Chapter

# Interstrand Crosslink Repair: New Horizons of DNA Damage Repair

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## Abstract

Since the dawn of civilization, living organisms are unceasingly exposed to myriads of DNA damaging agents that can temper the ailments and negatively influence the well-being. DNA interstrand crosslinks (ICLs) are spawned by various endogenous and chemotherapeutic agents, thus posing a somber menace to genome solidity and cell endurance. However, the robust techniques of damage repair including Fanconi anemia pathway, translesion synthesis, nucleotide excision and homologous recombination repair faithfully protect the DNA by removing or tolerating damage to ensure the overall survival. Aberrations in such repair mechanisms adverse the pathophysiological states of several hereditary disorders i.e. Fanconi Anemia, xeroderma pigmentosum, cerebro-oculo-facio-skeletal syndrome and cockayne syndrome etc. Although, the recognition of ICL lesions during interphase have opened the new horizons of research in the field of genetics but still the detailed analysis of conditions in which repair should occur is largely elusive.

**Keywords:** DNA damage repair, Interstrand cross links (ICLs), Homologous Recombination Repair, Translesion synthesis, Non-homologous end-joining repair, FA pathway

## 1. Introduction

There is an amalgam of various environmental, endogenous as well as chemotherapeutic agents that are continuously having a contact with the genetic material in living beings and making it a point of real concern throughout the globe. The attack of reactive oxygen as well as nitrogen species on DNA have contributed towards a large amount of defects and complex chemical structures that take place in DNA [1]. These damages give rise to a series of simple and bulky base modifications that distort the helical structure, abasic sites, the breaks in phosphodiester linkages along with the interstrand crosslinks (ICLs). These lead to various mutagenic changes in the genetic blueprint and become a reason of inhibition of the transcriptional or replicative machinery that induce activate apoptotic divisions or necrosis [2].

Interstrand cross-links (ICLs) are the anomaly that link the complementary strands of DNA by the covalent linkage between the bases. These are formed by the chemicals along with the two reactive electrophilic groups. It is a highly sequence-dependent reaction in which the two nucleophilic groups on the opposite strands are aligned geometrically and enable the dual reaction of the bifunctional cross-linking agent with it. This complex chemical reaction give rise to ICLs,

mono-adducts, intrastrand cross links as well as DNA-protein cross-links [3]. The ICLs are made with the help of reactive endogenous chemicals such as lipid peroxidation product known as malondialdehyde or aided with the reactive aldehyde group of an unpromptedly formed or the enzyme-derived abasic site in the DNA molecule with a normal base on the complementary strand [4].

A large amount of anticancer and chemotherapeutic agents such as mitomycin C (MMC), cisplatin, nitrosoureas and nitrogen mustards are notorious for introducing formidable blocks in the normal metabolic processes of DNA with ICLs and need repair for cell sustenance. ICLs are also caused by various antitumor agents that defects DNA through radical processes like C-1027, neocarzinostatin [5]. With the passage of time, the organisms have developed various complex mechanisms to alleviate these deleterious defects from the genome. The failure to remediate the defect can contribute towards cell death that can occur either through a mitotic catastrophe or the p53-dependent apoptotic pathway. In the mammalian cells, the repair mechanisms for ICLs repair are still ambiguous [6]. According to an estimation, about 40ICls that form in a mammalian genome can destroy a defective cell that lacks ability to be repaired.

The in vivo study gives an overview of the elimination of the ICLs in cellular DNA of both prokaryotes and eukaryotes. The model organisms are used for the clear understanding of the repair mechanisms. These include *E. coli* and yeast. The ICLs repair mechanisms in bacteria and yeast are replication dependent and independent while in vertebrates, they follow repairment pathway during replication of DNA [7]. Moreover, the recent study suggests the operation of replication independent ICL repair pathway in vertebrates.

The ICL repair pathway have been deduced from the relative sensitivity of the DNA repair defective cell lines to the cross linking agents. Pathways of ICL repair have mostly been inferred from the sensitivities of DNA repair defective cell lines to crosslinking agents. During the S phase of the cell division in vertebrates, the ICL repair is induced by the help of impeded replication forks. The process of ICL repair needs a nexus of multiple factors along with the structure specific endonucleases, for example TLS and HR. If a disturbance occurs during the repair, the genomic instability results that bring forth the birth of Fanconi anemia, a cancer prone ailment [8]. There is another ICL repair pathway that takes place in the G0/1 phase during the cell cycle which is a replication and recombination independent pathway [9]. In addition, the tolerance of ICLs in G1 as compared to S phase makes it an underappreciated pathway because there, the stalled replication fork possesses high toxicity. Contrarily, the toxicity of ICL in G1 can be depicted when it terminates the transcription of a gene playing a vital role.

The latest studies have proposed the role of NER proteins (as they cut one side of ICL) [7], Homologous recombination along translesion synthesis polymerases (Polζ, Rev1) that are involved in filling the gap for both type of cells undergoing replication as well as non-replicating ones [10]. The proteins involve in the ICL repair have a vital role in the pathophysiology of several hereditary diseases Proteins implicated in the repair of ICLs have a critical role in the pathophysiology of several hereditary disorders. In addition, cells deficient in the Fanconi Anemia (FA) pathway are highly sensitive to ICLs [11] and this pathway has been suggested to play an important role in mammalian ICL repair at replication forks promoting homologous recombination. There has been a series of continuous research on ICL lesions in the past decade and it covered the various aspects of ICLs be it as their identification, detection methods or their development along with the repair mechanisms and the exploitation of cross linkers in the laboratory. These have paved the way towards the better and more reliable understanding of ICLs in the complex biological samples. This chapter foregrounds the multiple aspects of the interstrand cross-link repairs with a reference to their pathophysiology and lesion repair mechanisms.

## 2. Basic biochemistry of ICL-generating agents

A large variety of natural and synthetic chemicals are notorious for bringing ICLs on the front and are regarded as the ICL inducers or inducing agents. In the same way, the metabolic byproducts formed in the cell also contribute towards ICLs formation. Their structure and function vary greatly but ICLs inducers are known for their bifunctional reactivity with both of the strands of DNA. The endogenous as well as exogenous sources of ICLs are summarized as follows:

### 2.1 Endogenous sources of interstrand cross links

The endogenous sources of ICLs comprises of the reactive aldehydes that are generated as a result of lipid peroxidation along with base excision repair (BER) [12]. There are other endogenous by products of lipid peroxidation, the α, β-unsaturated aldehydes or enals namely crotonaldehyde, acrolein, along with the 4-hydroxynonenal (4-HNE). These are formed as a result of oxidative stress [13]. Moreover, there are exogenous contributors as well namely cigarette smoke and automobile exhaust to expose with acrolein and croton-aldehyde. The DNA nucleobases interact with enals to give rise to exocyclic adducts. These adducts then interact with proteins. The incorporation of enals to dG is done with the help of Michael addition in which addition of $N^2$ -amine occurs to generate $N^2$-(3-oxopropyl)-dG adducts. The next stage is cyclization of N1 with the aldehyde, giving rise to $N^2$ -γ-hydroxypropano-dG adducts [14]. These products are also genotoxic to human beings. Shapiro and Leonard are famous for their earlier study of nucleosides reactions with glyoxal, chloroacetaldehyde, malondialdehyd along with related bis-electrophiles [14, 15]. The in vitro formation of ICL is attributed to the opening of the exocyclic 1, $N^2$-dG product that minimizes the steric hindrance and forms ICL on exposure towards an aldehyde [16].

Moreover, there are DNA lesions that are formed as a result of accumulated acetaldehyde in the cells. The acetaldehyde is produced as a result of alcohol metabolism with aldehyde dehydrogenase 2 (ALDH2) as a biocatalyst. The drug disulfiram if used, blocks the enzyme ALDH2 and accumulates the acetaldehyde in the cells. The lesions produced are DNA adducts, breaks in single or double-strands of DNA (DSBs), sister chromatid exchanges (SCEs), point mutations, along with crosslinks in DNA [17]. The DNA adducts like $N^2$-ethylidene-2′-deoxyguanosine, $N^2$-propano-2′-deoxyguanosine, $N^2$-ethyl-2′-deoxyguanosine, along with $N^2$-etheno-2′-deoxyguanosine are vital DNA damage agents that follow the accumulation of acetaldehyde in the cells. The acetaldehyde reacts with guanine and forms a crosslink precursor known as $N^2$ -propanoguanine (PdG) which in turn reacts with N2 amine of guanine in 5′-CpG sequence consequently forming acetaldehyde interstrand crosslinks (AA-ICL). In Asian continent, the irreparable detoxification of acetaldehyde is found more often and is linked with alcohol mediated cancers [18]. Moreover, cells in *Saccharomyces cerevisiae* don't have ability to repair ICLs and are acetaldehyde sensitive thus gives validation of acetaldehyde mediated ICLs [19].

The intestinal pathogens in human beings known as *Enterobacteriaceae* and other bacteria play a vital role in the progression of colorectal cancer. They produce colibactins that are genotoxic in nature and bring harm to human beings. With their structural chemistry still unknown, colibactins produce ICL dependent DNA double-strand breaks (DSBs) and activates the ICL repair pathways [20]. *Cellulo* also depicts another picture of the DNA damaging mechanism in which colibactin producing bacterial exposure towards the genomic DNA of cultured human cells made it susceptible to interstrand cross links. There are different changes observed in the intoxicated cells including the replication stress, the activation of

| ICL AGENTS | Source | Adducts formed/ Target DNA sequences | Clinical benefits | Elimination half-life of drug | Metabolism | References |
|---|---|---|---|---|---|---|
| Platinums | | | | | | |
| Carboplatin | Synthetic (made from cisplatin) | Adducts: G-Pt-G and Pt-GG DNA sequence: 5'-GC | Treatment of ovarian cancer | 1–2 hours | Kidney | [28] |
| Oxaliplatin | | | Treatment of colorectal cancer | 26 hours or 20 hours | Kidney | [29, 30] |
| Mitomycin | | | | | | |
| Mitomycin C | *Streptomyces caespitosis* | 5′-CG-3' | Treatment of Esophagal and bladder carcinoma | Alpha-half-life of 8.2 mins, beta-half-life of 51.8 mins | Hepatic | [10, 31] |
| Azinomycin | | | | | | |
| Azinomycin B | Streptomyces sahachiroi | 5′-GNC or 5′-GNT sequences | having antitumor activity against P388 leukemia in mice | N.A | N.A | [32] |
| Chloroethylnitrosoureas | | | | | | |
| Carmustine | Synthetic (nitrogen mustard) | G-C base pair | Treatment of multiple myelomas | 70 minutes | Liver | [33] |
| Nitrogen mustards | | | | | | |
| Cyclophosphamide | Synthetic | 5′ -GNC | Treatment of lymphoma, multiple myeloma and ovarian cancer | **3 to 12 hours**. | Liver | [34] |
| Ifosfamide | | | Treatment of sarcomas and organ cancers | 60–80% in 72 hours | Kidney | [35] |
| Chlorambucil | | | Treatment of Chronic lymphocytic leukemia, Hodgkins lymphoma and Non-hodgkin lymphoma | 1.5 hours | Liver | [36] |

**Table 1.**
*Exogenous agents of Interstrand cross-link lesions.*

ataxia-telangiectasia along with Rad3-related kinase (ATR), as well as the retrieval of Fanconi anemia protein D2 (FANCD2). Contrarily, FANCD2 knockdown or ATR inhibition decreases the survival capability of cells having an exposure towards colibactins. The evidence ensures that collectins mediated DNA defects in infected cells favors DNA ICLs [21].

### 2.2 Exogenous sources of ICLs

The other sources of ICLs are exogenous in nature. They have the same mechanism of bifunctional alkylating agents but differ in their preferences for sequences, topologically restrict the DNA and need certain processing within the cell to form functioning ICL inducers [9]. In spite of the fact that they have a history of damaging DNA, their innovative uses also aid in understanding the mechanisms they follow to contribute in various therapeutic applications.

These include psoralens that belong to the family of furocoumarins, being mutagenic are still a matter of contention with their photochemotherapeutic applications in inflammatory skin diseases like psoriasis, vitiligo and eczema [22]. The Psoralens generates adducts on interaction with pyrimidines, most often with thymine and give rise to ICLs at the sequences made up of d(TpA):d(TpA) residues [23]. The several derivatives of psoralen form multiple changes in the DNA helical structural framework and exhibit their toxic nature. The DNA duplex adducted with 4′- (aminomethyl)-4,5′,8-trimethylpsoralen (AMT) exhibited 561 unwinding and 531 bending into its major groove [24].

Another chemotherapeutic agent known as cis-platinum diamminedichloride i-e CDDP, cisplatin also induces ICLs. It makes an adduct with purines, most often at the N7 position of the guanines, hence ICL forms at d(GpC): d(GpC) sequences. This is employed in various head and neck cancers, esophageal, epithelial lung, colon, gastric, bladder along with ovarian and testicular tumors. About 90% of the total defects are formed by 1,2-IaCL and 1,3-IaCL along ICL making only 5% of the total DNA lesions [23].

Apart from these anticancer agents, one of prime importance is Adriamycin which is also termed as doxorubicin. It generates a great response against a range of tumors be it as breast tumors, acute leukemia, lymphomas, stomach, sarcomas, multiple myelomas or bone tumors. It is employed as a singly or in combined form [25]. The interaction of Adriamycin is clearly understood with the help of the *in vitro* transcription assays that demonstrates the drug-induced DNA adducts at the GpC sites [26]. The electrospray mass spectral analysis revealed details of GpC drug binding regions and gives the information that the cross links are favored by formaldehyde under the certain conditions [27]**. Table 1** illustrates the exogenous agents of Interstrand crosslink lesions.

## 3. ICL Repair genes and human disorders

The proteins involved in the repair of ICLs have vital role in pathophysiology of various hereditary disorders for example xeroderma pigmentosum (XP), cerebro-oculo-facio-skeletal syndrome (COFS), Fanconi Anaemia (FA), trichothyodistrophy as well as Cockayne syndrome (CS) [37]. FA is associated with aplastic anemia, cancers (often acute myelogenous leukemia) and bone marrow failure. The mutational changes in any *FANC* genes contribute towards genomic instability and the sensitivity against the ICL agents [38]. According to an estimate 18 genes are involved in FA and the products of genes collaborate for ICL repair during the S phase [39]. Apart from these, the defective NER pathways also result in several rare

autosomal-recessive diseases like XP, CS, TTD and COFS syndrome [40]. Moreover, there are 11 genes that are associated with NER pathways and the defect in these occur due to the mutations in these genes. XP is associated with pigmentation, photosensitivity as well as cancerous skin diseases. Another inherited syndrome known as CS is present in which there are several problems arises namely ocular defects, mental deficiency, extensive demyelination, short stature, photosensitivity, large hands, feet, as well as ears [37]. There are wide ranging clinical spectrum of CS and the patients acutely affected are categorized under COFS syndrome patients. TTD is associated with neuro-ectodermal symptoms and clear sulfur-deficient brittle hair [41]. These NER diseases are different from each other with respect to their physical characteristics involving cutaneous ailments.

Keeping in view the various DNA repair factors, ICL genes has found to be having a strong link with cancer. There are several genes that are revealed by next-generation sequencing and play a part in hereditary breast cancer as well as ovarian cancer syndrome (HBOC). These genes are *BRCA1, BRCA2, PALB2, BRIP1 and RAD51C* exhibiting a close link with HBOC in the ICL repair pathways [42]. The preventive medication strategy requires the early detection of the mutations happening in BRCA1 and BRCA2 genes to help in process of recovery.

## 4. Recognition of ICL lesions in mammalian cells

During the course of ICL damage, the UHRF1 protein comes to rescue at the site within a fraction of seconds [43]. These proteins identify ICLs with the help of its SET and RING finger associated (SRA) domain, the same domain notable for its recognition ability for the hemi-methylated DNA and employment of DNMT1 to ensure the maintenance of methylation signature in the cells of mammals [44]. The relative affinity of UHRF1 protein in response to hemi-methylated DNA as well as ICLs are somewhat similar and proposed that UHRF1 interacted with both of them through related mechanisms. The UHRF1 proteins are employed preceding the incorporation of FANCD2 to ICLs [43]. About 10 minutes are lagged between the assembling of UHRF1 and FANCD2 to ICLs. This strengthens the assumption of other proteins being employed or the other PTM events that might occur during this time interval. The proper mechanism of UHRF1 mediated FANCD2 repair is not clear but implicate a direct protein–protein interaction. There has also been a proposed role of UHRF1 in a nuclease scaffold [45]. It is also proposed that the rapid incorporation of UHRF1 to the ICLs paves the way for FA mediated repair of lesion later on. As ICLs vary in their structural framework, there is a probability that in addition to UHRF1, other ICL sensor proteins do exist in the same way.

## 5. Factors involved in ICL repair pathway

There are several proteins that take part in the ICL repair. Along with these, included 15 proteins that are not only specific to FA genes (A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, and P) but also to other repair pathways [46]. The important recombination factors like RAD51, the structure-specific endonucleases like MUS81/EME1 and XPF/ERCC1, translesion DNA polymerases and Holliday junction processing factors all contribute towards the repair of ICLs.

A rare human genetic disease known as FA, which is associated with pancytopenia, various developmental abnormalities and a high cancer risk [47]. The cells procured from FA patients depict the large amount of chromosomal breakage as well as the formation of radial chromosomes [48] that bring strength to the idea of

high genomic stability in the ICL repair-deficient cells. The classical FA pathway has FA core complex (consisting of A, G, FAAP20, C, E, F, B, L, and FAAP100), an E3 ubiquitin ligase activity and the catalytic activity dedicated to the RING domain comprising FANCL protein. The core complex also acts on monoubiquitination of FANC1/D2 complex and is stimulated by damaged DNA [49]. The next step is the utilization of other downstream effectors that are attracted by the activated complex. These comprises nucleases, homologous recombination factors and translesion polymerases to remediate the lesions [50]. Whereas the exact function of monoubiquinated FANCD2 is still ambiguous.

An ATP dependent DEAH domain helicase namely FANCM exhibit a DNA translocase activity. It combines with FAAP24 and forms a complex structure comprising a histone-fold complex i-e MHF1/MHF2. It is a significant part of activated FA pathway [51]. The biochemical analysis also proposed that FANCM/FAAP24 complex is responsible for stabilizing and remodeling the stopped replication forks of DNA [52]. The complex of FAAP24 plays a vital part in the checkpoint activation that also need ATR to begin its function [53]. However, FANCM takes part in recombination independent ICL remediation by stimulating ubiquitination of PCNA thus promotes the incorporation of other NER incision factors to the sites with ICLs [51].

The group of genes associated with FA comprises of FANCD1 (BRCA2), FANCJ, FANCN, as well as FANCO are the recombination factors that forms a connection with susceptibility for breast or ovarian cancer. The downstream processing of ICL require the employment of recombination factors, mostly when there are the double strand breaks in the DNA. The paralogous gene of FANCO (RAD51C) is RAD51 [54]. FANCO forms complex structures on interaction with RAD51B, RAD51D, XRCC2, as well as XRCC3. Another significance of these paralogs is the utilization of the recombinase RAD51 while managing a single stranded DNA [55]. RAD51 and its paralogs are vital to cells tolerant against ICLs and vice versa because they provide the homologous recombination in response to ICLs as well as the double strand breaks [56].

The endonucleases also pay a part in ICLs repair. Three important heterodimeric structure-specific endonucleases are MUS81/EME1, SLX1/SLX4 and XPF/ERCC1. SLX4 is often mutated in the complementation group consisting of FANCP [57]. The combination of SLX4 and SLX1 make up a heterodimeric nuclease. Its function is to resolve the Holliday junction formed during the remediation of ICls [58]. During the process, SLX4 act as a scaffold protein that combines the multi-activity nuclease complex comprising MUS81/EME1 as well as XPF/ERCC1. The latter acts in either of the NER pathway as well as ICL repair. The studies proposed that NER works independent of SLX4 with XPF/ERCC1 complex and the analysis of FANCP patients further strengthens the idea as they were resistant against the UV radiations [59]. Further studies suggest that XPF/ERCC1 activity requiring SLX4 involves the complete detaching in ICL repair. It is a replication dependent remediation of ICLs [60]. Digesting nuclease (SNM1A) then follows and digest the detached oligonucleotides [61]. This step is a better alternative as compared to the bypass step used for synthesis.

Moreover, the lately discovered nuclease FAN1 also has a significant part in remediation of ICL. The ubiquinated FANCD2 aids in employing FAN to ICL regions. This step is mediated with the ubiquitin-binding zinc finger domain that is present in FAN1 [62]. Another important domain of FAN1 exhibit 5′-3′ exonuclease activity as well as structure-specific endonuclease activity at 5′ [63]. FAN1 thus cuts the exposed ends of DNA along with DNA replication structures that hinders the process.

Other important participants in ICL repair are the translesion DNA polymerases. The blockage of normal replicative DNA polymerases is done before reaching the ICL regions. Other translesion polymerases in *Xenopus laevis* include Y-family polymerase Rev1 as well as B-family polymerase Pol ζ (Rev3/Rev7) have a significant part in complete removal of ICLs. These models also use replisome remodeling

machinery so that the extension of stalled DNA strand occur on one base before the ICL region [64]. On unwinding, Rev1's deoxycytidyl transferase of Rev1 incorporates cytosine on the complementary strand across the ICL region [65]. This is then succeeded by Pol ζ that extends the unpaired strand.

## 6. ICL lesion removal in quiescent G0/G1 phase

The comprehension of ICL repair is a difficult task because it has an implication on both strands of DNA. The cells in G0/G1 phase do not require homologous recombination for ICL repair [66]. Moreover, all eukaryotic organisms ranging from *Saccharomyces cerevisiae* to the human beings, require NER for the incisions of ICL. The single stranded gap is produced at the first step of NER by the oligonucleotide on ICL lesion. This can be bypassed with the help of translesion DNA polymerases REV1 just like the DNA polymerases (η, ι, κ, and ζ,). Both the DNA polymerases κ, and ζ, as well as REV1 are vital for this stage of NER [67].

## 7. ICL recognition and repair in proliferating S-phase

The repair of ICL faces several complications during the S phase. The data exhibits the formation of double stranded breaks by interaction with ICL causing agents [59]. The ICL induced Double stranded breaks can be repaired by HR rather than non-homologous end joining (NHEJ) method [68]. This brings to the conclusion that ICL-induced DSBs are linked with DNA replication forks. NER indicates ICLs in *S. cerevisiae* and NER function is important for ICL repair. So, all NER-mutants exhibit hyper sensitivity to the ICL causative agents. Contrarily, the cells deficient in *XPF-* as well as *ERCC1-* show immense hypersensitivity to the ICL agents (mitomycin C & nitrogen mustard) in mammals. The product of *XPF* as well as *ERCC1* make up an endonuclease which is hetero-dimeric in nature identifies and incise the single stranded branched structures [69]. Moreover, MUS81-EME1 along with XPF-ERCC1, the homologous structure specific endonucleases are also keen in repairing the ICL lesions [70]. MUS81-EME1 is notable for its binding with the double-stranded branched structures, flaps at 3′ end, as well as Holliday junctions [71]. Either of the two XPF-ERCC1 and MUS81-EME1 are responsible for ICL-induced double strand formation. Since, a multitude of nucleases are recognized recently being the key players in ICLs incision, the mechanism underlying the process need to be explored. We abridge the current knowledge about the ICL repair mechanism in S phase. HR repairs the ICLs induced DSBs. An experiment conducted in *S. cerevisiae*, gives an outline of hypersensitivity against ICL causative agents in *rad51*, *rad52, rad54*, *rad59*, as well as *mre11* mutants but not in case of *yku70* mutants. The hypersensitivity of *rad52 yku70* double mutants to ICLs is at par with that of rad52 mutants [72]. The HR deficient strains show the increase in accumulated DSBs successively on treating with ICL inducers as there lacks an ability to cure DSB which means that NHEJ is not a pre-requisite to remediate DSBs stimulated by ICLs. The mammals follow the same process in their cells. The HR deficient cells depict hypersensitivity against ICLs like cells having mutated paralogs of *RAD51*, *RAD54*, *RAD54B*, along with *BRCA2*, while it is not observed in cells deficient in NHEJ [73]. It significantly highlights the role of HR in repairing DSBs and re-initiating the halted replication forks of DNA. Fanconi anemia (FA) genes are key players in the remediation of ICL in eukaryotes. The proper role of FA gene products in biochemical reactions are still not identified properly, but are notable for their control of HR at the replication forks of DNA [74].

## 8. Interstrand crosslinks lesion repair mechanisms

Lesions in interstrand crosslinks epitomize an arduous challenge in genome maintenance pathways due to the compromise of genomic information present on both strands. Therefore, an application of non-damaged strand as a template for accurate repair in straightforward cut and patch mechanism is not feasible. In this regard, ICL repair employs the concerted and synchronized interaction of dynamics from numerous mechanisms of DNA damage repair, including NER, homologous recombination, mismatch repair, translesion synthesis, ataxia telangiectasia, Rad3 related and Fanconi anemia pathway. **Figure 1** illustrates the schematic mechanism of ICL repair [75].

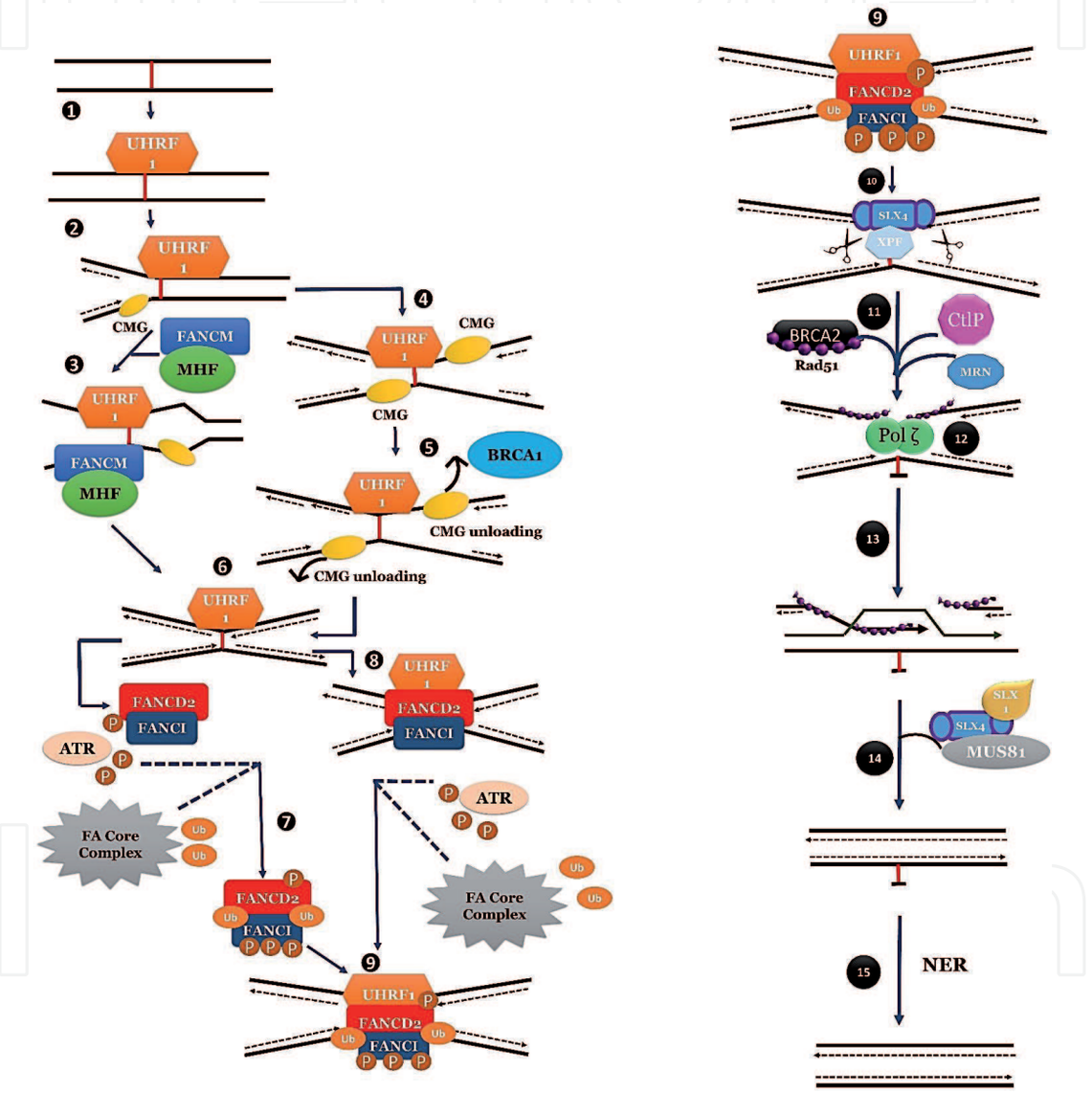


**Figure 1.**
*Schematic of ICL repair mechanism. (1) After the formation of ICLsin the cells, UHRF1 is recruited through its SRA domain immediately. (2) Single replication fork reaches at ICL. (3) Then Replication machinery is transversed through ICL by the help of FANCM/MHF complex and allowes the ICL for later repair. (4), (5) On an alternate basis FANCS or BRCA1 allows the unloading of CMG helicase complex, when second replication fork arrives at ICL. (6) Then replicative polymerase reaches at −1 position of ICL, leaving X shaped similar to the transverse mechanism. (7) Then ATR allows the phosphorylation of FANCD2/FANCI complex at multiple sites and meanwhile FA core complex mono-ubiquitinate at FANCD2/FANCI complex at K561 and K523 respectively. (8) The complex is then recruited to ICL at the replication fork. (9), (10) This ubiquitinated complex recruits SLX4/XPF on ICL in order to unhook the ICL. (11) Afterwards, CtIP an MRN complex resect the double strand breaks and BRCA2 facilitates the formation of RAD51 filament on single stranded DNA generated by resection. (12) Then Polζ carry out the polymerization step through the unhooked ICL. (13) Rad51 then facilitates the invasion of strand with subsequent extension of the other strand. (14) Lastly SLX4 and nucleases resolve the Holliday junction (15) and NER repair proteins remove the damaged nucleotides.*

### 8.1 Role of homologous recombination in ICL repair

The phenomenon of homologous recombination repair (HRR) employs homologous DNA sequences as template for repair and tolerance of DNA lesions that obstruct DNA replication in S-phase. Homologous recombination usually encompasses four step (i) double strand break recognition tailed by nucleolytic processing to produce 3′ single stranded ends of DNA, (ii) protein-mediated strand invasion of single-stranded DNA with homologous chromosome (iii) synthesis of DNA which regenerates degraded DNA using undamaged homologous chromosome as a template and (iv) resolution of Holliday junction intermediates. Usually the platinum drugs drive fruitful results in the treatment of BRCA1- and BRCA2- associated ovarian cancers [76]. However, the protein products of these two genes give rise to HR-mediated repair of DNA damage. A dynamic combination of BRCA1 and associated RING domain protein 1 (BARD1) exhibits ubiquitin ligase activity that is essential for the proper localization of RAD51, which is a central player in Homologous Recombination repair. Through BRCA2 mediated interaction with RAD51, it is specifically targeted to sites where recombination is initiated [77]. However, RAD51-deficient cells represent hypersensitivity towards ICL-inducing agents.

In this regard, the model organism, *Escherichia coli* has provided deep insights in the mechanisms involved in HRR of bacteria. Usually, RecA of bacteria has proven to be an effective protein in all major aspects of HRR due to its ability of forming nucleoprotein filament with both single and double stranded DNA. In *E. coli,* RecBCD complex- combination of nuclease/helicase, initiates the phenomenon of recombination by creating 3′-terminal single-stranded DNA substrate for the activity of RecA protein. RecBCD complex usually binds to the end of linear double stranded DNA and RecA in combination with single-stranded binding proteins (SSBP) allows an incessant formation of presynaptic filament on DNA. This nucleoprotein complex allows a rapid and efficient search for homology within the double-stranded DNA recipient, with subsequent formation of a joint molecule. After the formation of joint molecule, DNA PolI regenerates the sequence and the resultant Holliday junction is resolved by the action of RuvC protein that acts in concert with RuvAB proteins to coordinate the steps of branch migration and Holliday junction resolution [78].

In *Saccharomyces cerevisiae*, the incision of DNA is carried out by an anonymous nuclease. A yeast homologue of RecA, Rad51 works in conjunction with Rad52 dislocates the single-stranded DNA that is ostensibly covered by RPA. The subsequent nucleofilament works with Rad54 and Rad55/57 in DNA unwinding and strand annealing between donor DNA and incoming Rad51 nucleoprotein. The resolution of subsequent recombination intermediates is frequently carried out by assorted set of mechanisms including mus81-mms4 nuclease and Resolvase A [79].

### 8.2 Translesion DNA synthesis in DNA interstrand crosslinks

Translesion DNA Synthesis polymerases are considered essential for ICL repair in both S/G2 and G1 to bypass an ICL unhooked from one of the two cross-linked strands. The phenomenon of Translesion synthesis encompasses multiple polymerases with a dynamic ability to carry out an insertion of nucleotide across the lesion and others carrying out further extension. Based on genetic and biochemical studies, an assortment of polymerases has been implied in repair of ICLs. Usually translesion synthesis is a threefold step: (i) release of replicative polymerase after an interruption of normal bidirectional DNA with lesion, (ii) release of specialized translesion polymerase onto a site and starts the replication at a short distance past the lesion, (iii) the replacement of translesion polymerase with replicative DNA polymerase which continues the normal process of replication [80].

For HR-mediated repair of replication-dependent DSB and excision of ICL from the genome, this is vital to generate an intact template. In this regard, an assortment of polymerases allows the bypass of unhooked ICLs in vitro by using cross-linked DNA substrate model. In *Escherichia coli,* PolIV can easily bypass the unhooked ICLs of $N^2$-$N^2$-guanine in a non-mutagenic manner [81]. A set of human TLS polymerases entail Pol η, Pol ι, Pol κ, REV1, and Pol *ν* that tend to insert the complementary bases or evade anatomically varied ICLs. Competencies of such polymerase-catalyzed reactions is contingent upon the structure of ICL and the amount of double-stranded DNA around ICL.

The role of TLS polymerases in ICL repair is strongly supported by the study of genetics. In yeast, mutations in genes encoding subunits of Polζ i.e. Rev3, Rev7 or REV1 render cells hypersensitive to cross-linking agents [72]. Polζ is majorly important for the cross-linking resistance of non-replicating cells. However, to date *in vitro* studies have not been able to show bypass of ICL damage by Pol ζ-REV1, thus suggesting the other factors involved in lesion bypass. However, Pol η mutants are not sensitive for cross-linking agents [82].

In mammals, Pol ζ (comprising of REV3 and REV7 subunits) and REV1 are significant factors in ICL repair. However, the cells deficient in any of the aforementioned genes are highly sensitive to various cross linking agents [83]. REV1 act as TLS polymerase scaffold and thus facilitates the polymerase exchange with additional deoxycytidyl transferase activity that is involved in insertion of dCMP residues opposite to ICLs.

### 8.3 FA proteins and ICL repair

All Fanconi Anemia patients usually indicate hypersensitivity to cross-linking agents, signifying that FA pathway plays an indispensable role in distinguishing, beckoning or repair of lesions generated by agents. However, the precise role of FA proteins in response to ICLs is still in its infancy. FA pathway tends to participate in both replication-dependent and independent pathways of ICL repair. After an exposure of FA cells withy cross-linking agents, they accumulate chromosomal breaks and radial chromosomes [84] which is an outcome of defects in cellular responses to ICLs.

After recognition of ICL and signaling cell cycle arrest, FA pathways function to coordinate the repair of ICL. Approximately, thirteen Fanconi anemia proteins are essential for resistance against ICLs and the clampdown of chromosomal stability. Eight FA proteins tend to form a nuclear protein complex in order to mono-ubiquitylate FancD2 and FancI. This event is crucial for the cellular resistance to ICL agents. Disruption in FA core complex and ID complex tend to decrease ICL repair efficiency [85]. The depletion of FANCD2 prevents identification of post-incision product i.e. double-strand breaks (DSB). The programmed DSB that is promoted by FANCI-FANCD2 complex majorly leads to the formation of Rad51 filaments and thus allows subsequent repair via Homologous recombination. Notably, FA pathway has been associated with proteins involved in HDR, TLS and Nucleotide excision repair. However, the exact role of FA proteins in HDR provides a vague notion. Though, there exists an interaction between the conduits of FA-BRCA, as FANCD1 exhibits homology with BRCA2 and for this reason, numerous proteins of FA pathway unswervingly interact with BRCA1 and BRCA2. In this way, it is believed that FA pathway donot play a significant role in all Homology Directed repair mechanisms (HDR), because of having a role in the recruitment of repair proteins in ICL damage. Certainly, *in vitro* analysis recommend that FANCD1/BRCA2 play a momentous role in ICL repair [86]. FANCD2 allies with the Mre11-Rad50-Nbs1 (MRN) complex, that is considerably crucial for incision of DNA strands during double-strand breaks (DSBs), a preliminary step of all homology dependent processes [87].

In response to cross-linking agents, FANCD2 has been exposed to co-localize with Nucleotide Excision Repair component, XPF that affects the solidity of ubiquitylated FANCD2. After replication arrest, FANCD2 has also been shown to co-localize with Rev1 [88]and core complex components of FA i.e. FANCA and FANCG have been shown to be required for Rev1 foci formation [89]. Because of a dynamic ability to play an indecisive role in HDR and upstream process of TLS and NER, FA pathway orchestrates and regulate such repair mechanisms for a suitable removal of ICL damage. In this way, inactivation of FANCD2 affect both nucleolytic incision and translesion synthesis [90]. Recent investigations have examined the role of FA pathway in ICL repair by means of DNA substrates carrying site-specific ICLs in the supernatants of Xenopus.

Having a DNA substrate containing MMC-like ICL adducts significantly distorts DNA helix. The other study has stated that ICL repair can proceed through replication dependent and independent mechanisms [85]. In nutshell, ICL repair could take place in an absence of DNA replication in *Xenopus* extracts and upon transfection of an ICL- containing plasmid in G1-arrested mammalian cells is consistent with accumulating evidence for ICL repair in G1.

### *8.3.1 RUNX poly(ADP-ribosyl)ation and BLM interaction facilitate the Fanconi anemia pathway of DNA repair*

Fanconi anemia is considered as a universal genome maintenance network that orchestrates the repair of DNA interstrand crosslinks (ICL). The tumor suppressors RUNX1 and RUNX3 have been shown to regulate the FA pathway independent of their canonical transcription activities, by controlling the DNA damage dependent chromatin association of FANCD2. RUNX3 usually modifies by PARP-dependent poly(ADP-ribosyl) ation which in turn allows RUNX binding to DNA repair structures lacking transcription-related RUNX consensus motifs. After DNA gets damage, the increased interaction between RUNX3 and BLM facilitates the efficient FANCD2 chromatin localization. The mutations of RUNX-Walker motif in breast cancers have been impaired for DNA damage-inducible PARylation, thus unveiling an impending mechanism for FA pathway inactivation in cancers [91].

## 8.4 Suppression of NHEJ reduces ICL sensitivity

Even though Homologous Recombination promotes repair of double strand break in S-phase, an alternative mechanism, Non-homologous end joining (NHEJ) also exist to repair damaged DNA in all phases of the cell cycle. The phenomenon of NHEJ employs a simplest mechanism of splicing to rejoin the free end of DNA. The process involves the binding of KU70-KU80 heterodimers to the free double-stranded ends of DNA, thus allows the binding of DNA-dependent kinase subunit (DNA-PKcs) and initiates the activation of downstream steps [92]. DNA is processed to remove 5′-or 3’-ssDNA tails and the subsequent ends are directly rejoined by the activity of DNA ligase IV-XRCC4. Unlike HRR, in which homologous sequences proofread the repair process, NHEJ generates deletions, insertions and translocations in case of joining of incorrect ends.

In past, researches on mice and yeast has stated the notion that human cell lines defective in factors of Non-homologous end joining i.e. KU70, KU80, Ligase, DNA-PKcs or XRCC4, donot exhibit hypersensitivity towards ICL-inducing agents [93]. However, recent analysis has indicated that inhibition of NHEJ pathway in cell lines of FA patients can reduce the toxicity of ICL-inducing agents. For instance, in a knockout model of chicken or nematode, specific FA-like defects can be salvaged by the co-deletion of ligase IV or KU70. Moreover, through simultaneous inhibition

of NHEJ by PKcs inhibitor, NU7036 in FANCA- and FANCD2- deficient human cell lines, the high sensitivity to MMC can be rescued easily. Through analysis of mitotic spreads in these cell lines, a rare sight of uncharacteristic radial chromosomes was observed. These annotations direct that a key purpose of the FA conduit in repair of Interstrand crosslink lesions, is to subdue the forged ligation of ICL-induced Double Strand breaks amid non-homologous chromosomes.

HR and NHEJ pathway provides the complementary functions in the repair of *de novo* double strand breaks and the co-inhibition of these repair pathways leads to increased cell death [94]. However, Fanconi Anemia cells are not defective in HR per se, so the inhibition of NHEJ in FA cells still allows them to proliferate and repair double strand breaks. This is mainly due to the reason that FA pathway mainly endorses HR at stalled replication forks through stabilization of intermediate that is a prerequisite for unhooking and TLS. If still the replication fork is not stabilized, HR can befall but the generated free end of DNA likes to bound by KU70-KU80, as it has a very high affinity for the structures [95]. By inhibition of NHEJ pathway, the less active and less toxic FA-independent HR pathway can re-establish the replication fork.

## 9. Conclusion

The development of interstrand cross-links play a chief role in the mechanism of significant chemotherapeutic agents. Emerging evidences suggest that these ICL lesions may also be formed by environmental agents and unwanted byproducts of metabolic processes. A better understanding of these lesions could lead to the improvement of supplementary therapeutic agents and strategies. However, despite the efforts of considerable investigations, the mechanism of ICL repair is still an enigma. At the transcriptomic level, proteins involved in a number of repair pathways have been identified. However, the detailed analysis of conditions in which repair should occur is largely elusive. What's clear is that a repair of interstrand-cross links in eukaryotes involves multiple factors from NER and HRR pathways. Given the state of activities, it is ostensible that diverse experiments need to be done before we get a vivid picture of this important repair mechanism.

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