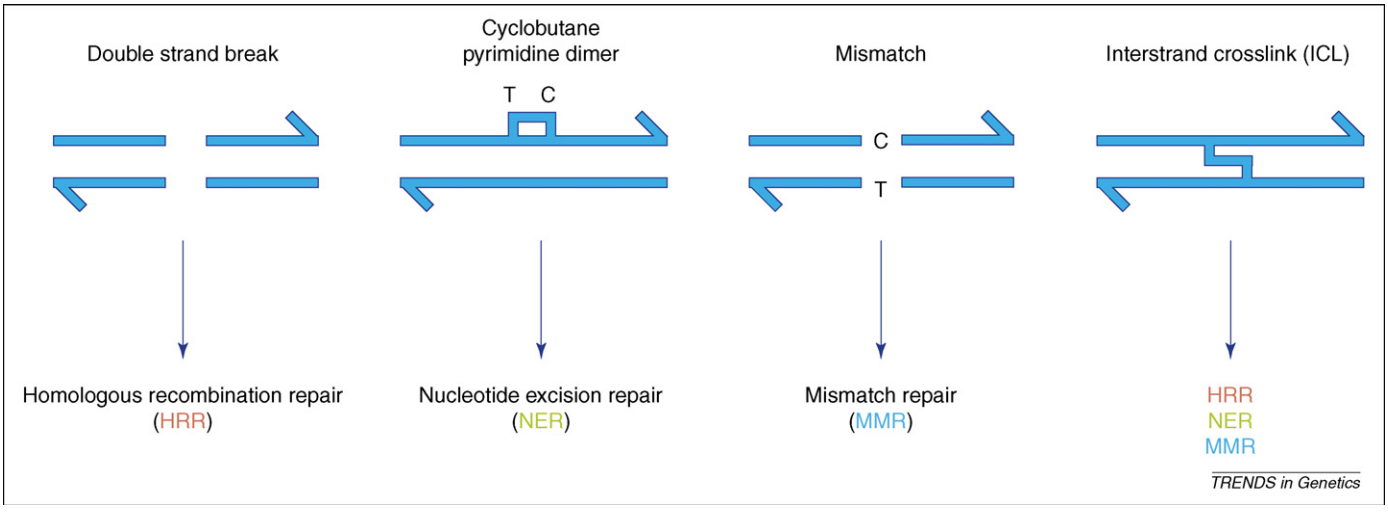


**Figure 1**. Interstrand crosslink repair uses the components of several repair pathways, including homologous recombination repair, nucleotide excision repair, and mismatch repair. Illustrated here are the canonical lesions recognized by each of these pathways.

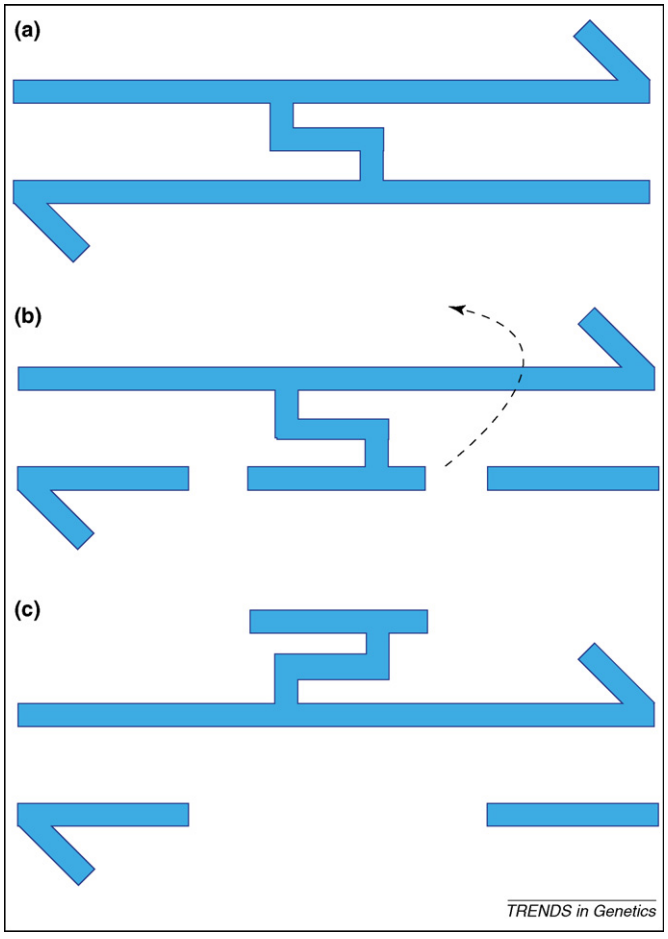


**Figure 2**. Interstrand crosslink (ICL) repair requires crosslink unhooking. (**a**) ICL strands. (**b**) Single-strand nicks are made 3′ and 5′ to the crosslink. 3′ ends are indicated by arrows. (**c**) These nicks allow for the crosslink to swing out from the helix, as indicated by the dashed arrow.

## ICL repair requires DSB repair through homologous recombination

Homologous recombination repair is an important feature of the cellular response to ICLs. ICL-induced HRR is thought to be initiated by a DSB that takes place at a stalled replication fork (Figure 3a,b) [15]. DSBs have long been recognized as a feature of ICL repair in *S. cerevisiae*, and more recent studies have also identified ICL-induced DSBs in mammalian cells [4,10–12]. Consistent with the hypothesis that these breaks occur at stalled replication forks, ICL-induced DSBs occur only in proliferating cells [4,10–12], and ICL repair in mammalian cells requires DNA replication [18].

The structure-specific nuclease mutagen sensitive 81–essential meiotic endonuclease 1 (Mus81–Eme1) has been implicated in the genesis of one-ended DNA DSBs at stalled replication forks [19–21]. Recent work has shown that Mus81–Eme1 is required for ICL-induced DSBs [22]. According to the model of Osman and Whitby [23], it is likely that the cut is made on the template strand for leading-strand synthesis after fork regression (Figure 3b). This cut would result in a broken double-strand with a 3′ tail (Figure 3c).

During HRR, 3′ tails are bound by replication protein A (RPA), which is later replaced by the recombination mediator RAD51 and related proteins. RPA is involved in the repair of DNA ICLs [24,25]. (Notably, it is also required for the positioning of XPF-ERCC1 during NER [26].) In addition, mutation of Rad51 or its paralogs – XRCC2, XRCC3, Rad51B, Rad51C and Rad51D – promotes sensitivity to crosslinking agents in vertebrate cells [27,28]. Another recombination factor, Rad54, is also involved in ICL repair. *Rad54*$^{-/-}$ cells and mice are hypersensitive to MMC [29,30]. Genetic ablation of the Rad54 paralog Rad54B also leads to MMC sensitivity [31]. Intriguingly, Rad54 interacts with Mus81 during ICL repair, although Rad54 is not required for the genesis of DSBs [22].

Most notable among recombination factors required for ICL repair are those with mutations related to Fanconi Anemia (FA) – for recent reviews, see Refs. [32–35]. Reviews by Thompson and colleagues [36,37] explain how a dual defect in translesion synthesis and HRR can account for FA mutants' generalized sensitivity to crosslinking damage, and how the FA mutations relate to a defect in HRR. Recent work has focused on the tumor suppressors BRCA1 (also known as FANCD2) and BRCA2, which are both implicated in FA [6]. Mouse embryonic fibroblasts (MEFs) deficient for BRCA1 are hypersensitive to MMC, and BRCA2 is required for replication-coupled repair of ICL DNA *in vitro* [14,38].

More direct evidence for recombination during ICL repair is derived from assays that measure sister chromatid exchange (SCE). It has long been recognized that SCE, which is a product of homologous recombination, is induced by crosslinking agents [39,40]. For example, SCE is rare during embryonic stem (ES) cell division, but treatment with MMC increases SCEs fourfold [22]. Furthermore, ICL-induced homologous recombination was recently shown to be deficient in *ERCC1* and *XPF* mutant cell lines using a plasmid-based assay [41]. This finding raises the possibility that XPF-ERCC1 functions during recombinational repair of ICL-induced DSBs rather than at the lesion itself.

## XPF-ERCC1 is implicated in homologous recombination

Evidence in *Drosophila* and mammalian cells supports a role for XPF-ERCC1 in homologous recombination. Like their mammalian orthologs, the *Drosophila* proteins MEI-9 (XPF) and ERCC1 participate in NER, and *mei-9* mutants show hypersensitivity to ICL-inducing agents [42]. Interestingly, *mei-9* was initially identified not because of its repair function, but in a screen for mutants exhibiting defects in meiotic recombination [43,44]. These defects manifest as a lack of crossovers: *mei-9* loss-of-function mutant females show a 95% decrease in meiotic crossovers, and *Ercc1* mutant females have a similar phenotype [43,45]. Clearly, MEI-9 and ERCC1 are involved in recombination outside of NER and ICL repair in *Drosophila*.

An additional factor has also been recognized in *Drosophila*. Mutation of *mus312* results in a meiotic crossover defect similar to that of *mei-9* and *Ercc1* mutants [46]. Moreover, *mus312* mutant flies exhibit enhanced sensitivity to the ICL-inducing agent HN2 [45,47]. The MUS312 protein was identified in a screen for MEI-9 interacting

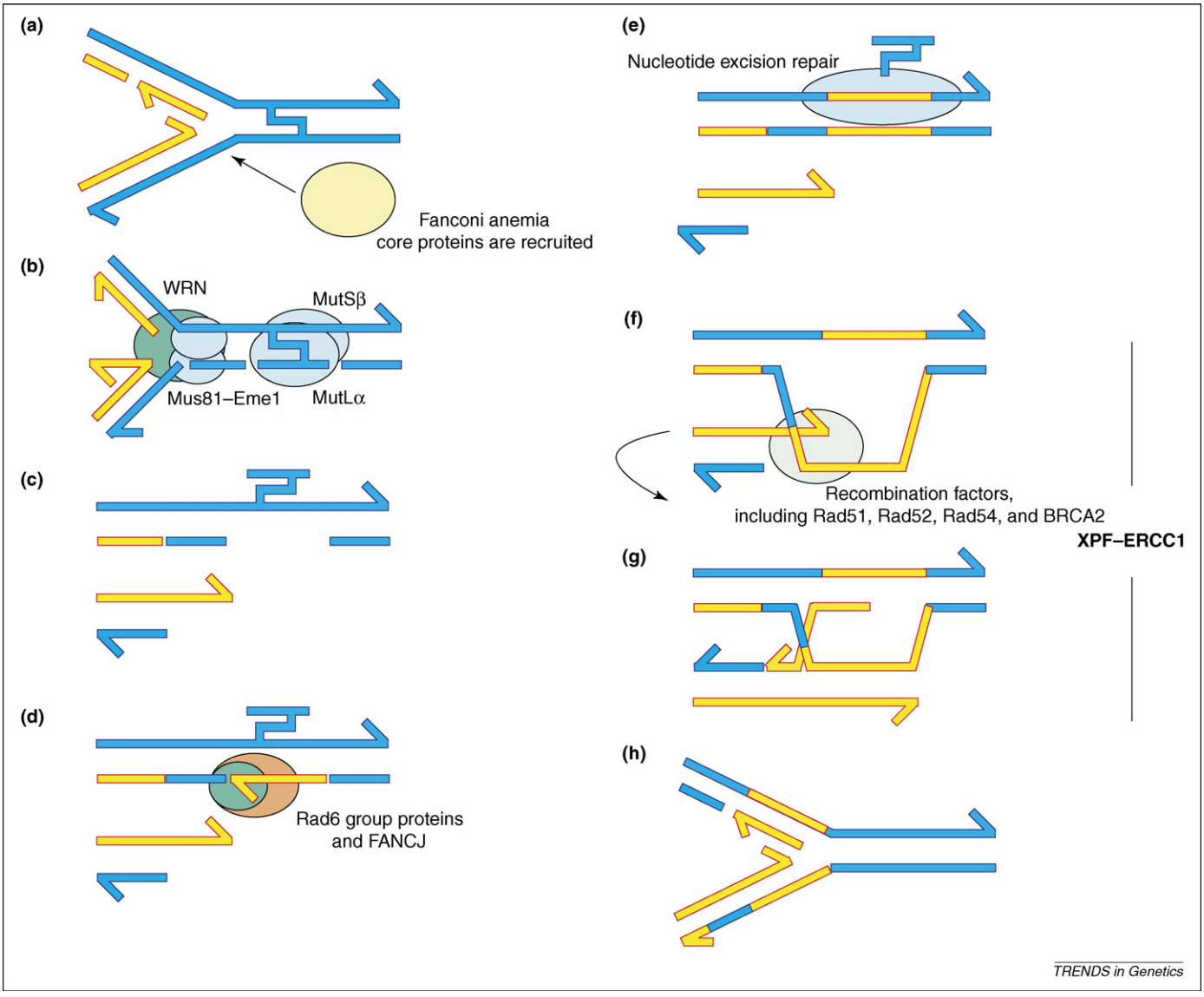


**Figure 3**. A model for interstrand crosslink (ICL) repair. Arrowheads indicate 3′ ends. Parental strands are shown in blue; newly synthesized DNA is shown in yellow. (**a**) The replication fork stalls at the crosslink. The Fanconi Anemia core proteins are recruited at this step [25,32,34,35]. (**b**) Werner syndrome protein mediates fork regression to produce a substrate for Mus81–Eme1. Mus81–Eme1 creates a one-ended double-strand break with a 3′ tail. The single-strand nick left behind can be one of the two required for crosslink unhooking. Alternatively, this nick is ligated to the 5′ end of the Okazaki fragment. The mismatch repair machinery nicks adjacent to the crosslink to mediate unhooking. (**c**) The unhooked crosslink swings away from the helix. (**d**) Recent work has shown the importance of translesion synthesis (TLS) factors belonging to the Rad6 epistasis group to ICL repair. TLS can act with the helicase FANCJ to complete the double strand at the site of the unhooked crosslink [4,35,41,59]. (**e**) Nucleotide excision repair removes the unhooked crosslink. (**f**) Recombination factors, such as Rad51, Rad52 and Rad54, promote invasion of the single-stranded 3′ tail. For the sake of clarity, the lower strands are flipped in the next panel (as indicated by the arrow). (**g**) The original replication fork is restored after strand invasion. A four-strand DNA junction is formed on fork restoration. Although the precise role of xeroderma pigmentosum complementation group F-excision repair cross-complementing rodent repair deficiency complementation group 1 (XPF-ERCC1) in recombination is unclear, we suggest that the XPF-ERCC1 nuclease mediates resolution of this junction. (**h**) Shown here is one of two potential outcomes of recombination junction resolution.

partners, and it seems that a physical interaction between MUS312 and MEI-9 is essential at least for the meiotic recombination function of these proteins [47]. Based on the genetic evidence, it has been hypothesized that MEI-9, ERCC1 and MUS312 participate in Holliday junction resolution [47].

XPF and ERCC1 have also been implicated in recombination in mammalian cells. XPF-ERCC1 physically interacts with the recombination factor Rad52 in human cell extracts, and the presence of Rad52 enhances XPF-ERCC1 nuclease activity *in vitro* [48]. Additional studies have shown a functional link between XPF-ERCC1 and recombination. Sargent *et al.* [49] measured recombination at an endogenous *APRT* locus that had been altered to form a direct tandem repeat. In cell lines lacking ERCC1, recombination at this locus commonly resulted in chromosome rearrangements, indicating unsuccessful processing of recombination intermediates. It has also been shown that XPF-ERCC1 is required for homology-driven gene replacement in ES cells [49,50]. Although the precise role of XPF-ERCC1 in recombination remains unclear, we suggest that it is this function that makes these two proteins essential for ICL repair.

## XPF-ERCC1 is required for ICL-induced DSB repair

Based on epistasis experiments, the fly proteins MEI-9 and ERCC1 are thought to function late in the meiotic recombination pathway, well after formation of the initiating

DSB [47,51]. We suggest that the same holds true for ICL repair. Crosslinking agents induce the same increase in DSBs in *XPF* and *ERCC1* mutant cells as in wild-type cells [10–12]. This finding indicates that XPF-ERCC1 functions downstream of the break. Moreover, ICL-induced DSBs have been shown to persist much longer in *Ercc1*$^{-/-}$ MEFs, *Ercc1* mutant cells and *XPF* mutant cells than in their wild-type counterparts, as measured by the presence of foci containing phosphorylated histone H2AX [11]. This finding shows that XPF-ERCC1 is required for repair of ICL-induced DSBs.

Additional evidence to support an ICL repair function for XPF-ERCC1 outside of unhooking can be derived from a modified comet tail assay, which is used to indicate the degree to which genomic DNA is compacted. The 'comet tail' reflects the mobility of DNA through a gel matrix. DNA that is less constrained is free to move through the gel, resulting in a longer tail. After exposure to ICL-inducing agents, *XPF* and *ERCC1* mutant cells exhibit shorter tails than wild-type controls [10]. This has been interpreted to mean that the ICL is not unhooked in the mutant lines, constraining the DNA to retard its migration. An alternative explanation for the shorter comet tail is that migration is impeded by the presence of aberrant DNA structures resulting from failed recombination. In fact, extensive chromosomal aberrations, presumably resulting from unsuccessful recombination, have been observed in MMC-treated *Ercc1*$^{-/-}$ ES cells and in *Ercc1* and *XPF* mutant CHO cells [11]. Included among these structures were large fusions and radial structures, which would certainly retard the electrophoretic mobility of DNA [11].

Interestingly, the most common type of aberration observed in this study was fusion between sister chromatids [11]. All of these fusions were within chromosomes rather than end-to-end and are thus likely to reflect sites of stalled recombination. Micronuclei and anaphase bridging, which are characteristic of chromatid nondisjunction, were also observed [11]. Given the accumulated evidence, we propose that XPF and ERCC1 do not act to unhook interstrand crosslinks but instead participate in the recombination steps of ICL repair (Figure 3f,g).

## If XPF-ERCC1 does not mediate unhooking, what does?

Although XPF-ERCC1 might not be required for unhooking, this step is nonetheless likely to be critical to ICL repair. The structure produced by formation of covalent bonds between nucleotides on opposite strands probably resembles a substrate for the MMR pathway more than it does the bulky mono-adducts repaired by NER; earlier work has shown that the MMR machinery can indeed bind these lesions [52,53]. Moreover, recent studies have shown that MMR proteins are required for ICL repair.

MMR is initiated in mammals by the binding of one of two MutS heterodimers, MutSα or MutSβ [54]. Whereas MutSα is required for the repair of base–base mismatches, and MutSβ for unpaired loops of several nucleotides, either heterodimer can initiate repair of single nucleotide loops [54]. Intriguingly, MutSβ is also required for ICL repair. Cell lines deficient in Msh2 (a component of both MutSα and MutSβ), but not those deficient in the MutSα-specific component Msh6, are unable to efficiently repair a crosslinked plasmid substrate [41]. In gel shift assays, MutSβ binds to a psoralen-induced crosslink, and based on *in vitro* experiments, MutSβ is necessary for incisions made proximal to a psoralen-induced ICL [55].

Another heterodimer, composed of homologs of MutL, is also required for MMR. Peng *et al.* [56] have recently shown that MutLα interacts with the FANCJ helicase, which is thought to participate in translesion synthesis at the unhooked crosslink [35]. Disruption of the MutLα–FANCJ interaction causes hypersensitivity to MMC and cell cycle arrest with 4C DNA content, which is characteristic of FA-associated ICL repair deficiency [56]. Intriguingly, MutLα was recently identified as an endonuclease capable of cleaving DNA on either side of a mismatch [57]. This function is dependent on the processivity factor proliferating cell nuclear antigen (PCNA), which is known to be required for ICL repair [24,57]. Given these findings, it seems possible that the MMR machinery, rather than XPF-ERCC1, is responsible for the single-strand nicking that results in crosslink unhooking (Figure 3b).

## A role for NER in ICL repair outside of unhooking

Although mutations in *XPF* or *ERCC1* cause extreme hypersensitivity to ICL-inducing agents, the increase in sensitivity caused by mutations in other NER genes is more modest [58]. However, sensitivity is not necessarily an accurate indicator of the relative importance of NER proteins to ICL repair. According to our model and others, ICL repair requires repair of a DSB and restoration of a replication fork. Failure to complete these steps is at least cytostatic, if not cytotoxic. Importantly, these steps do not themselves require removal of the unhooked crosslink. The cell is likely to continue to cycle in the presence of the unhooked crosslink, which, as a bulky mono-adduct, is a candidate substrate for the NER pathway. The persistence of such lesions could later prove mutagenic, cytotoxic or neither. Evidence to support a role for NER in ICL repair has recently been provided by Cipak *et al.* [14]. Although XPA is not required for *in vitro* DNA replication of an ICL substrate, a DNA lesion persisted after replication in the absence of XPA [14]. This finding shows that the NER pathway is not required for ICL-induced HRR, but also suggests that NER is responsible for complete repair, defined by removal of the unhooked nucleotide. We therefore contend that NER represents a step in ICL repair that, although important, is not an absolute requirement for tolerance to ICL-inducing agents (Figure 3e).

## Concluding remarks

Given its emerging clinical significance, interstrand crosslinking (ICL) repair is an important target for study. Forward genetic studies have proven invaluable for the identification of contributing factors, but do not always offer sufficient data to place these factors within a pathway. The endonuclease xeroderma pigmentosum complementation group F-excision repair cross-complementing rodent repair deficiency complementation group 1 (XPF-ERCC1), although obviously critical to ICL repair, might have been thus far mischaracterized. Rather than acting solely at the site of the lesion, we propose that it is critically involved in mediating ICL-induced recombination.

**Box 2. Questions for future research**

Several questions are raised by the model presented.

The recombination process that takes place during interstrand crosslink (ICL) repair is unlike canonical homologous recombination repair (HRR). Rather than repairing a two-ended double-strand break (DSB), it repairs a one-ended DSB associated with an ICL repair complex. If the repair of other lesions in metazoans follows a similar pathway, is xeroderma pigmentosum complementation group F-excision repair cross-complementing rodent repair deficiency complementation group 1 (XPF-ERCC1) similarly involved?

The *Drosophila* protein MUS312 is implicated in ICL repair. What is the function of this protein?

Recent evidence suggests that the mismatch repair (MMR) machinery might mediate crosslink unhooking. This possibility has not yet been thoroughly studied. Does MMR participate in unhooking?

If the MMR pathway is not responsible for unhooking, is XPF-ERCC1 (as previously thought)? Could it be involved at this step and at a later one?

We and others suggest that translesion synthesis can cooperate with nucleotide excision repair to repair the unhooked crosslink. This activity has not yet been shown (Figure 3d,e) [32,35]. Is translesion synthesis required?

Although we believe that the recombination function of XPF-ERCC1 has been overlooked in ICL repair models to date, we note that the accumulated evidence is not inconsistent with the previous interpretation, namely that XPF-ERCC1 is responsible for at least one of the nicks made during crosslink unhooking. Indeed, it could be that XPF-ERCC1 is required for three steps in ICL repair: unhooking, nucleotide excision repair of the unhooked crosslink, and recombination-mediated double-strand break repair. All three possibilities should provide fuel for future study. Although substantial progress has already been made, ICL repair will remain a fertile source of questions for some time (Box 2).

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