Published in final edited form as:

Nat Rev Cancer. ; 11(7): 467–480. doi:10.1038/nrc3088.

DNA interstrand crosslink repair and cancer

Andrew J. Deans^{*,‡} and Stephen C. West^{*}

^{*}London Research Institute, Cancer Research UK, Clare Hall Laboratories, South Mimms EN6 3LD, UK

Abstract

Interstrand crosslinks (ICLs) are highly toxic DNA lesions that prevent transcription and replication by inhibiting DNA strand separation. Agents that induce ICLs were one of the earliest, and are still the most widely used, forms of chemotherapeutic drug. Only recently, however, have we begun to understand how cells repair these lesions. Important insights have come from studies of individuals with Fanconi anaemia (FA), a rare genetic disorder that leads to ICL sensitivity. Understanding how the FA pathway links nucleases, helicases and other DNA-processing enzymes should lead to more targeted uses of ICL-inducing agents in cancer treatment and could provide novel insights into drug resistance.

The use of DNA interstrand crosslinking agents as a tool for chemotherapy was born out of the horror of the use of chemical weapons in the Second World War. In December 1943, the US Merchant Ship S.S. *John Harvey*, which was anchored in the Italian harbour of Bari, was bombed in a German air raid. On board was a secret and deadly cargo of around 60 tonnes of sulphur mustard bombs, which detonated and contaminated the nearby city and surrounding area. After the ensuing chaos had passed, physicians carrying out autopsies noticed that the chemical had specifically attacked the victims' white blood cells. With great insight, they hypothesized that these chemicals could have a more righteous action: for the treatment of leukaemia. In 1946, after the barbarity of the war had past, the first publication of 'Nitrogen Mustard Therapy' appeared¹.

Sixty-eight years and a few chemical modifications later nitrogen mustards such as cyclophosphamide and melfalan are still front-line chemotherapeutic agents in the treatment of leukaemia, as well as solid tumours. Nitrogen mustards were joined in the clinical treatment of cancer by mitomycin C (MMC) in 1956, platinum compounds such as cisplatin in 1971 and psoralens in 1989. Although these drugs all showed remarkable anti-cancer properties, it was not known that they function by inducing interstrand crosslinks (ICLs) until well after their introduction as chemotherapy. Only within the past few years has it become clear how cells can recognize, repair and ultimately develop resistance to ICL-inducing agents, and how this allows tumour regrowth.

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Correspondence to S.C.W. stephen.west@cancer.org.uk.

[‡]Present address: St. Vincent's Institute of Medical Research, Fitzroy, Victoria 3065, Australia

Competing interests statement

The authors declare no competing financial interests.

DATABASES

National Cancer Institute Drug Dictionary: <http://www.cancer.gov/drugdictionary> cisplatin | cyclophosphamide | filgrastim | melfalan | MMC | sargramostim | trastuzumab

FURTHER INFORMATION

Stephen C. West's homepage: <http://science.cancerresearchuk.org/sci/genrecombi/>

Fanconi Anaemia Mutation Database: <http://www.rockefeller.edu/fanconi/>

The separation of the two strands of a DNA double helix is essential for cellular processes such as replication and transcription. ICLs are extremely toxic to cells because they prevent this separation, by coordinated chemical reactions with bases on opposing strands. The resulting covalent linkage is usually irreversible. Covalent crosslinks between bases in the same DNA strand, which are known as intrastrand crosslinks, can also arise. Such damage can be bypassed by some DNA polymerases, making this type of lesion less toxic during replication than crosslinks between the two DNA strands.

The mechanism of action of all interstrand crosslinking drugs is broadly similar (BOX 1). Each ICL-inducing agent exhibits different base specificity and, as discussed below, the resulting structural changes in the DNA can result in rather different cellular responses. The structure of some of these drugs crosslinked to DNA bases, and their clinical usage, is highlighted in TABLE 1. In this Review we detail how complex networks of DNA repair processes are coordinated to repair ICLs. We also describe how defects in these repair processes can cause cancer but can also sensitize established cancers to ICL-based chemotherapy.

ICLs and cancer initiation

Although the clinical use of DNA crosslinking drugs in killing cancer cells is well established (FIG. 1), it is less clear whether DNA crosslinking presents a cause of cancer. One reason for this is that ICL-inducing drugs generally cause a wide variety of DNA lesions in addition to crosslinks. For example, cisplatin, the most widely used crosslinking drug, causes damage that is estimated to comprise of 90% intrastrand crosslinks (predominantly crosslinks between adjacent purine residues on the same strand of the DNA double helix) and less than 5% ICLs². MMC and alkylating agents, such as the nitrogen mustards, also typically only cause 5–10% ICLs³. Furthermore, exposure to such drugs is generally in a clinical setting — there is very little evidence to suggest exposure to high levels of natural sources of ICL-inducing agents.

The most well-characterized potential sources of ICLs in nature are the by-products of lipid peroxidation, including acrolein and crotonaldehyde R, β -unsaturated aldehydes^{4,5}, the concentrations of which could increase with a high-fat diet or with alcoholism^{6,7}. Other sources could include abasic sites, natural psoralens (such as those found in bergamot, celery and parsley) and even oestrogens^{8–12}. Pre-cancer phenotypes such as hyperplasia and cell atypia are found to be more severe in mice that are treated with photoactivatable bifunctional psoralens (which are able to form crosslinks) than when closely related, but monofunctional, agents are used¹³; this is despite the fact that bifunctional agents cause a similar number of point mutations to monofunctional agents when assayed *in vitro*¹⁴. One interpretation is that, although ICLs inhibit strand separation, their repair proceeds via a pathway that causes point mutations but also other tumour-promoting lesions, such as deletions and translocations.

Sadly, the Italian inhabitants of Bari harbour went on to have a much higher incidence of cancers, specifically leukaemias, in the aftermath of the S.S. *John Harvey* bombing¹⁵. An increased incidence of acute myeloid leukaemia (AML) is also seen in patients who undergo therapeutic treatment with ICL-inducing drugs^{16,17}. The blood seems to be specifically sensitive to both the carcinogenic and the chemotherapeutic effects of ICLs, and we have come some way in understanding the molecular details of these events by analysis of the genetic disorder known as Fanconi anaemia (FA).

Cancer that is associated with FA

FA is a rare inherited syndrome that is characterized by sub-fertility, congenital abnormalities, progressive bone marrow failure and a highly elevated risk of haematological and squamous cell cancers¹⁸. The disease is genetically heterogeneous, and 14 complementation groups have so far been identified after exhaustive genetic and functional complementation studies^{19,20}. All of the products of the FA genes are thought to act in a common cellular pathway, and at least seven (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG and FANCL) form a complex that is known as the FA core complex. Diagnosis of FA involves the cytogenetic analysis of lymphocytes that have been treated with ICL-inducing agents — FA-positive cells show highly increased levels of chromosome aberrations, including chromosome breakage and radial chromosome formation²¹. FA cells are also hypersensitive to ICL-inducing agents, and the management of cancer in patients with FA must strictly exclude treatment with these drugs.

Although the majority of reported cases of FA are due to mutations in *FANCA* (65%), *FANCC* (15%) or *FANCG* (10%) (see the [Fanconi Anaemia Mutation Database](#); see Further information), the range of both clinical outcome and cancer susceptibility in each of the groups is broadly similar (except for the *BRCA2* (also known as *FANCD1*) and partner and localizer of *BRCA2* (*PALB2*; also known as *FANCN*) subgroups, as detailed below). Bone marrow failure is the most common symptom, and this can be readily treated with human leukocyte antigen (HLA)-matched transplant. In this regard, the autologous transplant of gene-corrected stem cells may be possible in the near future²². Unfortunately, many patients with FA develop AML before a stem cell transplant can take place, with a >700-fold risk of developing AML compared with the general population²³. In one study, the actuarial risk of developing haematopoietic abnormalities was 98%, and the risk specifically for AML was 52% by the age of 40 years in patients with FA²⁴. Solid tumours are also present at early onset and represent a greater risk in patients with FA, with the most common being head and neck squamous cell carcinoma (HNSCC; 700-fold elevated risk), as well as oesophageal (2,300-fold increased risk) and gynaecological (>180-fold increased risk) cancers²³.

There are, however, some differences in the cancer susceptibilities that are associated with the various complementation groups. For example, patients with *PALB2* or *BRCA2* mutations seem to be prone to cancer earlier in life, and there is a high proportion of cases of medullo-blastoma in patients with *FANCD1* (REFS 25,26). Cancer mortality was close to 100% by 5 years of age for children with biallelic *BRCA2* mutations and in seven families with *PALB2* mutations^{27,28}. Other groups, such as those with mutations in *FANCM*, *FANCL* and *RAD51C* (also known as *FANCO*) have no cancer probands but this is probably due to the very low incidence of individuals with a mutation in these genes²⁹⁻³¹. Within the most prevalent complementation group there have been descriptions of highly cancer-prone families who have been associated with particular *FANCA* mutations, but a larger population-based study has shown that the most prevalent ‘hot spots’ within *FANCA* are actually founder mutations^{25,32}. Unfortunately, although the majority of the leukaemia predisposition is removed when patients receive donor transplants, patients with FA often go on to develop extremely aggressive HNSCCs with 40% cumulative incidence by the age of 40 years. Two-year survival rates in such individuals are less than 50%³³.

The origin of cancers in patients with FA has been a topic of debate, and given that some form of bone marrow failure precedes many sporadic leukaemias³⁴, it is one that may apply to some sporadic cancers. Although *Fanca*^{-/-}, *Fancc*^{-/-}, *Fancg*^{-/-}, *Fancm*^{-/-} and *Fancd2*^{-/-} mice all show mildly elevated tumour incidence³⁵, no research undertaking has determined whether this incidence increases with low-dose exposure to agents such as MMC. Furthermore, laboratory animals that are raised in a highly controlled setting may not be

exposed to environmental sources of ICLs. The severe genetic instability of FA cells (both mouse and human) exposed to interstrand crosslinking agents nonetheless suggests several potential routes to tumour development.

First, the genetic instability of FA cells would make them more prone to the translocation of oncogenes or the deletion of tumour suppressor genes. Interestingly, the incidence of point mutations seems to be somewhat decreased in FA cells, but genomic rearrangements, including a selection of specific and oncogenic translocations such as loss of 1q and 3q and monosomy on chromosome 7, are favoured³⁶⁻³⁸. It has been suggested that a single unrepaired ICL that arises spontaneously in DNA could be enough to induce an oncogenic translocation in an FA cell^{39,40}.

Second, the induction of apoptosis by unrepaired DNA damage leads to increased cell death in the haematopoietic system of patients with FA and in knockout mouse models^{41,42}. This leads to the depletion of the haematopoietic stem cell pool, which is analogous to the depletion that is seen in other DNA repair defective disorders⁴³. The repeated proliferation of the remaining haematopoietic stem cells in order to maintain tissue homeostasis leads to the rapid selection of tumorigenic clones. At least in mice, such clones have increased resistance to apoptosis-inducing cytokines, and these tumorigenic clones can accumulate excessive translocations⁴⁴. In this manner, the increased genomic instability and selective pressure for resistance to apoptosis may combine to drive tumorigenesis in patients with FA.

A third potential mechanism of tumour susceptibility in patients with FA has recently been suggested that may account for the unusually high incidence of HNSCCs⁴⁵. The accumulation of unrepaired ICLs induces a transient arrest at the G2 phase of the cell cycle, presumably until the ICLs have been removed⁴⁶. In FA cells, this G2 arrest is considerably extended and may make them more susceptible to Epstein–Barr virus (EBV) and human papilloma virus (HPV) infection — a potent source of oncogenic activation. These viruses preferentially integrate into regions of the genome that have stalled replication⁴⁷. In a sample of 25 American patients with FA-associated head and neck cancers, 85% of tumours were found to be positive for HPV DNA⁴⁸ (although a similar European follow-up study found only 10%⁴⁹). If other studies provide support for the link between the failure to repair ICLs and HPV-associated HNSCC, it may also explain why young cancer patients who receive ICL-inducing chemotherapy have an increased incidence of head and neck cancers in later life⁴².

The failure to repair ICLs and the consequent tumorigenesis in individuals with FA indicate that the FANC genes are essential for an ICL repair pathway, especially as inherited or targeted defects in many DNA repair genes can lead to ICL sensitivity and, in many cases, cancer-prone disorders that are similar to FA (TABLE 2). FA has thus become a model disease for the understanding of ICL repair, and the FA pathway is now thought to involve the coordination of several repair systems, including homologous recombination (HR), nucleotide excision repair (NER) and translesion synthesis (TLS). The essence of the ICL repair pathway is highlighted in FIG. 2, and the various elements are discussed below.

Activation of coordinated ICL repair

Seven of the FA gene products are known to form the FA core complex, which contains ubiquitin ligase activity owing to the FANCL component. Other proteins in the complex are orphans with no known functional domains but with many coiled-coil regions (reviewed in REF. 20). In some respects, the FA core complex is similar to the SKP1–CUL1–F-box (SCF) ubiquitin ligase complex, which is involved in cell cycle regulation. The FA core complex interacts with FANCM, which is a protein that belongs to the ERCC1/XPF family of structure-specific DNA-binding proteins⁵⁰. ICLs that are induced during S phase activate

the FA core complex to monoubiquitylate two further DNA-associated factors, FANCD2 and FANCI^{51,52}, leading to their retention on chromatin⁵³. In the absence of any one component of the FA core complex, ubiquitylation fails to take place — highlighting its importance as a central element of FA pathway activation.

ICL damage recognition requires FANCM when the damage is encountered by a replication fork⁵⁴ (FIG. 2). As such, this mechanism of ICL repair is inefficient or non-existent in cells in G1 and in non-cycling cells⁵⁵. The replication of DNA requires DNA unwinding ahead of the DNA polymerase, which is achieved by the helicase functions of the minichromosome maintenance complex (MCM2–7)⁵⁶. However, replication stalls when the replisome encounters an ICL because the DNA strands cannot be separated owing to the covalent linkage between the two DNA strands⁵⁷. The resulting structure is then recognized by FANCM, which contains both a degenerate inactive carboxy-terminal ERCC4 nuclease domain that binds to branched DNA structures *in vitro*⁵⁸ and an internal domain that is required for interaction with the FA core complex⁵⁹. FANCM obtains stability and specificity by interactions with another ERCC4 domain-containing protein, FAAP24, to form a FANCM–FAAP24 heterodimer⁵⁸. Recruitment of the FA core complex to chromatin by FANCM–FAAP24 is thus limited to phases of the cell cycle in which replication is active, and recruitment is also limited by the regulated degradation of FANCM during mitosis^{55,59}. Possibly because of the interaction of FANCM with the histone-like proteins MHF1 (also known as CENPS) and MHF2 (also known as CENPX), FANCM itself is constitutively associated with chromatin^{60,61}. As the other FA proteins only enter the chromatin on replication stalling, it is likely that the interaction of FANCM and the FA core complex is further regulated. In support of this, FANCM or FAAP24 depletion leads to the loss of FANCD2–FANCI monoubiquitylation, despite normal FA core complex assembly^{58,59}.

Unlike the polyubiquitylation signals that are important for protein degradation, the monoubiquitylation of proteins alters their activity, localization or ability to form protein–protein interactions^{62,63}. It is unknown whether monoubiquitylation of FANCD2–FANCI precedes chromatin binding, or merely stabilizes their interaction with damaged sites, but the vast majority of chromatin-associated FANCD2 is in the monoubiquitylated form⁶⁴. Recombinant FANCD2 or FANCI have both been shown to bind independently to branched DNA structures^{65,66}, suggesting that they might be capable of associating with sites of damage, such as those recognized by FANCM^{65,66}. One possibility is that FANCD2 and FANCI can transiently interact with damaged DNA, but that monoubiquitylation by the proximal FA core complex stabilizes their localization. Once the damage is repaired, the subsequent dissociation of the FA core complex would allow access to the deubiquitylating enzyme ubiquitin-specific peptidase 1 (USP1). Deactivation of the pathway following DNA damage removal seems to be equally important because the deletion of USP1 also causes an FA-like phenotype in mice⁶⁷ (although *USP1* does not seem to be associated with human disease).

Recently, two proteins were shown to specifically interact with the modified form of FANCD2: Fanconi-associated nuclease 1 (FAN1) and DNA polymerase ν (Pol ν ; also known as POLN)^{68–71}. Both proteins colocalize with FANCD2 in nuclear foci when cells are treated with agents such as MMC, but neither is required for monoubiquitylation. FAN1 has a UBZ4-type ubiquitin-binding zinc finger domain, which is required for interaction with modified FANCD2, as well as a VRR-nuclease domain. FAN1 degrades single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) exposed ends (5′–3′ exonuclease activity) and can cut DNA on the 5′ side of a ssDNA–dsDNA junction (known as 5′-flap endonuclease activity) and would therefore have an activity that is suited to cutting DNA that is adjacent to a stalled replication fork. Such cleavage is termed

‘unhooking’, and may convert the stalled fork into a double-strand break (DSB). This has been the most widely supported model for the initial step in ICL repair, and it can be adapted to include two replication forks encountering the ICL⁵⁷. The stalling of a replication fork by MMC can trigger the initiation of a new replication fork from a nearby origin⁷², which would approach the ICL from the other side. The frequency of double replication fork encounters with ICLs has never been experimentally determined, but could be presumed to increase as cells complete replication in late S phase. In the comprehensive model for ICL repair depicted in FIG. 3, we present mechanisms for ICL repair at each stage of the cell cycle. Given that nuclease-mediated ICL unhooking is necessary in some form at all stages of the cell cycle, followed by bypass of the crosslinked base pair by TLS DNA polymerases, it is likely that repair will require the actions of various nucleases and DNA polymerases.

Nucleases and polymerases in ICL repair

Hints regarding the importance of specific nucleases in ICL repair have come from individuals with biallelic mutations in *ERCC4* (which encodes XPF), *ERCC1* and *SLX4*. Hypomorphic mutations in *ERCC4* cause the ultraviolet (UV) radiation-sensitive disorder xeroderma pigmentosum (XP), whereas biallelic *ERCC4* or *ERCC1* mutations lead to a premature ageing syndrome known as XPF/ERCC1 progeroid syndrome that causes sensitivity to ICLs^{73,74}. *SLX4* mutations cause a more typical FA phenotype, and the gene has recently been designated *FANCP* on the basis of the identification of FA families with biallelic mutations in this gene^{75,76}. FAN1 and the heterodimeric nucleases MUS81–EME1, XPF–ERCC1 and SLX1–SLX4 are all required to prevent sensitivity to nitrogen mustards, MMC or platinum drugs^{50,77,78}. These three endonucleases are specific for 3′-flap structures, which, combined with the 5′-flap activity of FAN1, could result in the cleavage of the DNA backbone on both sides of an ICL. Interestingly, SLX1–SLX4, MUS81–EME1 and XPF–ERCC1 have been shown to form a ‘supercomplex’ that can be co-immunoprecipitated from cells⁷⁸; this makes it difficult to determine which particular nuclease promotes cleavage at an ICL. The possible involvement of these nucleases in downstream events, such as in the resolution of recombination intermediates, provides a further complication^{79–81}.

One possibility is that these different nucleases exist to deal with diverse forms of DNA crosslinks — such as crosslinks that distort the DNA double helix, as well as those that do not distort the DNA double helix — and there may be particular requirements for different nucleases according to the stage in the cell cycle at which an ICL is encountered. For example, it has been shown that the amount of unhooking present in G1 phase is directly influenced by the degree of ICL-induced helical distortion⁸². XPF–ERCC1 could cleave DNA adjacent to ICLs that substantially distort the DNA double helix in G1, in much the same way as they cut adjacent to damaged nucleotides that distort DNA during NER⁵⁰. SLX4 could also be recruited to distorting ICLs in G1 by its direct interaction with the mismatch repair (MMR) complex MUTSβ^{78,83,84}.

The importance of helix distortion in ICL repair has been addressed using cell-free systems and plasmids containing different types of ICLs. Lesions that result in little helix distortion are only repaired in replication-competent extracts, whereas the repair of helix-distorting ICLs can also be detected in non-replicating plasmid DNA^{57,85}. Genetic and toxicity studies suggest that even for highly distorting lesions, such as those that are induced by platinum, not all ICLs are repaired in G1 (REFS 86,87). One possibility is that these distorting lesions are not detected in the context of highly condensed heterochromatic DNA or in regions of the genome that are non-B-form DNA, such as upstream promoter elements⁸⁸. Some of this damage could also undergo a futile cycle of repair, involving NER and TLS enzymes that mediate the nucleolytic cleavage of ICLs during G1 but are unable to complete the removal

of the covalently linked bases⁸⁷ (FIG. 3). These cleavage events would persist into S phase, leading to the direct collapse of the replication fork when it encounters the nicked DNA. HR would then repair this collapsed fork in a scenario that is similar to that in which canonical S phase ICL repair is initiated.

The encounter of an ICL by the replication fork leads to nuclease recruitment by mechanisms that are different from those that occur in G1. FAN1 is recruited to stalled replication forks by monoubiquitylated FANCD2 (REF. 70), and SLX4 could be recruited directly to the vicinity of DNA damage by its tandem UBZ domains⁷⁶ that interact directly with K63-linked ubiquitin chains, which themselves represent markers of stalled replication^{76,89}. Finally, MUS81 and XPF are relocalized as part of the SLX4 complex⁹⁰. This regulated recruitment of nucleases by ubiquitylated proteins might suggest a mechanism that orients cleavage on the basis of the localization of the ubiquitylated protein in relation to the DNA substrate. In an environment such as that found at an ICL between two converging replication forks (FIG. 3), there are many potential nucleolytic substrates, so the careful regulation of DNA cleavage will be essential.

After unhooking, the nascent DNA strand must be extended past the lesion but normal replicative DNA polymerases are incapable of extension when the replisome is blocked by a crosslink⁹¹ (as opposed to the situation with base damage in which the MCM helicase and polymerase can be uncoupled⁹²). The replication fork may be stabilized by HR (see below) and lesion bypass will require the TLS polymerases (reviewed in REF. 93).

Xenopus laevis extracts that are capable of replication have provided a useful model system for studying the repair of a single platinum crosslink, and these studies indicate that the replicative polymerase stalls about 24 nucleotides before the crosslink. At this site, a switch to TLS is presumed to take place, and this may involve Pol ν , which extends the nascent strand to within 1 base pair of the ICL before unhooking takes place⁵⁷. Such a switch would be more difficult if the replication fork had collapsed much earlier owing to unhooking before replication. TLS requires the monoubiquitylation of the replication-associated factor proliferating cell nuclear antigen (PCNA) on K164, a step that also stimulates the activation of the FA pathway through FANCD2 recruitment^{94,95}.

After unhooking, the deoxycytosine monophosphate (dCMP) transferase REV1 can insert a cytosine into the nascent strand opposite the unhooked ICL^{96,97}. One of several other TLS polymerases may then be responsible for the extension of the nascent DNA beyond the crosslinked base pair still present in the DNA double helix. Although many of these polymerases can extend the nascent strand beyond the crosslink (reviewed in REF. 98), only depletion of Pol ζ seems to abrogate TLS extension in *X. laevis* extracts⁵⁷. Consistent with this summary, only Pol ν , REV1 and Pol ζ are essential for ICL repair, and, owing to the error-prone nature of the reactions they promote, their involvement is potentially mutagenic^{71,99}.

The role of HR in ICL repair

Extremely positive results have been obtained with the ICL-inducing agent carboplatin in early phase clinical trials for the treatment of triple-negative breast cancers — those that do not express HER2 (also known as ERBB2) or oestrogen and progesterone receptors and that are most likely to be associated with alterations in *BRCA1* or *BRCA2* (REF. 100). Platinum drugs are also beneficial when used as single agents in the treatment of *BRCA1*- and *BRCA2*-associated ovarian cancers¹⁰¹. The protein products of these two genes are important for HR-mediated repair of DNA damage. BRCA1, in combination with BRCA1-associated RING domain protein 1 (BARD1), exhibits ubiquitin ligase activity¹⁰² that is necessary for the proper localization of RAD51, a central player in HR; BRCA2 interacts

directly with RAD51 and promotes its specific targeting to sites where recombination is initiated^{103,104}. RAD51-deficient cells, like the tumour cell lines derived from patients with *BRCA1* and *BRCA2* alterations, are hypersensitive to ICL-inducing agents^{105,106}. This observation further supports a role for HR in ICL repair.

The process of HR during ICL repair requires the generation of a DSB, because RAD51 nucleoprotein filament formation requires a free DNA end and single-stranded regions in order to stimulate the search for homology on the adjacent DNA strand¹⁰⁷. γ H2AX is a biomarker of nuclear DSBs in patients receiving chemotherapy, and can be measured in hair follicles that are extracted at various times after treatment¹⁰⁸. Several potential sources of DSBs exist during the processing of an ICL. As discussed above, the nuclease-mediated unhooking of an ICL can occur either before encountering the replication fork — leading to the collapse of the replication fork into a one-ended DSB — or after the switch to TLS and the progression of the replication fork to within 1 base pair of the ICL⁵⁷. Alternatively, the stalled replication fork may regress to form a ‘chicken foot’ structure at the site of the ICL to generate a free dsDNA end (FIG. 3). FANCM has been shown to promote fork regression *in vitro*¹⁰⁹, and chicken foot structures have been shown to exist *in vivo* and *in vitro*^{110,111}. Replication fork stabilization after formation of a chicken foot structure occurs through the recombination of the nascent regressed arm into homologous sequences ahead of the fork (FIG. 3).

Although the process of HR at a two-ended DSB (such as that caused by ionizing radiation) is well characterized (reviewed in REF. 107), there are important differences when HR takes place at a stalled replication fork. First, the association of the replication complex with the nascent DNA molecules that are adjacent to the break may facilitate recombination with the correct template sequences rather than with related, but incorrect, target sequences. Second, the MRE11–RAD50–nibrin (NBS1; known as the MRN complex) nuclease, which is normally required for end resection, is inhibited by RAD51, probably because extensive resection at a replication fork is unnecessary or potentially deleterious¹¹². The involvement of HR factors that function specifically in ICL repair indicate that there may also be important structural differences in the types of DNA intermediates that form during the repair process. For example, cells that are deficient in the RAD51-paralogue RAD51C (also known as FANCO) are sensitive to ICL-inducing agents, and this protein may have a more important role in the repair of a one-ended, rather than a two-ended, DSB^{113,114}. *RAD51C*, like *BRCA1*, *BRCA2* and BRCA1-interacting protein 1 (*BRIP1*; which encodes FANCF), was recently shown to be a breast and ovarian cancer predisposition gene in heterozygous carriers, and it causes an FA-like syndrome when homozygously mutated^{31,115}. The HR step of ICL repair (and probably other replication-fork stalling lesions) may thus be one of the most important tumour suppressive roles of *BRCA1* and *BRCA2*.

The completion of HR requires ‘second end capture’ and extension of the template strand by a DNA polymerase. The identity of the polymerase that carries out this DNA synthesis step is currently unknown, but after encountering a single replication fork this polymerase will promote replication restart. In the ‘late-S phase model’, when two forks converge on an ICL (FIG. 3), second end capture and extension will lead to the completion of replication and the formation of a double Holliday junction (DHJ) that can be dissolved by the BTR complex (comprised of the BLM RecQ helicase, topoisomerase III α (TOPOIII α), RecQ-mediated genome instability 1 (RMI1) and RMI2) in a reaction that avoids sister chromatid exchanges (SCEs)^{116,117}. BLM is mutated in Bloom’s syndrome, a disorder that is similar to FA in many respects. Consistent with this, the BTR complex associates with the FA core complex, providing a direct link between the upstream signalling of ICL damage recognition and the downstream resolution of the HR products^{59,118}. A characteristic feature of Bloom’s syndrome cells is an elevated frequency of SCEs, and these levels are further increased by

treatment with MMC¹¹⁹. It was recently shown that, in the absence of BLM, DHJs are resolved by the MUS81–EME1, SLX1–SLX4 and GEN1 endonucleases that promote SCE formation⁸¹. Strikingly, genetic exchanges between homologous chromosomes are also highly elevated in BLM-deficient cells, resulting in loss of heterozygosity¹²⁰, a hallmark feature of tumorigenesis. High doses of ICL-inducing agents in wild-type cells also lead to increased SCE levels¹²¹, suggesting that the nucleolytic cleavage pathway of Holliday junction resolution occurs when BLM function is normal but when the levels of DNA damage are saturating. By promoting the BLM-mediated pathway during ICL repair, there is less chance of both SCE formation and crossing over between homologous chromosomes. The high tumour incidence in patients with Bloom's syndrome (which includes a broader range of cancer types than those associated with FA) reflects the importance of this component of the repair process.

Suppression of NHEJ reduces ICL sensitivity

Although HR promotes error-free DSB repair in S phase, an alternative mechanism that is known as non-homologous end joining (NHEJ) exists to repair broken DNA in all phases of the cell cycle. NHEJ uses a simple splice mechanism to rejoin the two free ends of DNA. The process is initiated by binding of the KU70–KU80 heterodimer to the free dsDNA ends and involves the activation of downstream steps by the binding of DNA-dependent protein kinase catalytic subunit (DNA-PKcs)¹²². DNA is processed to remove any 5′- or 3′-ssDNA tails, and the resulting end is directly rejoined with a similarly processed sequence by DNA ligase IV–XRCC4 (reviewed in REF. 123). Unlike HR, in which homologous sequences are used to proofread the repair process, NHEJ is capable of generating deletions, insertions and translocations when incorrect ends are rejoined.

Human cells that are defective for KU70, KU80, DNA-PKcs, DNA ligase IV or XRCC4, are insensitive to MMC, platinum or other ICL-inducing drugs, confirming studies in yeast and mice showing that the NHEJ pathway is not required for ICL repair^{124,125}. However, recent reports have shown that the inhibition of the NHEJ pathway in cell lines derived from patients with FA can reduce the toxicity of ICL-inducing drugs. For example, in chicken or nematode knockout models, specific FA-like defects can be rescued by the co-deletion of KU70 or ligase IV^{126,127}. Furthermore, in *FANCA*- and *FANCD2*-deficient human cells, the high sensitivity to MMC can be rescued through the simultaneous inhibition of NHEJ by the DNA-PKcs inhibitor NU7026 (REF. 127). Analysis of mitotic spreads from these cells shows that the atypical radial chromosomes that are seen in FA cells are only rarely found when NHEJ is inactivated. These observations indicate that a major function of the FA pathway in ICL repair is to suppress spurious ligation of ICL-induced DSBs between non-homologous chromosomes (FIG. 3).

For the repair of *de novo* DSBs (such as those caused by ionizing radiation) HR and NHEJ provide complementary functions, and co-inhibition of HR and NHEJ leads to increased cell death^{128–130}. However, FA cells are not defective in HR per se, so the inhibition of NHEJ in FA cells still allows them to proliferate and repair DSBs (FIG. 4). This is because the FA pathway only promotes HR at stalled replication forks, by stabilizing the intermediate that is required for unhooking and TLS. HR can still occur if the replication fork is not stabilized, but the free DNA end that is generated is more likely to be bound by KU70–KU80, which has a very high affinity for such structures^{131,132}. KU70–KU80 promotes NHEJ with the correct adjacent sequence only some of the time, but causes the formation of radial structures if it promotes ligation with sequences on another broken chromosome¹³³. By inhibiting the NHEJ pathway, the less active, but also less toxic, FA-independent HR pathway can re-establish the replication fork (FIG. 4). Ongoing work aims to test the efficacy of NHEJ inhibitors in diluting the severity of FA-like phenotypes in FA mouse

models, but these drugs could have a wider use in the protection from ICL toxicity in the blood of patients treated with chemotherapies.

Modulating ICL sensitivity: protect and destroy

As platinum and alkylating agents currently represent some of our most successful chemotherapeutic agents, the ability to change cell sensitivity to these drugs could have enormous therapeutic implications. As outlined in TABLE 1, the therapeutic window for ICL-based chemotherapy is restricted by dose-limiting toxicities in the blood: therefore, being able to specifically activate haematopoietic ICL resistance could theoretically raise the maximum tolerated dose. In patients with FA, a potential strategy to prevent developmental cytopenias may be the use of inhibitors of NHEJ (such as the DNA-PKcs inhibitor NU7026), but it remains to be seen whether such an approach would also protect the blood of wild-type patients undergoing chemotherapy. Current clinical practice involves the use of recombinant cytokines granulocyte colony-stimulating factor (G-CSF; known as filgrastim) and granulocyte-macrophage CSF (GM-CSF; known as sargramostim) to promote the mobilization of haematopoietic stem cells to try and recover the cells lost by ICL-induced apoptosis¹³⁴. These agents are normally given in the recovery phase, and most ICL-induced cytopenias can be rescued in this manner. However, when given concurrently with crosslinking drugs, these cytokines may actually potentiate cytopenia¹³⁵, as stem cells that are induced to proliferate may be more sensitive to DNA damage than quiescent cells¹³⁶. It is therefore reasonable to suggest that the prevention of cytopenias may benefit from an understanding of how tumour cells become resistant to ICLs, and in applying this knowledge to promote resistance in the non-diseased cells of the blood.

One mechanism of resistance to ICL-inducing drugs is the accelerated removal of DNA adducts. Such activities have been detected in ovarian and testicular tumours that become resistant after chronic cisplatin exposure, and this is often associated with elevated levels of XPF-ERCC1, MUS81-EME1 and FA proteins¹³⁷⁻¹⁴¹. FANCF-dependent resistance to cisplatin leads to increased cross-resistance to MMC¹³⁹, and larger studies have identified a general pattern of cross-resistance after exposure to just one type of ICL-inducing drug¹⁴². The development of methods that increase ICL repair in normal tissue might lead to effective chemoprotection.

Conversely, as resistance to ICL-inducing drugs develops, targeted inactivation of ICL repair represents a tumour control strategy. An indirect mechanism of downregulating elevated ICL repair in tumour cells is mediated by the inhibition of extracellular signalling pathways, although little is understood about this mechanism. For example, the HER2 antibody trastuzumab can suppress the repair of cisplatin adducts in cell cultures¹⁴³ and can potentiate cisplatin and melphalan treatments in breast cancer¹⁴⁴⁻¹⁴⁶. Trastuzumab may function indirectly to affect cell cycle checkpoints, and the direct targeting of checkpoint kinases has also shown some clinical promise.

The checkpoint kinase CHK1 suppresses exit from G2 phase into mitosis by phosphorylating several key cell cycle proteins¹⁴⁷. This G2 cell cycle checkpoint is necessary to allow time to repair DNA damage, particularly when the G1 phase, p53-dependent cell cycle checkpoint is absent, as it is in most tumour cells. The absence of both cell cycle checkpoints allows p53-mutant tumour cells to enter mitosis even though they have extensive DNA damage. These cells die by a process known as mitotic catastrophe — death occurs not by the defined pathway of apoptosis, but by the loss of genetic material that is essential for survival¹⁴⁸. This strategy may be particularly potent in re-establishing sensitivity to ICL-inducing drugs in tumours because the mechanism of death is apoptosis-independent and the catastrophe can be accentuated by larger amounts of unrepaired DNA

damage. In this manner, strategies that include CHK1 inhibition, or MAPK inhibition (MAPK participates in a parallel pathway to CHK1 in G2) have shown promise in re-establishing cisplatin sensitivity in *in vitro* models^{149,150}.

Concluding remarks

Over the past decade, rapid advances have been made in understanding how ICL damage is repaired following chemotherapy. We now know that ICL repair involves a complex coordination of NER, HR and TLS, as well as the suppression of NHEJ. But information is still missing, and this has meant that the translation of knowledge into the improved clinical use of ICL-damaging agents remains in its infancy. Some investment has been made in improving cell killing by these drugs, such as for use in BRCA1-deficient breast cancers¹⁰¹, but the core dose-limiting toxicity of ICL-inducing agents — the damage to the haematopoietic system that was ironically first seen in the casualties of the Bari disaster nearly 70 years ago — has generally been ignored. Overcoming this toxicity will allow greater therapeutic windows, and reduced side effects in patients. Increased tumour cell killing by using higher doses of ICL-inducing drugs may also limit the development of tumour resistance. Further investigation into FA, and other model diseases, may uncover the last remaining players in ICL repair, but a concerted effort to understand the precise mechanistic details of events that occur when the FA pathway is activated will undoubtedly provide key insights regarding the use of DNA crosslinking agents in chemotherapy.

Acknowledgments

This research was supported by a grant to A.J.D. and S.C.W. from the Fanconi Anaemia Research Fund. Work in S.C.W.'s laboratory is supported by the European Research Council, the Louis-Jeantet foundation, the Breast Cancer Campaign, the Swiss Bridge Foundation and Cancer Research UK.

Glossary

Lipid peroxidation	The oxidation of fats and oils to form radicals capable of crosslinking DNA. It can occur in foods before they are eaten or can take place in the body.
Radial chromosome	A structure thought to result from the fusion of the broken arms of non-homologous chromosomes. Such chromosomes cannot be properly segregated in most cells, resulting in either chromosome breakage or a failure in cell division.
Homologous recombination	(HR). RAD51-mediated pairing and exchange of genetic information between homologous DNA sequences.
Nucleotide excision repair	(NER). Nucleolytic removal of a damaged nucleotide by sequential action of 5' - and 3' -endonucleases followed by polymerase-mediated filling of the resulting gap.
Translesion synthesis	(TLS). A DNA damage tolerance process that allows replication past DNA lesions. If the normal replicative polymerase cannot insert a base owing to damage in the template strand, it is often replaced by a lower fidelity translesion polymerase.
Replisome	The active and assembled structure that contains the enzymes required for DNA replication.

Mismatch repair	(MMR). A strand-specific mechanism for editing mismatched bases inserted in the daughter strand during replication. This damage is repaired by recognition of the deformity caused by the mismatch.
Futile cycle	A DNA repair process that is repeatedly initiated, but a subsequent step re-establishes the damaged state.
Sister chromatid exchanges	(SCEs). An exchange of genetic information between the two daughter strands of a replicated chromosome. This in itself does not result in loss of genetic information, but high SCE levels indicate that other recombination events such as the exchange of genetic material between homologous chromosomes could also occur and so lead to a loss of heterozygosity.
Cytopenias	Loss of one or multiple types of white or red blood cells, which is often caused by the depletion of stem cell pools.
Cell cycle checkpoints	A control mechanism that prevents cell cycle continuation in the presence of damaged, unreplicated or incompletely segregated DNA.

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At a glance

- DNA interstrand crosslinks (ICLs) may arise following exposure to environmental mutagens, and are potentially toxic when induced in large numbers by chemotherapeutic drugs. ICL-based chemotherapy is one of the most widely used forms of cancer treatment, particularly in the treatment of leukaemias.
- Fanconi anaemia (FA) is a disorder that results in sensitivity to ICLs, which is caused by the mutation of one of at least 14 different genes. Although their functions have not been completely elucidated, there is substantial evidence to suggest that these genes, including the *BRCA2* (also known as *FANCD1*) breast and ovarian cancer tumour suppressor, participate in a common pathway of ICL repair.
- Homologous recombination (HR)-mediated repair of ICLs is promoted by the FA pathway. FA cells that are exposed to ICL-inducing agents, or chronically treated wild-type cells, may use alternative pathways of repair that lead to deleterious genetic aberrations such as radial chromosomes.
- Structure-specific nucleases and translesion polymerases participate in the coordinated removal of the ICL and the resumption or completion of DNA replication. There is increasing evidence that non-replication-associated repair of ICLs also takes place — however, this is insufficient to remove all ICL damage.
- The modulation of ICL repair could improve chemotherapy outcomes. For example, the dose-limiting toxicities of ICLs mostly affect the blood system, so increasing the ability to repair ICLs in blood cells could prevent anaemia phenotypes. Alternatively, targeted downregulation of ICL repair in tumours could improve ICL-mediated tumour killing.

Box 1**Mechanism of interstrand crosslink formation by commonly used chemotherapeutic agents**

The mechanisms of action of the four main classes of crosslinking drugs (alkylating agents, platinum, mitomycin C and furocoumarins, and psoralens) are broadly similar in that two chemically active leaving groups are required. In bifunctional nitrogen mustards and platinum compounds these leaving groups are acquired inside the cell by the sequential displacement of two chloride ions by water molecules (see part **a** of the figure). This activated form of the drug can react with bases on each DNA strand, most commonly the *N*⁷-position of guanosine or adenosine (highlighted in orange in the figure)¹⁵¹. Mitomycin C and psoralens are natural products originally derived from fungal sources that form DNA crosslinks more directly. Mitomycin C and psoralens both contain planar rings that are activated by photon-mediated cycloaddition or cycloreduction, respectively, to attack DNA bases on opposing strands (see parts **b** and **c** of the figure). Monoadducts, in which the agent remains linked to one rather than two DNA strands, can also form if a water molecule accepts the second free leaving group. Psoralen is particularly useful for targeted tumour therapy, as its crosslinking ability is only activated in response to treatment with ultraviolet A (UVA) radiation.

Figure 1. How ICLs kill tumour cells

Interstrand crosslinks (ICLs) can block the progression of the replication fork by inhibiting the progression of the replisome. ICLs can stall transcription. ICLs may distort the structure of chromatin and prevent the access of DNA-interacting proteins. Tumour cell death can be induced by p53- and FAS ligand-dependent apoptosis (programmed cell death) or p53-independent mitotic catastrophe (death when apoptosis is absent but vital DNA integrity is lost).

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Figure 3. ICL repair at different stages of the cell cycle

In cells in G1 phase of the cell cycle, nucleotide excision repair (NER) can remove a subset of interstrand crosslinks (ICLs). Excision by XPF-ERCC1 is followed by translesion synthesis, and excision of the 'flipped out' ICL. However, some lesions cannot be bypassed in this manner and a futile cycle of repair involving excision and ligation occurs. During S phase, a partially processed intermediate may be encountered by a replication fork, which leads to the collapse of the replication fork and the formation of a one-ended double-strand break (DSB). These collapses are probably avoided by FANCM-mediated fork regression and stabilization of the fork by the Fanconi anaemia (FA) pathway. This FA-stabilized homologous recombination (HR) intermediate (highlighted by beige circles) allows the nascent leading strand of DNA synthesis (indicated by a green arrow) to be extended by translesion synthesis polymerases (Pol; black dashed arrow). Unhooking of the ICL by

coordinated incision by the 5' - and the 3' -flap endonucleases allows extension past the lesion. HR is then completed and replication can be re-established. If a second replication fork has encountered the ICL from the opposite direction, unhooking on the 3' side cuts the leading strand template. The extension of the 'left-hand' leading strand would lead to its joining with the lagging strand of the right-hand fork, followed by second end capture and extension of the right-hand leading strand and the left-hand template strand. This process would complete replication without the re-initiation of lagging strand synthesis but would generate a double Holliday junction. This structure is resolved by the BLM-containing complex or may in some instances be cut by structure-specific nucleases (not shown). FAN1, Fanconi-associated nuclease 1.

Figure 4. Suppression of ICL sensitivity by inhibition of non-homologous end Nature Reviews Cancer joining

In wild-type cells, interstrand crosslinks (ICLs) can be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ). The Fanconi anaemia (FA) pathway, however, promotes HR-dependent stabilization of the replication fork and DNA repair. In FA cells, double-strand breaks (DSBs) are frequently created that can be repaired by either HR or NHEJ. Because the DSB is one ended, NHEJ does not have a natural substrate to rejoin so these breaks can remain unrepaired (generating chromatid breaks) or may ligate with a DSB in a different chromosome (generating radial chromosomes). Suppression of NHEJ by deleting a component of this pathway or by using DNA-dependent protein kinase catalytic subunit (DNA-PKcs) inhibitors promotes the repair of the DSBs by FA-independent pathways of HR (dashed arrow).

Table 1
Crosslinking agents used in the clinic

Drug	Clinical application	Dose-limiting toxicity	Example solution structure
Platinums			
Cisplatin	Testicular, ovarian and non-small-cell lung cancer	Central nervous system, renal and gastrointestinal toxicity ¹⁵³	Cisplatin ¹⁵²
Carboplatin	Ovarian cancer	Neutropenia ¹⁵⁴	
Oxaliplatin	Colorectal cancer	Neuropathy ^{135, 155}	
Satraplatin	Prostate and breast cancer	Thrombocytopenia and neutropenia ^{156, 157}	
Picoplatin	Phase II trials for relapsed lung cancer and Phase I and II trials for the treatment of solid tumours, and prostate, colorectal and small-cell lung cancer	Thrombocytopenia and neutropenia ¹⁵⁸	
Nitrogen mustards			
Cyclophosphamide	Lymphoma	Neutropenia ¹⁶⁰	Carmustine ¹⁵⁹
Melphalan	Multiple myeloma, melanoma and ovarian cancer	Leukopenia and thrombocytopenia ¹⁶¹	
Chlorambucil	Chronic lymphocytic leukaemia	Pancytopenia and neurotoxicity ¹⁶²	
Ifosfamide	Non-small-cell lung cancer	Leukopenia, thrombocytopenia and renal toxicity ¹⁶³	
Others			
Mitomycin C	Oesophageal and bladder cancer	Leukopenia and thrombocytopenia ^{165, 166}	Psoralen ¹⁶⁴
Psoralen plus ultraviolet A radiation	Cutaneous T cell lymphoma	Dermatitis ^{167, 168}	
Pyrrolobenzodiazepines	Phase II trial for solid tumours	Fatigue and thrombocytopenia ¹⁶⁹	

Table 2
Genes associated with ICL sensitivity

Gene	Disease	Cancer types	Functions of gene product
<i>FANCA</i> , <i>FANCG</i> , <i>FANCF</i> , <i>FANCC</i> , <i>FANCE</i> and <i>FANCB</i>	FA	AML and HNSCC	Forms the FA core complex that is required to activate FANCL
<i>FANCL</i>	FA	None reported (FANCL mutations account for only two cases of FA)	Ubiquitin ligase that monoubiquitylates FANCD2–FANCI
<i>FANCD2</i> and <i>FANCI</i>	FA	AML and HNSCC	Binds DNA, promotes DNA damage signalling and the recruitment of repair enzymes
<i>USP1</i> and <i>WDR48</i> (which encodes UAF1)			Heterodimer required for the deubiquitylation of FANCD2 and the completion of ICL repair
<i>BRIP1</i> (which encodes FANCI)	FA	AML and breast * cancer	3′-5′ DNA helicase with preference for branched DNA substrates
<i>FAN1</i>			DNA 5′-3′ exonuclease, and 5′-flap endonuclease. Specifically binds the monoubiquitylated form of FANCD2
<i>REV1</i> , DNA polymerase ν and DNA polymerase ζ			Translesion synthesis polymerases that are required for DNA synthesis at the site of ICLs
HELQ			Helicase function required for the completion of ICL repair
<i>FANCM</i>	FA	None reported (only two cases of FA with FANCM mutations)	5′-3′ translocase and branch migration activity. Structure-specific DNA junction binding and recruitment of FA core complex and BLM. Checkpoint activation
<i>APITD1</i> (which encodes MHF1), <i>STRA13</i> (which encodes MHF2) and <i>C19orf40</i> (which encodes FAAP24)			FANCM accessory factors
<i>PALB2</i> (which encodes FANCN)	FA and BOC	AML, medulloblastoma, neuroblastoma, breast and ovarian * cancer and pancreatic * cancer	Stabilizes and promotes localization of BRCA2 to DNA damage sites
<i>BRCA2</i> (which encodes FANCD1)	FA and BOC	AML, ALL, medulloblastoma, breast and ovarian * cancer and prostate * cancer	Required for loading of RAD51 recombinase onto DNA
<i>BRCA1</i>	BOC	Breast and ovarian * cancer	Ubiquitin ligase activity towards histone H2A and CTIP
<i>SLX4</i> (which encodes FANCP)– <i>SLX1</i>	FA	HNSCC (one case reported)	Structure-specific endonuclease
<i>ERCC4</i> (which encodes XPF)– <i>ERCC1</i>	XP and XFE	Skin cancer, HNSCC * and lung cancer *	DNA 5′-flap endonuclease
<i>MUS81-EME1</i>			DNA 3′-flap endonuclease
<i>RAD51</i>			Recombinase that searches for homology in DNA templates and promotes strand exchange
<i>RAD51C</i> (which encodes FANCO)	FA and BOC	None reported (four cases of FA with <i>RAD51C</i> mutations), breast and ovarian cancer *	Required for HR
<i>NBS1</i>	NBS	B cell lymphoma	Required for HR. Component of the MRE11–RAD50–NBS1 (MRN) complex
<i>BLM</i>	BS	A broad range of cancers are	5′-3′ DNA helicase, inhibits RAD51 strand invasion and

Gene	Disease	Cancer types	Functions of gene product
		increased	promotes dissolution of Holliday junction intermediates
<i>TOP3A</i> , <i>RMI1</i> and <i>C16orf75</i> (which encodes RMI2)			Required for Holliday junction resolution by BLM
<i>ATR</i>	<i>SS</i>	None reported	Senses accumulation of RPA-coated ssDNA. It is a kinase and phosphorylates many targets that are required to activate cell cycle checkpoints and promote repair

ALL, acute lymphocytic leukaemia; AML, acute myeloid leukaemia; *APITD1*, apoptosis-inducing, TAF9-like domain 1; *ATR*, ataxia-telangiectasia and Rad3-related; *BLM*, Bloom's syndrome RecQ-helicase like; BOC, breast and ovarian cancer susceptibility; *BRIP1*, BRCA1-interacting protein 1; BS, Bloom's syndrome; FA, Fanconi anaemia; *FANL*, Fanconi-associated nuclease 1; *HELQ*, helicase, POLQ-like; HNSCC, head and neck squamous cell carcinoma; HR, homologous recombination; ICL, interstrand crosslink; NBS, Nijmegen breakage syndrome; *NBS1*, nibrin; *PALB2*, partner and localizer of BRCA2; *RMI*, RecQ-mediated genome instability; SS, Seckel syndrome; ssDNA, single-stranded DNA; *STRA13*, stimulated by retinoic acid 13; TOP3A, topoisomerase III α ; XFE, XPF/ERCC1 progeroid syndrome; UAF1, USP1-associated factor 1; *USP1*, ubiquitin specific peptidase 1; *WDR48*, WD repeat domain 48; XP, xeroderma pigmentosum.

* Documented in heterozygotes only.