Repair of DNA Interstrand Cross-links Produced by Cancer Chemotherapeutic Drugs

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1 DNA Interstrand Cross-linking Drugs

It has been clear for over 50 years that bifunctional reactivity is an essential prerequisite for the potent cytotoxic and antitumour activity of agents such as the nitrogen mustards [1]. DNA was later identified as a target for these drugs [2, 3], and the covalent modification of DNA almost certainly accounts for the antitumour activity of these drugs [1]. The fact that a bifunctional covalent reaction with DNA (cross-linking) is essential for the toxicity of these agents is evident from studies employing monofunctional analogues; for drugs such as the nitrogen mustard's mechlorethamine and melphalan, their monofunctional counterparts are many orders of magnitude less toxic [4, 5]. Cross-links can be formed on the same strand of DNA (intrastrand), between the two complementary strands of DNA (interstrand), or between a base on DNA and a reactive group on a protein (DNA–protein). For the bifunctional alkylating drugs (e.g. the nitrogen mustard class and mitomycin C), it is clear that the interstrand cross-link (ICL), although accounting for only a small proportion of the total DNA adducts, is the critical cytotoxic lesion [6, 7]. For the platinum drugs (e.g. cisplatin and carboplatin) the majority (>80%) of DNA adducts are intrastrand cross-links, although the <5% of ICLs are critical cytotoxic lesions [8].

Drug-induced ICLs, which are generally irreversible, prevent the separation of the two strands of DNA which is essential for cellular processes such as replication and transcription. Since both DNA strands are involved, ICLs pose problems for the cellular DNA repair machinery and it is clear that there is a

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co-ordination of ICL-induced cellular responses, including cell cycle arrest, DNA damage repair and cell death [9]. Different human tumour types differ in their inherent sensitivity to DNA cross-linking agents, and this appears to be the result, at least in part, of their differing abilities to repair specific types of drug-induced DNA damage [10, 11]. Increased repair of ICLs is clearly also a critical mechanism of clinical acquired resistance to agents such as the nitrogen mustards, chlorambucil and melphalan. This has been shown in chronic lymphocytic leukaemia [12], and in multiple myeloma [11]. More recently, this has also been demonstrated for platinum drugs in ovarian cancer [13]. In addition, the capacity to repair ICLs appears to decline with age in normal cells, which may be a factor in the poor tolerance of chemotherapy in the elderly [14].

Although there are many ICL anticancer drugs approved for clinical use, relatively few of these agents have been widely employed in the most detailed mechanistic studies of ICL repair. In fact, one of the cross-linking agents most commonly employed in such studies is not used in cancer treatment. Of the anticancer agents, the original nitrogen mustard mechlorethamine [chemically, 2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine] is by far the best characterised. This agent cross-links preferentially between opposed guanines in the sequence 5′-GNC-3′/3′-CNG-5′ [15], and this cross-linking represents only a small fraction of the total DNA lesions that this drug produces (<5%), the remainder being monofunctional alkylations at guanine N7 and adenine N3 [1]. Cisplatin also cross-links between guanine N7 positions in the DNA major groove, but in this case in the sequence 5′-GC-3′/5′-CG-3′ [16]. Another anticancer agent commonly used in mechanistic studies is mitomycin C. This natural product molecule requires metabolic reduction in order to generate the reactive species, which produces cross-links in the DNA minor groove through reaction with the N2 position of guanines, cross-linking the opposed guanines in the sequence 5′-GC-3′/5′-CG-3′ (up to 13% of total adducts are ICLs) [17].

The non-anticancer compounds that have been studied in great detail are the psoralens [18], particularly 8-methoxypsoralen. Following 405 nm visible radiation, the formation of DNA monoadducts is favoured, whereas ultraviolet A (UVA) (365 nm) is required to convert these to abundant ICLs (up to 40% of the total adducts). The basis of the activity of psoralens is UVA-induced reactivity at 5′-AT-3′/5′-TA-3′ base pairs to form ICLs. Because of the more complex, multi-ringed structure of the psoralens, they form asymmetric cross links that bear a furan-ringed side and a pyrone-ringed side.

One property of the ICLs produced by drugs such as the nitrogen mustards and platinum drugs is that they significantly distort the structure of the DNA [16, 19]. Pyrrolobenzodiazepine dimer-based drugs such as SJG-136 (SG2000) have been rationally designed as highly efficient minor groove ICL agents [20, 21]. Interstrand cross-linking is primarily between the two guanine N2 positions in the sequence 5′-purine-GATC-pyrimidine-3′, and an important property is that the ICL produces minimal distortion of the normal DNA structure. Because of the high specificity and efficiency of ICL formation, substrates containing single SJG-136 ICLs are proving useful in detailed mechanistic studies of ICL repair [22, 23].

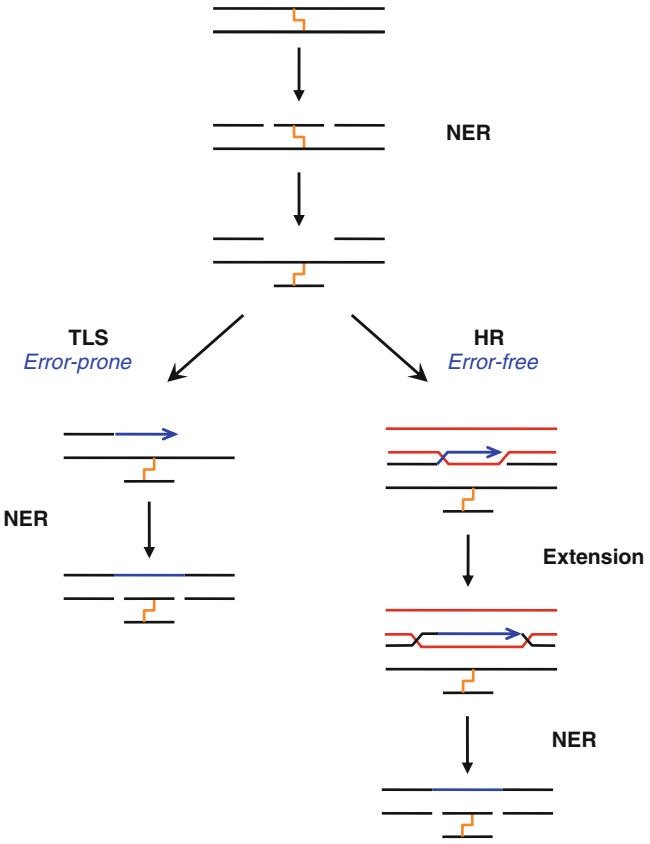

Fig. 1 A basic model for ICL repair based on understanding from bacteria and yeast. The NER pathway recognises the lesion and makes incisions around the lesion, unhooking the interstrand cross-link. This substrate can then be processed by two different pathways. The first is an error-prone process involving TLS, and the second is an error-free process dependent on HR. Both pathways result in DNA synthesis across the lesion, followed by a second round of NER to fully remove the cross-link

2 Basic Model of ICL Repair Based on Bacterial and Yeast Studies

Given the physical constraints ICL lesions impose on the DNA double helix, their repair requires the co-ordination of multiple repair pathways. A general model of ICL repair has been proposed based on studies in bacteria and yeast [24, 25]. Early genetic studies involving epistasis analysis of bacterial or yeast mutants sensitive to a range of DNA damaging agents, identified three major groups of genes involved in ICL repair, corresponding to the nucleotide excision repair (NER), homologous recombination (HR) and the translesion synthesis (TLS) DNA synthesis pathways [26–29]. Collectively, a model has emerged in which two parallel, but non-redundant, pathways are implicated in the repair of ICLs (Fig. 1). Both of these pathways require the pivotal, initial action of the

NER machinery, making incisions around the ICLs. This "unhooked" ICL-repair intermediate then undergoes further processing by two independent pathways involving either HR or TLS. Both pathways lead to DNA synthesis across the tethered ICLs, filling the gap created by the incisions. Subsequently, a second round of NER is required to fully remove the remaining ICL adduct. These ICL repair pathways have both been partially reconstituted *in vitro* using purified bacterial proteins [30–36].

It has become evident that many DNA repair pathways are well-conserved in higher eukaryotes. The available evidence suggests that the models outlined above for bacteria and yeast ICL repair are relevant to higher eukaryotes, although several key differences do exist. The following section attempts to summarise the current understanding of ICL repair in mammalian cells by using the model depicted in Fig. 1 as a framework. Evidence for the involvement of different repair pathways in the distinct steps of ICL repair will be provided. Unique features of mammalian cell ICL repair not found in lower eukaryotes will be highlighted.

3 Recognition of ICL Lesions in Mammalian Cells

In order for the repair process to begin, sites where DNA has been damaged must first be recognised. A number of mechanisms have been proposed for cross-link recognition in higher eukaryotes. This is likely due to the disparity in the assay systems and cross-linking agents used, although this could also reflect the fact that different systems are required for recognising the distinct chemical properties of different ICL types. Furthermore, the impact of the presence of ICLs on DNA structure and helical density would also affect its recognition as a lesion [37]. This section will summarise the literature on damage recognition of ICLs in vertebrate cells.

3.1 NER and ICL Lesion Recognition

The NER pathway appears to be essential for the repair of ICLs in both bacteria and yeast. Therefore, it has been postulated that NER also functions in vertebrate ICL repair. In the context of ICL repair, given that the two strands are covalently linked by the presence of the cross-link, no base pairs could be "flipped out"; therefore binding to the undamaged single-stranded DNA is impossible in principle. However, it is likely that distorting ICL lesions would result in a degree of unwinding that could provide an entry site for XPC binding adjacent to the ICL lesion [38]. Evidence of the involvement of XPC in the damage recognition of psoralen ICLs exists whereby both XPC-hHR23B and XPA-RPA can bind to triplex forming oligonucleotide with a psoralen cross link [39]. XPC proteins were found to be recruited rapidly to sites of a laser-induced damage "stripe" containing psoralen ICLs in G1 phase human cells [40]. However, a conflicting report on the involvement of XPC proteins in recognising cisplatin adducts exists, whereby XPC cells were not more

sensitive to cisplatin than the wild-type cells [41]. Furthermore, the mechanism of XPC recognition of mitomycin C ICLs remains unclear, given the modest distortion they induce without significant unwinding of the DNA. It is a possibility that the recognition of ICLs by XPC requires an interacting partner, such as the high mobility group protein B1 (HMGB1) that has recently been demonstrated to play a role in facilitating XPC in the recognition of psoralen-triplex ICLs [42].

3.2 Transcription-Coupled Mechanism of ICL Recognition

Transcription-coupled nucleotide excision repair (TC-NER) has been proposed to play an important role in the repair of ICLs, especially during G1 phase, as an ICL represents an absolute block to the RNA polymerases [43]. TC-NER has been described to be linked to ICL repair in the early 1990s when it was demonstrated that the repair of ICLs was more efficient in transcribed regions of an active gene [44–46]. Furthermore, host-cell reactivation experiments using expression plasmids containing ICLs placed in between the promoter and downstream reporter gene showed reduced ICL repair efficiency in cells defective in TC-NER [47–49]. However, it is important to note that the host-cell reactivation system is heavily transcriptionally biased as the readout of the assay is dependent on transcription. Therefore, although TC-NER is capable of repairing ICLs, the actual importance of TC-NER in contributing toward the repair of ICLs in cells remains to be determined although genetic studies also provide some evidence of TC-NER's involvement in ICL repair as both CSA and CSB defective cells were found to be sensitive to cisplatin [41].

3.3 Recognition of ICLs by Replication Forks

It has been proposed by a number of groups that the repair of ICLs in mammalian cells is replication-dependent during S-phase [9, 25, 50–53]. The replication-dependent repair of ICLs was first evident in observation that psoralen/UVA treatment only induced cell cycle arrest when synchronised human skin fibroblasts pass through S-phase, regardless of where in the cell cycle the cross-linking agent was initially administered [54]. This implies that the recognition of ICLs occurs exclusively in S-phase, and the replication fork arresting at the site of an ICL triggers the cellular repair response. It was also noticed that the repair of ICLs during S-phase results in generation of DNA double strand breaks (DSBs), which is not evident in stationary yeast or CHO cells [5, 55]. This leads to the proposal that replication-dependent repair of ICLs during S-phase involves the DSB repair pathway.

It has been proposed more recently that the replication-dependent repair of ICLs occurs when two forks converge on a single ICL (Fig. 2) [56, 57]. The convergence of two replication forks was observed by electron microscopy when ICL-containing plasmid substrates were replicated in the presence of *Xenopus* egg extract. The

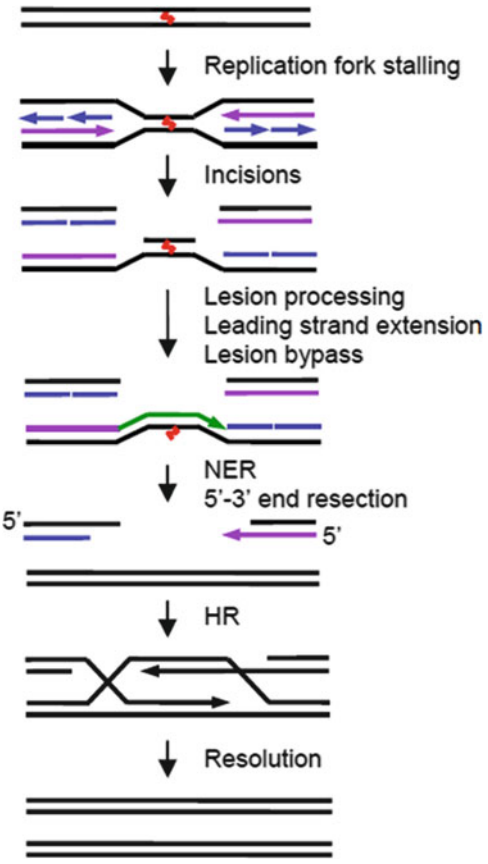

Fig. 2 Converging fork model of ICL repair. Based upon data from in vitro studies using *Xenopus* egg extracts with a plasmid-based system that favours converging replication forks [56, 57]. Replication fork stalling at the site of ICL is likely to provide a signal for monoubiquitination and activation of FANCD2-FANCI which orchestrates the repair of ICLs. The initiation of repair is thought to involve dual incisions around the ICL on one DNA strand. The "unhooked" ICL repair intermediate undergoes further processing, before the leading strand extends and TLS polymerase bypasses the remaining adduct that allow the restoration of a DNA template. Following a second round of incision, most likely involving NER that fully removes the ICL lesion, the HR machinery can utilise the DSB ends to re-establish the replication fork and complete DNA synthesis

repair of ICLs in such a context was found to be entirely replication-dependent. Using this system, it was observed that the converging replication forks initially stall 20–40 nucleotides from the lesion before one of the leading strands advances to within one nucleotide from the ICL. Subsequent dual incisions of the ICL result in the uncoupling of the two sister chromatids and lesion bypass DNA synthesis. The authors proposed that the double fork collision model of ICL repair is advantageous to cells as lesion bypass can readily occur from a nascent leading strand, preventing prolonged lag time between the incision and HR steps before the DNA synthesis is completed (Fig. 2). The absolute replication-dependence of ICL repair remains controversial as another similar *in vitro* study observed that replication-independent repair of ICL occurs [58]. Furthermore, the possibility of two forks arriving at the ICL is likely to be low in the *in vivo* setting, and is excluded in the situation when there are two ICLs formed in between neighbouring origins of firing. It has also been shown that ICL-induced checkpoint signalling would inhibit origin firing and slow fork elongation, limiting the possibility of two forks converging on an ICL [59]. Moreover, a two-sided DSB would be generated following incision of the two forks. This would be a potential substrate for non homologous end joining

(NHEJ) although it has been shown that NHEJ has a limited role in the repair of DSBs induced by ICLs [5]. ICLs have also been shown to induce sister chromatid exchanges (SCEs), which do not commonly occur in the context of repair of two-sided DSBs [53].

4 Unhooking of ICLs in Mammalian Cells

Following the recognition of ICLs as lesions, the repair machinery acts to make incisions on either side of the ICL to "unhook" the lesion. This represents a pivotal step, regardless of the mechanism of unhooking, as this relieves the torsional stress an ICL imposes on the DNA helix and permits processing of the repair intermediates by downstream pathways. A number of nucleases have been suggested to play a role in the unhooking step of ICLs.

Given the role of XPF-ERCC1 in making 5′ incision during NER, which is found to be essential in ICL repair in yeast and bacteria, this structure-specific endonuclease has long been implicated in the unhooking of ICLs. However, the extreme sensitivity of many *XPF* and *ERCC1* defective cell lines to cross-linking agents, compared to cells bearing mutations in other components of the NER apparatus, supports a role of XPF-ERCC1 in ICL repair processes other than NER [5, 60–62]. Purified XPF-ERCC1 proteins were able to make incisions on ICL placed on a duplex with splayed arm structure [63]. Incisions were observed on both the 5′ side and 3′ side of the ICL. The 3′ incision was stimulated when the cross-link was moved further away from the splayed arms. The inability of XPF-ERCC1 proteins to make incisions around the same psoralen ICL placed on a linear DNA duplex suggests that the splayed arm structure mimicking a stalled replication fork provides the substrate for XPF-ERCC1 recognition, further supporting the replication-dependent model of ICL repair. However, XPF-ERCC1 incision on DNA substrates containing a site-specific SJG-136 ICL has been found to be lesion specific [23].

Another structure-specific endonuclease related to XPF at the sequence level, Mus81-Eme1, has also been implicated in taking part in the unhooking step of ICLs. Mouse embryonic stem (ES) cells disrupted of MUS81 or EME1 were found to be mildly sensitive to mitomycin C [64, 65]. Based on the observation that *mus81*$^{-/-}$ MEFs showed suppression of DSBs [65], whereas *ercc1*$^{-/-}$ MEFs accumulate DSBs [62], several authors have proposed that ICL repair is initiation by MUS81-dependent incision, possibly on the leading strand template of the replication fork, followed by a second XPF-ERCC1 dependent incision 5′ to the ICL, where the net result is unhooking [62, 65, 66]. However, the observation that XPF-ERCC1 depleted human cells accumulated more MUS81-dependent DSBs argues against an initiating role for Mus81 in the unhooking [23]. Given that MUS81-dependent DSBs occur as a late response to ICLs, it is suggested that MUS81-dependent incision occurs when the XPF-ERCC1 incision fails or in the situation of a converging fork.

Another structure-specific endonuclease, the SLX1 and SLX4 heterodimer, is implicated in ICL repair as cells depleted of SLX4 has been shown to be hypersensitive

to cross-linking agents, and SLX4 has recently been identified to be a factor mutated in the complementation group P form of Fanconi anaemia (FA) (see below). However, given the very mild sensitivity of cells depleted of SLX1, the catalytic subunit of the heterodimer, it is unlikely that the Holliday junction resolvase SLX1–SLX4 acts to incise ICLs. The involvement of SLX4 in ICL repair may come from its interaction with XPF-ERCC1 and MUS81-EME1 [67–70]. It is postulated that SLX4 acts as a scaffold protein for recruiting these structure specific endonucleases to the sites of ICLs.

Most recently, a nuclease that is associated with the FA pathway, FAN1, has been identified as a factor that is required for resistance to mitomycin C [71–74]. It has not yet been demonstrated whether purified FAN1 is involved in the unhooking reaction. However, given that FAN1 depleted cells are also defective in HR repair as directly measured by the GFP reporter assay, it has been suggested that FAN1 is involved in the HR repair of DSBs induced by cross-linking agents [72, 73]. Consistently, FAN1 depleted cells also show increased chromosome aberration and radial chromosome formation.

5 Post-incision/excision Gap-Filling

Regardless of the mechanism which leads to unhooking of an ICL, the repair intermediate generated requires further processing in order to restore an undamaged template before the removal of the remaining ICL on the opposite strand. In the canonical NER reaction, the incision step is followed by the removal/excision of the released oligonucleotide to create a gap on the incised strand opposite the lesion before DNA synthesis could take place. In the context of ICLs, the incised oligonucleotide remains attached to the non-incised strand via the cross-link. Several studies have demonstrated that the downstream DNA repair synthesis opposite the remaining ICL lesion would be more efficient if the tethered oligonucleotide is displaced and/or removed [31, 75, 76]. This would involve the concerted action of a helicase and/or exonuclease.

The SNM1/PSO2 gene was initially identified in two independent screens in budding yeast [28, 77, 78]. Cells mutated for *PSO2* showed high sensitivity to cross-linking agents, but not to other forms of DNA damage, including that produced by monofunctional alkylating agent, IR or UV light. Three human orthologues of yeast Pso2 were identified: SNM1A, SNM1B (also known as Apollo) and SNM1C (Artemis) [79–81]. Structurally, SNM1A has been suggested to be the human homologue of Pso2 as there is greater degree of homology in the overall domain structure between Pso2 and mammalian SNM1A [82], and only complementing human SNM1A (hSNM1A) proteins in yeast *pso2* mutant cells would partially rescue the hypersensitivity to HN2 [83]. Purified hSNM1A protein demonstrates 5′ exonuclease activity on both double- and single-stranded DNA [83, 84], and it has been demonstrated recently that hSNM1A is capable of removing the tethered oligonucleotide from a 5′ incision initiated by XPF-ERCC1 pausing at the cross link [23].

Intriguingly, hSNM1A has been shown to interact with the mono-ubiquitinated form of PCNA [85], suggesting that the processing of hSNM1A might be associated with downstream TLS reactions.

TLS polymerases have been shown to play an essential role for gap-filling following unhooking and subsequent processing of ICLs [75]. The monoubiquitination of PCNA at lysine 164 by Rad6–Rad18 complex following replication fork stalling appears to be pivotal and allows the switch from replicative polymerases to TLS polymerase [86, 87]. The involvement of Rad18 in ICL repair in vertebrate cells is evident from the hypersensitivity of Rad18 and Rev3 cells to mitomycin C, mechlorethamine and cisplatin in chicken DT40 and mouse cells [88]. Recently, a number of TLS polymerases have been shown to be involved in the repair of ICLs, including Pol ζ [88–90], Rev1 [91–93], Pol η [48, 49, 94], Pol κ [76], Pol ι [75] and Pol ν [95–97]. However, the role and the regulation of the polymerases in performing lesion bypass have remained poorly defined. Mechanistic studies using *Xenopus* egg extracts showed that Pol ζ is not involved in inserting a nucleotide opposite a cisplatin ICL but is required for extension beyond the inserted nucleotide [57]. A detailed analysis of *in vitro* lesion bypass activities of translesion polymerases has shown that the Y-family polymerases, Pol κ, Pol ι and Pol η are capable of inserting a nucleotide opposite a cisplatin lesion, whereas Polζ alone or in complex with Rev1 is much less efficient [98]. Interestingly, Pol ζ complex failed to extend insertion products by Rev1 *in vitro*, whereas Pol η could perform translesion nucleotide insertion and a further extension of two nucleotides downstream [98]. The contrasting results of the poor *in vitro* efficiency of Pol ζ in lesion bypass in comparison to the more important role in TLS demonstrated in the *in vivo* context highlights the importance of the yet-to-be fully elucidated regulation of lesion bypass events during ICL repair.

5.1 HR in Resolving ICL-Induced DSBs

The presence of an ICL represents a blockage to replication fork progression that leads to replisome instability. If a single replication fork encounters an ICL, the collapse of the replication fork will result in one-ended DSBs, and the resolution of such DSBs and the re-establishment of replication forks require the break-induced repair the component of the HR pathway. In the situation when two replication forks converge on an ICL, dual incisions around the ICL result in the formation of two-ended DSBs, which are utilised by the HR machinery to complete replication and repair (Fig. 2). In higher eukaryotes, the involvement of HR in ICL repair has been demonstrated by the hypersensitivity of cells defective in HR to cross-linking agents. CHO cells defective in XRCC2 and XRCC3 showed hypersensitivities to mechlorethamine and mitomycin C, and are defective in the resolution of DSBs generated [5, 99]. Similar results were also seen in chicken DT40 cells in which knockout of five Rad51 paralogues were all sensitive to mitomycin C and cisplatin [100]. In contrast, Ku knockout chicken DT40 cells defective in NHEJ were resistant to cross-linking agents [101]. It was also observed that Rad54 and Rad54B

protect cells from mitomycin C in mouse ES cells [102]. Consistently, mouse cells defective in Rad51 interacting partners, BRCA1 and BRCA2 are extremely sensitive to a range of cross-linking agents [103–105]. The increase in sensitivity of these cells to cross-linking agents is associated with a suppression in ICL-induced Rad51 foci, indicating defective HR [103]. Consistently, *in vitro* repair of ICL-containing plasmids using *Xenopus* egg extracts has provided support for the involvement of Rad51-mediated HR in resolving ICL-induced DSBs, and in this system almost all ICL repair is HR-dependent [106]. The involvement of HR in ICL repair is further supported by the induction of SCEs by many cross-linking agents [107]. It has also been observed that the presence of a psoralen ICL on one homologous duplex stimulates short tract gene conversion events with associated crossovers [108].

A number of studies have implicated XPF-ERCC1 in HR during repair of ICLs. Yeast Rad1/Rad10 is involved in crossover generation through the processing of SSA recombination intermediates, removing terminal non-homologous sequences [109]. This function appears to be evolutionarily conserved in mammalian cells. In response to ionising radiation, XPF-ERCC1 is required for trimming 3′ ends extending beyond the annealed regions of microhomology during MMEJ [110]. In CHO cells, XPF-ERCC1 is needed for accurate resolution of recombination intermediates following strand invasion, involving 5′ incision at a single-stranded to double-stranded transition structure, which would otherwise block branch migration [111–113]. It was also found that in addition to Rad51 paralogues (RAD51D, XRCC2 and XRCC3), proteins from the FA network and REV3, XPF-ERCC1 were required for the homology-directed repair of psoralen ICLs involving DSBs in host-cell reactivation assays [114]. The role of XPF-ERCC1 has been suggested to be late during HR, as XPF-ERCC1 deficiency does not impair Rad51 foci formation but results in defective HR completion as measured in a reporter construct [115]. However, whether such a reporter system provides a direct measurement of HR specifically induced by ICLs remains questionable, given that XPF-ERCC1 deficient cells are already defective in HR within the constructs used in the absence of cross-linking agent treatment [115].

The role of HR in ICL repair is further supported by its interaction with the FA pathway. One FA factor, BRCA2, also known as FANCD1, is an HR factor. The following section will discuss the involvement of the FA pathway in ICL repair.

6 Fanconi Anaemia Pathway and ICL Repair

FA is an inherited recessive disease, caused by mutations in at least 15 different genes [116]. Mutations of FA genes are autosomal recessive with an exception of FANCB, which is X-linked [117]. In addition to developmental abnormalities and bone marrow failure, FA patients are also predisposed to cancer development, especially leukaemia and carcinomas [118]. Cells derived from these patients show genomic instability with increased radial formations. In addition, the hallmark of FA is cellular hypersensitivity to cross-linking agents, a trait that has been used as a

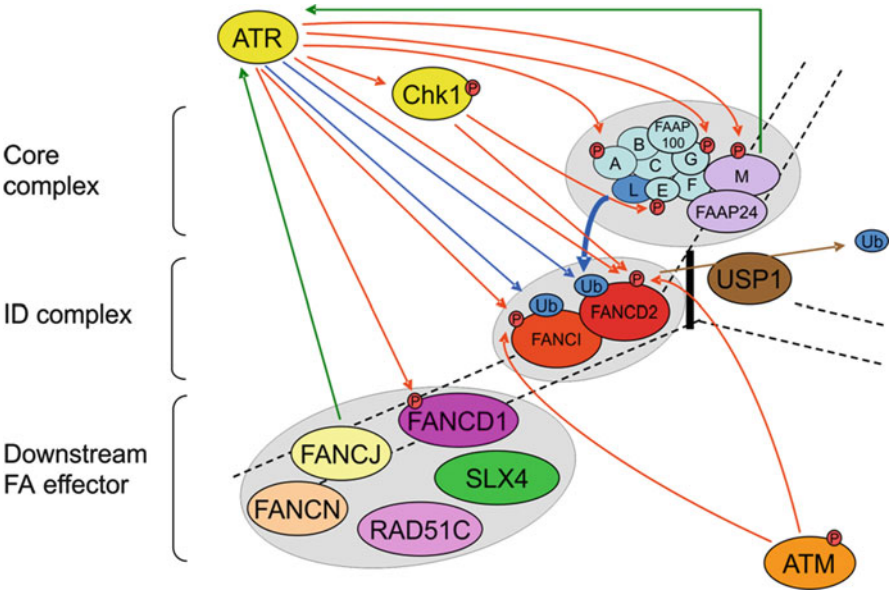

Fig. 3 A schematic representation of the FA pathway and its post-translational modifications. FA pathway becomes activated following detection of ICLs (*black solid line*) by FANCM-FAAP24 at sites of stalled replication fork (*dashed lines*). FANCM directly interacts with FANCF to recruit the FA core complex. The E3 ligase activity of FANCL subunit within the core complex monoubiquitinates FANCD2 and FANCI (ID complex), which becomes activated and associates to damaged chromatin. The activated ID complex recruits downstream FA effectors and possibly other factors (not shown) to facilitate repair of ICLs. The deubiquitination of the ID complex is essential for the completion of the FA pathway and ICL repair. The checkpoint kinases, ATM, ATR and Chk1 regulate the activity of the FA pathway by phosphorylating a number of FA factors (*red arrows*). ATR also regulates the monoubiquitination of the ID complex (*blue arrows*). The FA pathway, in turn, regulates the ATR pathway whereby FANCM has been found to be essential for activating the ATR pathway (*green arrow*). Adapted from [119], and [116]

diagnostic marker for FA patients [118]. The 15 gene products of FA have been found to be implicated in a common pathway involved in both DNA repair and checkpoint signalling, especially those involved in ICL repair (Fig. 3) [116, 119]. The monoubiquitination of FANCD2 and FANCI proteins at lysine 561 and lysine 523, respectively, appears to be the central event of the pathway [120, 121]. This occurs constitutively during S-phase and responds to the presence of DNA damage. Along with other accessory factors including FAAP24 and FAAP100, eight of the FA proteins (FANCA, B, C, F, G, L and M) form the FA core complex. FANCL is the catalytic E3 ligase that directly ubiquitinates FANCD2-FANCI [122]. There appears to be additional role of FANCM outside of the core complex, as FANCM deficiency only partially inactivates FANCD2 monoubiquination [123]. The remainder of the FA proteins are downstream effectors of the activated FANCD2-FANCI proteins. These include FANCD1/BRCA2, FANCN/PALB2, FANCJ/BRIP1 and RAD51C that have roles in facilitating homologous recombination (HR) [124], and

SLX4/FANCP, a recently identified scaffold protein that interacts with multiple nucleases [125–127]. It has been demonstrated that the FANCD2-FANCI deubiquitination is essential for the completion and recycling of FA pathway, and the USP1/UAF1 deubiquitinating enzyme complex plays this role [128, 129]. Inactivation of the deubiquitination of FANCD2 due to loss of USP1 leads to increased sensitivity to ICLs [130].

Although FA cells are typified by their extreme sensitivity to cross-linking agents, the exact role of FA factors in the repair of ICLs remains poorly understood. However, biochemical evidence exists showing a role of FA proteins in ICL repair. It has been shown that FANCA, FANCC and FANCG bind to psoralen cross-linked DNA in a complex with human α-Spectrin II *in vitro* [131]. By using chromatin-immunoprecipitation, Shen et al. further showed that FA proteins are recruited to sites of psoralen ICLs on episomal DNA that was ectopically introduced into cells, and there appears to be differential mechanisms of recruitment of the multiple FA proteins [132]. The cross-link association of FANCD1, FANCJ and FANCN requires replication, whereas the recruitment of FANCD2, FANCI and the core complex appears to be independent of replication [132]. Consistently, it has also been shown that FA recruitment to plasmids containing a site-specific ICL occurs regardless of DNA replication [58].

In vitro data using a site-specific ICL in plasmid DNA with *Xenopus* egg extracts have shown that the FANCD2-FANCI monoubiquitination is essential for the replication-dependent repair of ICLs [56, 57]. Inactivation of FANCD2-FANCI affected both the nucleolytic incision step and insertion of nucleotide opposite the tethered ICL during TLS [56]. It has been shown that monoubiquitination of FANCD2-FANCI is required for its nuclear foci formation at sites of ssDNA gaps and DSBs for orchestrating repair [120]. The newly identified nuclease, FAN1, has been shown to be recruited to sites of ICLs by FANCD2 [72–74, 133]. Interestingly, it has recently been shown that the monoubiquitination of FANCD2 following mitomycin C treatment occurs independently of XPF-ERCC1 [66]. However, chromatin association and deubiquitination of FANCD2 requires the presence of XPF-ERCC1, providing a link between the nucleolytic processing step of ICL repair and the FA pathway [66].

A clinical feature of FA patient cells is the increased radial structures generated between non-homologous chromosomes following treatment with cross-linking agents [134]. This is a result of reduced HR activity, accompanied by increased non-allelic homologous recombination, or use of f regions of microhomology as templates for replication fork restart, or increased NHEJ activities. It has been demonstrated recently that Ku deletion renders FANCC or FANCD2 mutant cells more resistant to cross-linking agents and reverses impaired homologous recombination in *Caenorhabditis elegans*, chicken DT-40 cells and human patient derived cell lines. The observation indicates that the FA pathway is involved in channelling the repair of ICL-induced DSBs away from inappropriate engagement with NHEJ and promoting HR during ICL repair [135, 136].

The link between the FA pathway and HR during ICL repair has been provided by the *in vitro* observation that BRCA2-defective cell extract could not fully remove psoralen ICLs present on plasmids, although unhooking of the ICLs was unaffected [137].

The mechanistic interaction between the FA pathway and HR core components appears to take place at several levels and remains poorly understood. The activated FANCD2 appears to be a critical regulator for HR activity. FANCD2 was found to recruit and co-localise with essential HR factors at sites of DNA damage, including BRCA1, BRCA2 and Rad51 [120, 138–140]. The interaction between FANCD2 and BRCA2 appears to require a role of FANCG independent of the core complex [141]. Furthermore, FANCD2 also co-localises with MRN complex following mitomycin C treatment [142]. Conversely, FANCD2 was found to be regulated by the MRN complex, providing a negative feedback loop in controlling FA activation during ICL repair [143].

The FA pathway also directly participates in checkpoint signalling in response to ICLs. It was demonstrated that the FA pathway plays an important role in activating the ATR/Chk1 checkpoint signalling as depleting FANCD2 inhibits phosphorylation of Chk1 and replication of an undamaged plasmid *in trans* [58]. This indicates that FA acts upstream of the ATR/Chk1 signalling. In addition, the FA pathway also appears to be regulated by checkpoint pathways. The monoubiquitination of FANCD2 on K561 in response to ICLs is mediated by ATR [144], and has been found to be dependent on the phosphorylation of FANCD2 on Ser 331 by Chk1 [145].

7 Clinical Relevance and Future Prospects

As our understanding of the complex molecular mechanisms involved in the repair of ICLs in human cells and the critical determinants of cellular sensitivity to damage of this type increases, so does the potential to develop sensitive screens to predict clinical response. Methods that allow the determination of formation and repair of ICLs in patient-derived material have clearly shown that repair of ICLs is an important determinant of inherent sensitivity or acquired resistance in the clinic. For example, using a modification of the single cell gel electrophoresis (comet) assay [146], the formation and repair of melphalan-induced DNA ICLs in plasma cells from melphalan naïve and melphalan-treated patients (i.e. those who had relapsed after a melphalan-conditioned autologous stem cell transplant or oral melphalan therapy) was examined [11]. Although similar levels of cross links were observed in cells from both melphalan naïve and treated patients, marked differences in repair (unhooking) of the cross-links were observed. Whereas cells from naïve patients showed no repair, those from treated patients exhibited efficient repair. A similar result has been found more recently for cisplatin-induced ICLs in ovarian cancer [13]. Cells from newly diagnosed patients were generally deficient at unhooking cisplatin ICLs, whereas cells taken following platinum-treatment (including from the same patients following therapy) showed enhanced ICL unhooking.

It has generally been assumed that the repair of different types of drug-induced ICL will be by a common mechanism or mechanisms. Indeed, resistance to melphalan due to enhanced removal of melphalan-induced ICLs in chronic lymphocytic leukaemia resulted in cross-resistance to other nitrogen mustard drugs [12]. Similarly, disruption of the FA-BRCA pathway in cisplatin sensitive ovarian tumours

resulted in sensitivity to mitomycin C [147]. However, this may not always be the case since the clinical acquired resistant myeloma cells described above, which are able to unhook the ICLs produced by melphalan, were not able to unhook the ICLs produced by cisplatin [11]. Conversely, the ovarian cancer cells from platinum-treated patients, which could unhook cisplatin-induced ICLs, could not unhook those produced by melphalan. Although the molecular basis for these differences remains unclear the results have important clinical implications and indicate that inherent sensitivity to DNA ICL agents can be the result of a specific DNA repair defect, and acquired resistance can be ICL agent class specific.

Key proteins involved in this specific process can also be considered as novel therapeutic targets, whose inhibition could increase sensitivity to cross-linking drugs in tumours normally inherently resistant or which have acquired resistance following initial therapy. Conversely, strategies to activate ICL repair in normal tissue, such as bone marrow, could protect from drug toxicity. Since the cellular response to DNA ICLs is co-ordinated to include cell cycle arrest, DNA damage repair and cell death [9], alternative strategies to sensitise tumours to ICL agents can target DNA damage response pathways such as the MAPK pathway [148] or the checkpoint kinase Chk1 [149].

Cisplatin and carboplatin have shown synergistic activity with the antimetabolite gemcitabine *in vitro* [150]. Recently, it has been demonstrated clinically that in patients with platinum-*resistant* ovarian cancer the combination of carboplatin and gemcitabine can be active, with the *in vivo* demonstration using the comet assay of a significant reduction in the repair (unhooking) of carboplatin-induced cross-links following the addition of gemcitabine [151]. Similar data have been observed with the clinical combination of gemcitabine and the cross-linking agent treosulfan [152]. Fludarabine has also been shown to inhibit the repair of cisplatin- and oxaliplatin-induced DNA cross-linking [153, 154]. The combination of fludarabine and the novel minor groove interstrand cross-linking agent SJG-136 has also been found to be synergistic in B-CLL cells, even in samples derived from patients with fludarabine resistance [155]. The combination resulted in higher levels of SJG-136-induced ICLs and it was demonstrated that fludarabine suppressed transcription of ERCC1 and inhibited SJG-136-induced ERCC1 transcription when given in combination, offering a mechanistic rationale for the synergistic interaction.

It is also becoming established that a significant proportion of tumours are defective in homologous recombination repair (e.g. BRCA1- and BRCA2-deficient) which confers sensitivity to cross-linking agents such as cisplatin and inhibitors of poly(ADP-ribose)polymerase (PARP), an enzyme required for repair of endogenous DNA damage [156]. The combination of PARP inhibitor and platinum drug can be more effective than either drug alone [157].

Another indirect mechanism to inhibit repair of some ICLs is through targeting extracellular signalling pathways. For example, inhibition of the EGFR (Her1) pathway by gefitinib resulted in synergy with cisplatin in human breast cancer MCF7 cells and was shown to inhibit the unhooking of the ICLs produced by cisplatin [158]. Similarly, incubation of breast cancer cell lines with the anti-HER2 antibody trastuzumab (Herceptin) delayed the repair of ICLs produced by cisplatin, with no effect on the repair of intrastrand cross-links [159]. In each of these examples the

effect is dependent on the cross-linking agent used but clearly shows that rational combinations of DNA ICL agents and targeted therapies can be found.

The modified comet assay, in addition to having an important role in mechanistic studies of ICL formation and repair *in vitro*, *in vivo* and in clinical material, is becoming established clinically as a pharmacodynamic endpoint for ICL drugs in early phase clinical trials of novel agents (e.g. SJG-136) [160], or novel targeting strategies involving ICL. For example, in a clinical trial of the prodrug CB1954 and the synthetic nicotinamide cofactor analogue, EP0152R, ICLs resulting from NQO2-reduced CB1954 were detected in biopsies demonstrating successful prodrug activation [161]. The DNA damage produced by DNA ICL agents, including mechlorethamine and cisplatin, can also result in phosphorylation of the histone protein H2AX resulting in γ-H2AX foci [162, 163]. The peak γ-H2AX response was detected 2–3 h after the peak of DNA ICL formation, and detection of γ-H2AX foci by immunofluorescence confocal microscopy was found to be up to ten times more sensitive than detection of cross links using the comet assay [163]. In addition, mechlorethamine or cisplatin-induced γ-H2AX foci persisted longer in cells that were defective in either ICL unhooking or homologous recombination. Together these data raised the possibility that the measurement of γ-H2AX foci could be used as a sensitive pharmacodynamic marker of DNA damage by ICL agents in the clinic and recently this assay has been utilised as a pharmacodynamic endpoint in Phase I studies of the novel agent SJG-136 [164].

Novel cross-linking agents continue to be developed in an attempt to produce more selective, less toxic drugs. Agents that produce cross-links in the minor groove of DNA are of particular interest. For example, the sequence selective ICLs produced by the pyrrolobenzodiazepine dimer SJG-136 in the minor groove of DNA produce minimal distortion of the normal DNA structure and, as a result, it appears to evade some of the recognition and repair mechanisms used for the processing of the distorting cross-links produced in the major groove by conventional drugs. SJG-136-induced ICLs persist in tumour cells able to efficiently unhook ICLs produced by drugs such as cisplatin and melphalan. This highly potent agent has significant antitumour activity in animal models [21, 165] and is currently in clinical development.

It is possible to generate pyrrolobenzodiazepine dimer-based ICL agents with increased potency compared to SJG-136, down to sub-picomolar GI_{50} values *in vitro* [20, 166, 167]. It remains to be established whether agents of this type will find a role in the clinic since their use as unmodified single agents may be limited *in vivo* by issues of delivery and toxicity. The fact that these molecules have multiple potential sites of attachment and that they can be inactivated with appropriate modification suggests that PBDs may also have a potential role in other strategies aimed at targeting and releasing highly cytotoxic agents directly at a tumour site, where production of potent and difficult to repair ICLs is desirable. Examples of such approaches are antibody-directed or gene-directed prodrug therapies (ADEPT and GDEPT) and hypoxia-activated prodrugs which are currently using prodrugs based on more conventional cross-linking agents [168, 169]. One important application is as the "warhead" component of antibody drug conjugates, an area that is fast emerging as one of the principal approaches in the field of monoclonal antibody cancer therapeutics [170].

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