Advances in Understanding the Complex Mechanisms of DNA Interstrand Cross-Link Repair

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DNA interstrand cross-links (ICLs) are lesions caused by a variety of endogenous metabolites, environmental exposures, and cancer chemotherapeutic agents that have two reactive groups. The common feature of these diverse lesions is that two nucleotides on opposite strands are covalently joined. ICLs prevent the separation of two DNA strands and therefore essential cellular processes including DNA replication and transcription. ICLs are mainly detected in S phase when a replication fork stalls at an ICL. Damage signaling and repair of ICLs are promoted by the Fanconi anemia pathway and numerous posttranslational modifications of DNA repair and chromatin structural proteins. ICLs are also detected and repaired in nonreplicating cells, although the mechanism is less clear. A unique feature of ICL repair is that both strands of DNA must be incised to completely remove the lesion. This is accomplished in sequential steps to prevent creating multiple double-strand breaks. Unhooking of an ICL from one strand is followed by translesion synthesis to fill the gap and create an intact duplex DNA, harboring a remnant of the ICL. Removal of the lesion from the second strand is likely accomplished by nucleotide excision repair. Inadequate repair of ICLs is particularly detrimental to rapidly dividing cells, explaining the bone marrow failure characteristic of Fanconi anemia and why cross-linking agents are efficacious in cancer therapy. Herein, recent advances in our understanding of ICLs and the biological responses they trigger are discussed.

Interstrand cross-links (ICLs) are lesions that covalently link two bases on the complementary strands of DNA. These lesions are formed by chemicals with two reactive electrophilic groups. The formation of ICLs is highly sequence-dependent because two nucleophilic

groups on opposite strands must be aligned geometrically to enable the bifunctional cross-linking agent to react twice. A consequence of this complex chemistry is that cross-linking agents form not only ICLs but also monoadducts, DNA-protein cross-links, and intrastrand cross-

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links. This makes it challenging to study the biological impact of ICLs except via methods using site-specifically adducted synthetic duplex oligonucleotides.

ICLs prevent separation of the two DNA strands, which is a prerequisite for transcription and replication. Hence, ICLs act as an absolute block to essential cellular processes and are particularly detrimental to rapidly dividing cells. This has led to the extensive use of cross-linking agents as potent anticancer therapies. Remarkably, in contrast to these exogenous ICLs, the existence of endogenous ICLs has never been formally proven in mammalian tissues, likely because only a few ICLs can be tolerated by a cell. Indeed, it has been shown that 1–2 ICLs can be lethal to a repair-deficient yeast cell, whereas about 20–40 ICLs are lethal in repair-deficient mammalian cells (Magana-Schwencke et al. 1982; Phillips 1996). This has led to much speculation about the identity of the endogenous lesions that drove the evolution of well-conserved mechanisms of ICL repair.

Pathways of ICL repair are still not completely defined. Historically, mechanistic studies were largely driven by genetics owing to the availability of cell lines specifically sensitive to cross-linking agents and a strong link between defects in ICL repair and several genome instability disorders, most notably Fanconi anemia. ICL repair is presumed to occur via different mechanisms depending on the phase of the cell cycle (i.e., during DNA replication or outside of S/G₂ phase). In the past decade, there has been substantial progress on each of these fronts: identifying endogenous ICLs, developing methods to detect ICL lesions in complex biological samples, elucidating the mechanisms of ICL repair, and exploitation of cross-linking agents in the clinic. This article elaborates recent developments in the study of ICLs and their repair.

ENDOGENOUS ICLs

The best recognized cross-linking agents are all exogenous chemicals, such as the cancer chemotherapeutics nitrogen mustards, cisplatin, or mitomycin C and psoralen, which are used to treat skin disorders. Clearly, we did not evolve

repair mechanisms for lesions induced by drugs developed in the last century. Recently, both synthetic and genetic approaches have been used to identify several sources of endogenous cross-linking agents. These agents arise from normal cellular metabolism and are summarized in Table 1. A number of aldehydes produced endogenously form ICLs in vitro (Stone et al. 2008; Guainazzi and Schärer 2010; Huang et al. 2010a; Garaycochea et al. 2012). Endogenous production of aldehydes is strongly influenced by ingestion of dietary lipids and alcohol. This illustrates that endogenous DNA damage burdens can be altered through dietary changes. Abasic sites are extremely abundant endogenous lesions caused by spontaneous hydrolysis of the glycosidic bond in DNA. They exist in equilibrium between a ring-open aldehyde and ring-closed hemiacetal. The former is able to form ICLs by reacting with the exocyclic amino group of adenine or guanine residues on the opposite strand (Dutta et al. 2007; Guan and Greenberg 2009; Johnson et al. 2012b). These represent a potentially tremendously important class of endogenous ICLs because of the abundance of abasic sites. Nitric oxide, a signaling molecule important for vasoregulation that is produced as a by-product of nitrous acid, can cross-link guanine residues on the opposite strands (Kirchner et al. 1992; Guainazzi and Schärer 2010). Nitrous acid is a by-product of nitrates used in preservation of processed meats, again linking endogenous DNA damage burdens with diet.

METHODS TO SYNTHESIZE, DETECT, AND QUANTITATE ICLs

Synthesis of site-specific lesions in duplex DNA has been essential for defining the chemical and biological impact of specific DNA adducts. This approach is especially critical in the study of ICLs because all cross-linking agents, when used to create random damage in DNA, induce a myriad of lesions, of which ICLs are typically rare (<10%). A major recent advance in the field has been the development of improved strategies to synthesize site-specific ICLs in duplex oligonucleotides (reviewed in Guainazzi

Table 1. Endogenous sources of DNA interstrand cross-links

ICL-inducing compounds	Target in DNA	Synthetic model	Endogenous sources	References
Aldehydes: <i>Trans</i> -4-hydroxynonenal, acetaldehyde, malondialdehyde, acrolein, formaldehyde, crotonaldehyde	5'-GC—non-distorting; 5'-CG—distorting	Stabilized trimethylene ICL between two N ² -G	Lipid peroxidation; metabolism of dietary components including coffee, ripe fruit, and alcohol	Stone et al. 2008; Huang et al. 2010a; Garaycochea et al. 2012
Nitric oxide, nitrous acid	5'-GC < 5'-CG—distorting	Nitrous acid-induced ICL between two N ² -G	Cell signaling; acidification of dietary nitrates	Shapiro et al. 1977; Harwood et al. 2000
Oxidized abasic lesion: 5'-(2-phosphoryl-1,4-dioxobutane)	A on the opposite strand; 3' to the abasic site	Photolabile precursor built into an ss oligonucleotide	Hypoxic conditions	Guan and Greenberg 2009
Ring-open aldehyde form of an abasic site 3' to a C residue	G opposite the C	AP site built into an ss oligonucleotide; ICL stabilized by reduction with NaCNBH ₃	Spontaneous hydrolysis of purines or BER repair intermediates	Dutta et al. 2007; Johnson et al. 2012a

ICL, interstrand cross link; ss, single strand; BER, base excision repair.

and Schärer 2010, and summarized in Tables 1 and 2). There are currently methods to synthesize site-specific ICLs arising from endogenous aldehydes or nitric oxide as well as common chemotherapeutic agents such as platinum drugs and nitrogen mustards (see Table 2). This has led to advances in our knowledge about the stability of ICLs and the amount of helical distortion these lesions introduce in DNA. This, in turn, may be important for determining whether these lesions are recognized by DNA repair machinery and are, therefore, repaired outside of S/G₂ phase of the cell cycle.

ICLs are challenging to detect and measure in biological samples. In addition to their relative rarity compared with other types of lesions, many ICLs are unstable and do not withstand isolation methods (Stone et al. 2008; Johnson et al. 2012b). Also, ICLs have a potent impact on cells that are replicating or transcribing their DNA. Hence, ICLs are not abundant in viable cells. Methods used to detect ICLs in cells or tissue samples include denaturing electrophoresis and, more recently, mass spectrometry and alkaline COMET assay. These methods and their strengths and weaknesses are elaborated in Table 3.

The creation of localized damage in subnuclear domains of cells is the *in vivo* equivalent of site-specific lesions. Local damage has been extremely useful for studying the recruitment of DNA repair proteins to sites of DNA damage (Volker et al. 2001). This enables identification of the sequential steps of repair mechanisms. The method depends on the use of a laser to induce a narrow path of DNA damage. 4,5'-8-trimethylpsoralen intercalates into DNA and can react with two nucleophilic groups upon photoactivation with UV-A. This was elegantly exploited for the study of ICL repair by creating psoralens conjugated with visible dyes or doxigenin (Thazhathveetil et al. 2007). Monolayers of cells are treated with conjugated psoralens followed by photoactivation with a UV-A laser, creating covalent DNA adducts, including ICLs, only in the path of the laser. Alternatively, a near infrared laser can be used for two photon activation of psoralen (Duquette et al. 2012).

Mass spectrometry (MS) is the most sensitive and specific method for detecting and quantifying DNA lesions. The only limitation is that, ideally, one should include an isotopi-

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Table 2. Common cross-linking agents and their target sequences

ICL-inducing agent or group	Target sequence	Example agents ^a	Site-specific adduct available for study?	Cancers ^a treated with agents	References
Nitrogen mustards	5'-GNC	Cyclophosphamide, Melphalan, Mechlorethamine, Chlorambucil, Ifosfamide, Bendamustine	Ring-opened formamido-pyrimidines with improved stability are used to cross-link dsDNA	Lymphoma, multiple myeloma, melanoma, ovarian, CLL, NSCLC	Ojwang et al. 1989; Millard et al. 1990; Rink and Hopkins 1995; Guainazzi et al. 2010
Platinum compounds	5'-GC	Cisplatin, Carboplatin, Oxaliplatin, Satraplatin, Picoplatin	Cisplatin reacted with an oligonucleotide containing a unique guanine can be annealed to complementary ssDNA	Testicular, ovarian, NSCLC, ovarian, colorectal, prostate, breast	Jamieson and Lippard 1999
Mitomycin C	5'-CG		Efficient formation of ICLs following treatment of duplex DNA with MMC	Esophageal, bladder	Tomasz 1995
Psoralen	5'-TA	Furocoumarins from plants and fungi	Psoralen intercalates into DNA and requires UV-A photoactivation to covalently bind DNA	Cutaneous T-cell lymphoma	Cimino et al. 1985; Thazhathveetil et al. 2007
Chloro-ethyl nitrosoureas	G-C base pair	Carmustine	3-(2-chloroethyl) thymidine can be synthesized in an oligonucleotide and annealed to complementary sequence ^b		Fischhaber et al. 1999; Hentschel et al. 2012

CLL, chronic lymphocytic leukemia; NSCLC, nonsmall cell lung carcinoma.

^aThe color coding indicates which tumor is treated with which drug.^bThymidine was more stable than adducts containing either guanine or cytosine.

cally labeled internal standard throughout sample processing to enable subtraction of artificial generation or loss of DNA damage. Thus, although MS has been applied to the measurement of chemotherapy-induced ICLs, it has not yet been applied to endogenous lesions because of their elusive identity and/or their chemical instability.

The COMET assay is frequently used to measure a variety of types of DNA lesions and

DNA repair intermediates. The method is unique in that it can be applied to single cells. Cell membranes are lysed and the nuclear DNA spread by electrophoresis under alkali conditions, creating a “comet tail” pattern of DNA. Longer tails represent more breaks in the chromosomal DNA, whereas shorter tails represent DNA that cannot be unraveled. This can be used to indirectly measure ICLs that prevent DNA unwinding.

Table 3. Methods to detect and measure ICL lesions and their repair

Method	End point measured	Advantages	Disadvantages	References
Local damage with psoralen + UV-A	Covalent addition of psoralen to chromatin in a portion of a cell nucleus	Used to identify proteins that co-localize with ICLs to determine the order of events during ICL repair	Can only be used in cells cultured in monolayers; cannot prove the lesions are ICLs	Thazhathveetil et al. 2007; Majumdar et al. 2008
Mass spectrometry	Levels of mitomycin C or other specific ICL lesions in genomic DNA	Highly specific if using tandem MS; highly sensitive if using isotopically labeled internal standard; applicable to cells, tissues, or body fluids	Limited to a single lesion per analysis and those for which synthetic standards are available	Paz et al. 2008
Alkaline COMET assay	DNA damage that restricts the electrophoretic mobility of DNA	Single-cell measurement of DNA damage; sensitive	Subjective quantification; cannot be used on lesions that are unstable in alkali; cannot be used on tissues; genome contains multiple types of DNA damage	Olive et al. 1991; Wu et al. 2009; Spanswick et al. 2010

GENES IMPLICATED IN ICL REPAIR

A large number and diverse spectrum of genes are implicated the response to and repair of ICLs. These have largely been defined by deleting the gene and determining if the deletion renders cells sensitive to cross-linking agents (Table 4). Another way in which these genes have been identified is by defining new complementation groups of Fanconi anemia (FA). FA is a heterogeneous disease characterized by congenital anomalies, bone marrow failure, and high risk of acute myeloid leukemia (Auerbach 2009), currently consisting of 16 complementation groups. The FA proteins work coordinately to facilitate replication-dependent ICL repair (see below and Fig. 1). Intriguingly, virtually every protein that plays an enzymatic role in ICL repair also plays a role in at least one other DNA repair mechanism. This has made it extremely challenging to decipher the specific biological effect of ICL lesions (i.e., it is impossible to knock out ICL repair completely and uniquely). Conversely, defects in most of the

other DNA repair/tolerance mechanisms (homologous recombination, nucleotide excision repair, mismatch repair, translesion synthesis, and base excision repair) render cells hypersensitive to cross-linking agents. This often cannot be completely ascribed to the fact that cross-linking agents generate a variety of lesions in addition to ICLs, and implicates proteins from each of these pathways in the repair of ICLs.

In general terms, ICL repair occurs through sequential excision of the lesion from one strand, then the other. This prevents the creation of multiple double-strand breaks (DSBs). During replication, the ICL is thought to be unhooked from the lagging strand template via two incisions 5' and 3' of the incision (Fig. 2). The 5' cut creates a DSB, which must be repaired by HR to reestablish the replication fork. HR-mediated repair of this single DSB entails DNA end resection to create a 3' overhang able to invade and capture sequence information from the lagging strand template. Before that can happen, a translesion polymerase

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Table 4. Genes associated with ICL sensitivity

Gene name	Homologs	Function in ICL repair	ICL repair mechanism
<i>FANCA</i>	Mouse (<i>Fanca</i>)	Core complex member	Replication based
<i>FANCB</i>	Mouse (<i>Fancb</i>)	Core complex member	Replication based
<i>FANCC</i>	Mouse (<i>Fancc</i>)	Core complex member	Replication based
<i>FANCD1/BRCA2</i>	Mouse (<i>Brca2</i>)	Loading RAD51 onto DNA	Replication based
<i>FANCD2</i>	Mouse (<i>Fancd2</i>); <i>Drosophila</i> (<i>Fancd2</i>); <i>C. elegans</i> (<i>fdc-2</i>)	DNA binding; promotes DNA damage signaling and repair protein recruitment	Replication based
<i>FANCE</i>	Mouse (<i>Fance</i>)	Core complex member	Replication based
<i>FANDF</i>	Mouse (<i>Fancf</i>)	Core complex member	Replication based
<i>FANCG</i>	Mouse (<i>Fancg</i>)	Core complex member	Replication based
<i>FANCI</i>	Mouse (<i>Fanci</i>); <i>C. elegans</i> (<i>fnci-1</i>); <i>Drosophila</i> (<i>Fanci</i>)	DNA binding; promotes DNA damage signaling and repair protein recruitment	Replication based
<i>FANCJ/BRIP1/BACH1</i>	Mouse (<i>Brip1</i>); <i>C. elegans</i> (<i>dog-1</i>)	3'-5' DNA helicase; preferred substrate is branched DNA	Replication based
<i>FANCL</i>	Mouse (<i>Fancl</i>); <i>Drosophila</i> (<i>Fancl</i>)	Ubiquitin ligase responsible for monoubiquitination of FANCD2-FANCI	Replication based
<i>FANCM</i>	Mouse (<i>Fancm</i>); <i>Drosophila</i> (<i>Cg7922</i>); <i>S. cerevisiae</i> (<i>MPH1</i>); <i>S. pombe</i> (<i>mfh1</i>); Archae- <i>Haloferax volcanii</i> (<i>Hef</i>)	5'-3' translocase; branch migration activity; binds DNA in a structure-specific manner and recruits the core complex along with BLM; involved in activation of checkpoint	Replication based
<i>FANCN/PALB2</i>	Mouse (<i>Palb2</i>)	Assists in BRCA2 localization to DNA	Replication based
<i>FANCO/RAD51C</i>	Mouse (<i>Rad51c</i>); <i>Arabidopsis</i> (<i>RAD51C</i>); <i>C. elegans</i> (<i>rad-51</i>) ^a ; <i>Drosophila</i> (<i>Spn-D</i>); <i>S. cerevisiae</i> (<i>DMC1</i>);	Involved in homologous recombination	Replication based
<i>FANCP/SLX4/BTBD12</i> SLX4 binds SLX1	Mouse (<i>Slx4</i>); <i>Drosophila</i> (<i>Mus312</i>) <i>C. elegans</i> (<i>slx4/him-18-slx1</i>) <i>S. cerevisiae</i> (<i>SLX4</i>); <i>S. pombe</i> (<i>slx4-eme1/mms4</i>)	Scaffold protein for endonucleases	Replication based
<i>FANCQ/ERCC4/XPF</i> XPF hetero-dimerizes with ERCC1	Mouse (<i>Xpf-Ercc1</i>); <i>Arabidopsis</i> (?- <i>ERCC1</i>); <i>C. elegans</i> (<i>xpf-1 ercc-1</i>); <i>Drosophila</i> (<i>Mei-9 Ercc1</i>); <i>S. cerevisiae</i> (<i>RAD1-RAD10</i>); <i>S. pombe</i> (<i>rad16-swi10</i>)	Structure-specific endonuclease with a preference for 3' flaps	Replication based and non-replication based

Continued

Table 4. Continued

Gene name	Homologs	Function in ICL repair	ICL repair mechanism
<i>APITD1/MHF1</i> <i>STRA13/MHF2</i> <i>C19orf40/FAAP24</i>	Mouse (? , ? , <i>Faap24</i>); <i>S. cerevisiae</i> (<i>MHF1</i> , <i>MHF2</i> ,?)	Accessory factors for FANCM	Replication based
<i>USP1/WDR48-UAF1</i>	<i>C. elegans</i> (<i>uaf-1</i>)	Deubiquitination of FANCD2	Replication based
<i>FAN1</i>	Mouse (<i>Fan1</i>); <i>C. elegans</i> (<i>fan-1</i>)	5'-3' exonuclease, 5'-flap endonuclease. Binds to monoubiquitinated FANCD2	Replication based
<i>MUS81-EME1</i> MUS81 hetero-dimerizes with EME1	Mouse (<i>Mus81-Eme1</i>); <i>Arabidopsis</i> (<i>MUS81-?</i>); <i>C. elegans</i> (<i>mus-81</i> <i>f56a6.4</i>); <i>Drosophila</i> (<i>Mus81-Mms4</i>); <i>S. cerevisiae</i> (<i>MUS81-MMS4</i>); <i>S. pombe</i> (<i>mus81-eme1</i>)	Structure-specific endonuclease with a preference for 3' flaps	Replication based
<i>SNM1A</i>	<i>C. elegans</i> (<i>mrt-1</i>); <i>S. cerevisiae</i> (<i>SNM1/PSO2</i>)	5' exonuclease	Replication based
<i>SNM1B</i>	<i>S. cerevisiae</i> (<i>SNM1/PSO2</i>)	5' exonuclease	Replication based
<i>BRCA1</i>	Mouse (<i>Brca1</i>); <i>Arabidopsis</i> (<i>BRCA1</i>); <i>C. elegans</i> (<i>brc-1</i>); <i>Drosophila</i> (<i>Muc14A</i>); <i>S. cerevisiae</i> (<i>RAD18</i>)	Histone remodeling via ubiquitin ligase activity targeting H2A and CTIP	Replication based
<i>RAD51</i>	Mouse (<i>Rad51</i>); <i>Arabidopsis</i> (<i>RAD51</i>); <i>C. elegans</i> (<i>rad-51</i>) ^a ; <i>Drosophila</i> (<i>Spn-A</i>); <i>S. cerevisiae</i> (<i>RAD51</i>); <i>S. pombe</i> (<i>rhp51</i>);	Homology search for template DNA during homologous recombination; promotes strand exchange	Replication based
<i>REV1</i> DNA polymerase ζ DNA polymerase ν	Mouse (<i>Rev1</i> , <i>Rev3l</i> , <i>Poln</i>); <i>Arabidopsis</i> (<i>REV1</i> , <i>ATREV3</i> ,?); <i>C. elegans</i> (<i>rev-1</i> , <i>y37b11a.2</i> ,?); <i>Drosophila</i> (<i>Rev1</i> , <i>Mus205</i> ,?); <i>S. cerevisiae</i> (<i>REV1</i> , <i>REV3</i> ,?); <i>S. pombe</i> (<i>rev1</i> , <i>rev3</i> ,?)	Translesion polymerases required for bypass of an ICL	Replication based and non-replication based
<i>HELQ</i>	Mouse (<i>Helq</i>)	Helicase required for ICL repair	Replication based
<i>BLM</i>	Mouse (<i>Blm</i>); <i>C. elegans</i> (<i>him-6</i>); <i>Drosophila</i> (<i>Blm</i>); <i>S. cerevisiae</i> (<i>SGS1</i>); <i>S. pombe</i> (<i>rqh1</i>)	DNA helicase (5'-3') important for Holliday junction dissolution and inhibition of RAD51 strand invasion	Replication based
<i>RMI2</i>	Mouse (<i>Rmi2</i>); <i>S. cerevisiae</i> (<i>RMI2</i>)	Coordinates with BLM for Holliday junction resolution	Replication based
<i>MRE11</i>	Mouse (<i>Mre11a</i>); <i>C. elegans</i> (<i>nrx-1</i>); <i>Drosophila</i> (<i>mre11</i>); <i>S. cerevisiae</i> (<i>MRE11</i>); <i>S. pombe</i> (<i>rad32</i>)	Component of the MRN complex (<i>MRE11-RAD50-NBS1</i>), role in homologous recombination	Replication based

Continued

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Table 4. *Continued*

Gene name	Homologs	Function in ICL repair	ICL repair mechanism
<i>NBS1</i>	Mouse (<i>Nbn</i>); <i>C. elegans</i> (<i>xnp-1</i>); <i>Drosophila</i> (<i>Nbs</i>); <i>S. cerevisiae</i> (<i>XRS2</i>); <i>S. pombe</i> (<i>nbs1</i>)	Component of the MRN complex (MRE11-RAD50-NBS1), role in homologous recombination	Replication based
<i>RAD50</i>	Mouse (<i>Rad50</i>); <i>C. elegans</i> (<i>rad-50</i>); <i>Drosophila</i> (<i>Rad50</i>); <i>S. cerevisiae</i> (<i>RAD50</i>); <i>S. pombe</i> (<i>rad50</i>)	Component of the MRN complex (MRE11-RAD50-NBS1), role in homologous recombination	Replication based
<i>ATR</i>	Mouse (<i>Atr</i>); <i>C. elegans</i> (<i>atl-1</i>); <i>Drosophila</i> (<i>Mei-41</i>); <i>S. cerevisiae</i> (<i>MEC1</i>); <i>S. pombe</i> (<i>rad3</i>)	Kinase important for signaling DNA damage to activate cell-cycle checkpoints through sensing of single-stranded DNA	Replication based
<i>CTIP/RBBP8</i>	Mouse (<i>Ctip</i>); <i>C. elegans</i> (<i>com-1</i>); <i>S. cerevisiae</i> (<i>SAE2</i>); <i>S. pombe</i> (<i>ctp1</i>)	End resection during homologous recombination	Replication based
<i>TOPIIIα</i>	Mouse (<i>TopIIIα</i>); <i>C. elegans</i> (<i>top-3</i>); <i>S. cerevisiae</i> (<i>TOP3</i>)	Topoisomerase involved in relaxing supercoiled DNA during homologous recombination	Replication based

Collis et al. 2006; Penkner et al. 2007; Youds et al. 2008; Meier et al. 2009; dos Santos and Van Houten 2010; Harris et al. 2010; Lestini et al. 2010; Yan et al. 2010a; Cherry et al. 2011; Deans and West 2011; McQuilton et al. 2012.

? Denotes no known homolog for one member of a complex.

^aIn *C. elegans*, there are two isoforms of rad-51 that are orthologous to *S. cerevisiae*, Rad51 and Dmc1.

must extend leading strand synthesis past the unhooked ICL to create a duplex molecule amenable to HR. Similarly, in nonreplicating cells, ICLs are thought to be repaired via unhooking from one strand, likely via nucleotide excision repair, followed by translesion synthesis (TLS) to fill the gap, then a second round of NER to completely remove the lesion. More details are provided in the following sections.

FA Pathway

Many of the genes that are important for protecting the genome from ICLs were discovered as encoding Fanconi anemia (FA) proteins. FA is a rare genetic disease characterized by congenital skeletal and renal anomalies, growth retardation, and bone marrow failure all of varying severity, and a high risk of acute myeloid leukemia. Diagnosis of FA is based on challenging the patient's cells with a cross-linking agent and measuring chromosome aberrations, in particular radial chromatid structures, indicative of faulty ICL repair. Currently, there are 16 FANC com-

plementation groups: *FANCA*, *FANCB*, *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ*, *FANCL*, *FANCM*, *FANCN*, *FANCO*, *FANCP*, and *FANCQ*. Many of the proteins encoded by these genes are important for cell signaling in response to replication stress caused by DNA damage (Fig. 1). Other proteins are directly implicated in DNA repair. For example, *FANCD1/BRCA2* is required for homologous recombination, whereas the most recently identified complementation group (*FANCQ*) encodes XPF, an endonuclease essential for nucleotide excision repair (NER) and ICL repair (Bogliolo et al. 2013; Kashiya et al. 2013). The FA pathway plays an important role in DNA damage sensing and signaling during S/G₂ phase of the cell cycle in cells with ICLs (Fig. 1).

When a replication fork encounters an ICL, polymerization is arrested. This leads to the recruitment of *FANCM* to the stalled fork, where it binds unwound DNA. *FANCM* is a highly conserved member of the XPF-heterodimeric 3'-flap endonuclease family, but does not have nuclease activity as a result of changes in active

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Figure 1. (Continued) Structure-specific endonucleases: MUS81-EME1 and ERCC1-XPF/FANCD2-FANCI are recruited to the damage site via interaction with FANCP/SLX4. The core complex and FANCD2-FANCI are recruited to the chromatin where they facilitate resolution of the repair intermediate by TLS polymerases (REV1-Pol ζ -Pol η) and the homologous recombination machinery, including FANCD1/BRCA2, RAD51, FANCN/PALB2, FANCO/RAD51C, and BRCA1. (B) Details of the protein-protein interactions important for recruitment of FA proteins to sites of DNA damage. The core complex is recruited to chromatin via interaction of FAAP20 with RNF8 and the FANCC interaction with BRCA2/FANCD1. RNF8-UBC13 polyubiquitinates histone H2A, marking the site of ICL damage. FANCD2 and FANCI are recruited to chromatin via interaction between FANCD2 and FANCE of the core complex. FAAP20 also interacts with the TLS polymerase REV1. Dissolution of the complex is dependent on UAF1/USP1 deubiquitinating FANCD2 and FANCI. (C) Events that occur independently of the FA core complex. Recruitment of FANCM, MHF, and FAAP24 to stalled forks does not require the core complex to be present or monoubiquitination of FANCD2 or FANCI. In addition, RPA loading and ATR activation do not require the core complex members. Finally, homologous recombination, in particular the formation of 53BP1 and BRCA1 foci, FANCD2 recruitment to the chromatin, DSB end resection by MRN-CtIP, and RAD51 loading on the resected end, do not require the core complex.

site residues (Niedernhofer 2007). FANCM is recruited to chromatin with FAAP24 (another homolog of ERCC1, the heterodimeric partner of XPF), which assists FANCM binding to ssDNA (Ciccio et al. 2007), and a histone-fold protein complex called MHF, which stimulates replication fork remodeling (Yan et al. 2010b). Remodeling is accomplished by the ATP-depen-

dent translocase activity of FANCM, which promotes migration of Holliday junctions and replication fork branch points (Gari et al. 2008a,b; Xue et al. 2008; Rosado et al. 2009). FANCM, FAAP24, and MHF are part of the FA core complex and are required for downstream events, including FANCD2 monoubiquitination (see below). Recent evidence suggests that the func-

Figure 2. Current model for replication-dependent ICL repair. As a replication fork approaches an ICL, the fork stalls 20–40 nucleotides from the lesion. Two incisions are made on the lagging strand template. The first incision creates a single-ended double-strand break. The identity of the nuclease making this incision is currently not known. Then FANCP/XPF-ERCC1 completely unhooks the ICL from the lagging strand template with a second nick. Both endonucleases are recruited to the damage site by FANCP/SLX4. Now translesion polymerases are able to bypass the unhooked ICL using the leading strand as a primer. Different TLS polymerases may be required to bypass various unhooked ICLs, whereas Pol ζ-REV1 is adept at extending mismatches created by bypass insertion. This DNA synthesis is required to enable homologous recombination-mediated repair of the broken end.

tions of FANCM and FAAP24 are not fully epistatic in ICL repair (Wang et al. 2013).

FANCM-dependent translocation causes accumulation of RPA, the ssDNA-binding protein, at the ICL damage site (Huang et al. 2010b; Vare et al. 2012). RPA localization to chromatin is required for ATR activation and activation of the DNA damage checkpoint (Zou and Elledge

2003; Ben-Yehoyada et al. 2009). ATR, once activated, phosphorylates CHK1 in response to ICL damage (Cui et al. 2009), leading to activation of the kinase activity of CHK1 and blocking entry of the cell into mitosis. Checkpoint activation in response to ICLs during DNA replication requires the presence of the FA core complex (detailed below) but may also occur

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independently of replication (Ben-Yehoyada et al. 2009; Shen et al. 2009a).

ATR and its downstream kinases phosphorylate several FA proteins, amplifying damage signaling. FANCE is phosphorylated by CHK1 (Wang et al. 2007). FANCD2 and FANCI are phosphorylated by ATR (Andreassen et al. 2004; Smogorzewska et al. 2007). ATR also phosphorylates and activates the MRN complex (MRE11-RAD50-NBS1), which must resect the DSB created when the ICL is excised from the replication fork to generate long 3' overhangs. These overhangs are necessary for the initiation of HR-mediated repair of the broken fork. The MRN complex interacts with CtIP, which is also required for DNA end resection (Sartori et al. 2007). Depletion of CtIP impairs recruitment of γ H2AX, RPA, ATR, and FANCD2 to local sites of ICL damage (Duquette et al. 2012), suggesting that end resection is an early event in ICL repair and critical for damage signaling.

The FA core complex includes FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM, FAAP20, FAAP24, and MHE. However, only FANCM, MHE, and FAAP24 have actually been shown to bind DNA (Fig. 1). Correct core complex assembly is necessary for proper downstream signaling, such as monoubiquitination of FANCD2 and FANCI, and HR-mediated restoration of the replication fork. The majority of the core complex members function to stabilize the complex via important protein–protein interactions. For example, FANCE nuclear localization depends on its interaction with FANCC (Léveillé et al. 2006). In addition, FANCB and FANCL interact and the FANCL–FANCA interaction is dependent on FANCB, FANCG, and FANCM, but not FANCC, FANCE, or FANCF (Kitao et al. 2006). In addition, FAAP20 is important for maintaining core complex stability, in particular through its interaction with FANCA and FANCD2 via the UBZ domain of FAAP20 (Yan et al. 2012). Some subunits of the core complex interact with other factors involved in ICL repair, for example, FAAP20 interacts with the translesion polymerase REV1 (Kim et al. 2012; Leung et al. 2012).

Once the FA core complex is assembled, phosphorylated FANCD2–FANCI is mono-

ubiquitinated by the FANCL subunit of the core, which is an E3 ligase (Smogorzewska et al. 2007). Maintenance of the posttranslational modifications of FANCD2 and FANCI depends on both proteins being modified (Smogorzewska et al. 2007). Monoubiquitination of FANCD2 and FANCI is clearly a critical step in ICL repair as evidenced by the high conservation of these proteins (Collis et al. 2006; Deans and West 2009; Lee et al. 2010; Sugahara et al. 2012) and the fact that deubiquitination is required to complete ICL repair (Kim et al. 2009). FANCD2 monoubiquitination appears to be upstream of its chromatin translocation, as these events can be uncoupled (McCabe et al. 2008; Bhagwat et al. 2009). FANCD2 monoubiquitination does not depend on nucleolytic processing of the ICL (McCabe et al. 2008; Bhagwat et al. 2009) but is required for it (Knipscheer et al. 2009).

Recruitment of the FA core complex to chromatin is mediated by several protein–protein interactions (Fig. 1B). For example, recruitment of the core protein FAAP20 to chromatin requires polyubiquitination of histone H2A near the site of ICL damage, which is executed by RNF8-UBC13 (Yan et al. 2012). Recruitment of FANCD2^{Ub}–FANCI^{Ub} to the chromatin is dependent on the interaction of FANCD2 with core protein FANCE (Léveillé et al. 2006). Once FANCD2 translocates to the chromatin it promotes histone H3 mobility following cross-link damage, a process stimulated by FANCI, suggesting a role for FANCD2–FANCI in chromatin remodeling (Sato et al. 2012).

Several events in this signaling cascade and ICL repair appear to be independent of the FA core (Fig. 1C). These include recruitment of MHF–FANCM–FAAP24 to stalled forks, RPA loading, ATR activation, recruitment of 53BP1–BRCA1, and subsequently FANCI/BRIP1/BACH1 to the chromatin, resection of the double-strand break (DSBs) created at the stalled fork by MRN–CtIP, RAD51 filament formation, and recruitment of FANCD1/BRCA2–FANCN/PALB2 to the DSB. FANCI interacts with MUTL homologs of the mismatch repair pathway, and it has been suggested that this interaction is critical for proper repair (Peng et al. 2007). Finally, USP1/UAF1 is the deubiquitinating enzyme

responsible for removing the monoubiquitin from FANCD2 and FANCI to terminate the DNA damage signaling (Nijman et al. 2005; Cohn et al. 2007; Oestergaard et al. 2007). The entire process of FA signaling, checkpoint activation, and replication restart can take several hours to complete (Vare et al. 2012).

Structure-Specific Nucleases

Excision of DNA adducts typically requires two incisions on the same strand of DNA, 5' and 3' of the lesion. This is not sufficient to completely excise an ICL from DNA, but it is adequate to enable DNA synthesis past the lesion (Ho and Schärer 2010). Hence nucleases play a key role in ICL repair. There are multiple structure-specific nucleases that contribute to ICL repair, including XPF-ERCC1, MUS81-EME1, SLX4-SLX1, and three *exo*/*endonucleases*: FAN1, SNM1A, and SNM1B. At this point in time, it remains unclear precisely which nuclease is required for each of the multiple incisions and resections in ICL repair, and to what extent there is redundancy between the various enzymes.

XPF-ERCC1 is an endonuclease that nicks double-stranded DNA adjacent to a 3' single-strand region. It is essential for NER of bulky monoadducts, but XPF and ERCC1 mutants are significantly more sensitive to cross-linking agents than other NER mutants, suggesting a role in ICL repair distinct from NER. Furthermore, mutating residues in ERCC1 that are critical for interaction with XPA severely compromises NER, but does not affect ICL repair, suggesting that ERCC1-XPF engages in distinct DNA repair pathways through specific protein-protein interactions (Orelli et al. 2010). Recently, XPF was identified as a complementation group of Fanconi anemia (FANCD2) (Bogliolo et al. 2013; Kashiyama et al. 2013). In the absence of XPF-ERCC1, ICL-induced replication-dependent DSBs accumulate (Niedernhofer et al. 2004; McCabe et al. 2008; Vare et al. 2012), indicating that at least one incision occurs at ICL sites in the absence of XPF-ERCC1. Furthermore, chromatin localization of FANCD2 is attenuated in the absence of XPF-ERCC1 (McCabe et al. 2008; Bhagwat et al. 2009), suggesting that nu-

cleolytic processing of ICLs is necessary for the stable recruitment of FA proteins to repair foci. Cumulatively, studies to date indicate that XPF-ERCC1 is required to make one of the incisions near an ICL to unhook the ICL from one of the DNA strands. This is essential for enabling translesion synthesis past the ICL and ultimately HR-mediated replication restart (Al-Minawi et al. 2009). Hence, as stated above, in the absence of this nuclease, DSBs accumulate in replicating cells treated with cross-linking agents. XPF-ERCC1 likely also plays a role in ICL repair outside S/G₂ phase of the cell cycle as part of the NER machinery (see below).

MUS81-EME1 is a highly conserved endonuclease (Table 4) related to XPF-ERCC1. In mammalian cells, deletion of MUS81 or EME1 causes hypersensitivity to cross-linking agents (Abraham et al. 2003; Hanada et al. 2006). The formation of DSBs in response to cross-linking agents is dependent on MUS81-EME1 (Hanada et al. 2006; Hanada et al. 2007; Wang et al. 2011). This suggests that these two endonucleases make incisions on different intermediates in ICL repair, with XPF-ERCC1 possibly acting in the primary sites of ICL repair, and MUS81-EME1 being active at stalled and/or regressed replication. Consistent with this notion, MUS81-EME1 has a defined role in Holliday junction resolution (Chen et al. 2001), establishing complex DNA junctions as a favored substrate for this endonuclease. These studies strongly implicate MUS81-EME1 as important for converting replication forks stalled at ICLs to DSBs to initiate HR-mediated repair.

Another related endonuclease of critical importance to ICL repair is FANCP/SLX4-SLX1 (Kim et al. 2011; Stoeckler et al. 2011). *Slx4*-null (*btbd12*^{-/-}) mice mimic many of the key features of FA (Crossan et al. 2011). SLX4-SLX1 is highly conserved, with homologs in yeast, worms (SLX4/HIM-18-SLX1), *Drosophila* (MUS312), and humans (BTBD12) (Andersen et al. 2009; Fekairi et al. 2009). Like MUS81-EME1, the endonuclease activity of SLX1 is important for Holliday junction resolution during G₂ (Svendsen et al. 2009). SLX4 is likely to have SLX1-independent functions in ICL repair. Cells depleted of SLX4 are hypersen-

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sitive to cross-linking agents and have impaired HR (Muñoz et al. 2009). SLX4 contains a ubiquitin-binding zinc finger (UBZ) that interacts with monoubiquitinated FANCD2 and is required for recruitment of SLX4 to DNA-damage foci and suppression of cross-link sensitivity (Yamamoto et al. 2011). In addition to SLX1, SLX4 binds other nucleases, especially XPF, MUS81, and possibly SNM1B (Andersen et al. 2009; Fekairi et al. 2009; Salewsky et al. 2012). It is this scaffold function, recruiting other nucleases and targeting their activity to sites of ICL repair, which is believed to be the key function of SLX4 in ICL repair (Muñoz et al. 2009). Mutation analysis of SLX4 indicates that the interaction of SLX4 with XPF-ERCC1 is the only interaction that is absolutely essential for ICL repair (Kim et al. 2013).

FAN1 (Fanconi anemia-associated nuclease 1) is a structure-specific endonuclease and 5' exonuclease (Kratz et al. 2010; Liu et al. 2010; MacKay et al. 2010; Smogorzewska et al. 2010). FAN1-depleted cells are hypersensitive to cross-linking agents and the protein associates with monoubiquitinated FANCD2 and FANCI through its UBZ domain (Huang and D'Andrea 2010; Liu et al. 2010), strongly suggesting a role in ICL repair. At which stage in ICL repair FAN1 acts is currently unknown, but it does not appear to be critical for an initial incision. FAN1 deficiency is not associated with FA, as patients with a microdeletion in 15q13.3 (which includes FAN1) have no detectable FAN1 protein yet, do not display any of the characteristic symptoms (Trujillo et al. 2012). Interestingly, patients with point mutations in *FAN1* instead suffer from the chronic renal disease karyomegalic interstitial nephritis (Zhou et al. 2012).

Other nucleases implicated in ICL repair are hSNM1A and B, human homologs of the yeast Snm1/Pso2 protein, named for its identification in screens for mutants sensitive to nitrogen mustard and psoralen. Yeast *SNM1/PSO2* mutants are hypersensitive to cross-linking agents but not UV-C or ionizing radiation, suggesting an exclusive role in ICL repair. Like FAN1, Snm1/Pso2 has 5'-exonuclease and structure-specific endonuclease activity (Tiefenbach and Junop

2012). Mammalian cells have three Snm1/Pso2 homologs SNM1A, B, and C, with C being implicated in end-joining of DSBs (Cattell et al. 2010). SNM1A and SNM1B mutants are hypersensitive to cross-linking agents, and the double mutant is even more sensitive (Yan et al. 2010a), suggesting only partial redundancy in ICL repair. Both proteins have 5' exonuclease activity; however, SNM1A is more active on high molecular weight DNA (Sengerová et al. 2012). Ectopic expression of hSNM1A suppresses the sensitivity of yeast *pso2* mutants to cross-linking agents (Hazrati et al. 2008) showing functional conservation. Biochemical studies suggest that SNM1A might have a role in digesting a duplex around an ICL, thereby possibly generating an intermediate in ICL repair that can be processed more readily by TLS (Wang et al. 2011). Depletion of SNM1A leads to accumulation of ICL-dependent replication-induced DBSs similar to what is observed in ERCC1-deficient cells. Indeed, SNM1A and ERCC1 are epistatic with respect to minor groove ICLs, suggesting that the two proteins act in a common pathway (Wang et al. 2011). SNM1B, in turn, is epistatic with FANCD2 and FANCI (Mason and Sekiguchi 2011) and coimmunoprecipitates with FANCP/SLX4 (Salewsky et al. 2012). These studies strongly implicate both nucleases in ICL repair but what their relative contributions are remains to be established.

TLS Polymerases

One of the major recent advances in our understanding of ICL repair has been the realization that TLS polymerases are essential for ICL repair in both S/G₂ and G₁ to bypass an ICL unhooked from one of the two cross-linked strands. This is vital to generate an intact template for HR-mediated repair of a replication-dependent DSB and excision of the ICL from the genome (see below). Consistent with this notion, a number of polymerases are capable of bypassing unhooked ICLs in vitro using model cross-linked DNA substrates. *E. coli* Pol IV (but not Pol II) can bypass unhooked N²-N²-guanine ICLs in a non-mutagenic manner (Kumari et al. 2008). A number of human TLS polymerases, including Pol η,

Pol ι , Pol κ , REV1, and Pol ν , insert a base opposite and/or bypass structurally diverse ICLs. The efficiency of these polymerase-catalyzed reactions is dependent on the structure of the ICL and the amount of double-strand DNA surrounding the ICL. In general, ICLs embedded in fully duplex DNA are only very inefficiently bypassed by TLS polymerases, whereas those surrounded by only a short duplex (2–5 base pairs) are efficient substrates for polymerases (Minko et al. 2008a; Yamanaka et al. 2010; Ho et al. 2011; Klug et al. 2012).

A role of TLS polymerases in ICL repair is also strongly supported by genetics. In *S. cerevisiae*, mutations in genes encoding Pol ζ subunits Rev3 and Rev7 (McHugh et al. 2000; Sarkar et al. 2006) or REV1 (Larimer et al. 1989; Sarkar et al. 2006) render cells hypersensitive to cross-linking agents. Pol ζ is particularly important for cross-link resistance in nonreplicating cells in this organism (McHugh and Sarkar 2006). However, to date in vitro studies have been unable to show bypass of ICL damage by Pol ζ -REV1 (Minko et al. 2008b; Ho et al. 2011), suggesting that other factors could be involved in lesion bypass. In contrast, Pol η mutants (the only other TLS polymerase in yeast) are not sensitive to cross-linking agents (Grossmann et al. 2001; Wu et al. 2004; Sarkar et al. 2006).

In mammals, Pol ζ (consisting of the REV3 and REV7 subunits) and REV1 are key factors in ICL repair, as cells deficient in either one of these genes are exquisitely sensitive to cross-linking agents (Nojima et al. 2005; Gan et al. 2008). REV1 functions as a TLS polymerase scaffold and facilitates polymerase exchange (Sharma et al. 2013) and has additionally a deoxycytidyl transferase activity that may be involved in inserting a dCMP residue opposite an ICL (Minko et al. 2008b). Pol ζ is unique in its ability to extend from distorted primer-template termini, such as those formed by an insertion of a nucleotide at a lesion by another TLS polymerase.

One reason why mutations in or REV1 or Pol ζ render cells hypersensitive to cisplatin or MMC is that these two enzymes are involved in replication-dependent and -independent ICL repair pathways. The sensitivity to cross-linking agents caused by mutations in the REV3 sub-

unit of Pol ζ is epistatic to mutations in the Fanconi anemia pathway in chicken DT40 cells (Sonoda et al. 2003; Niedzwiedz et al. 2004; Nojima et al. 2005), suggesting that it is a result of defect in replication-dependent ICL repair. A role of Pol ζ and Rev1 in replication-dependent ICL repair is also strongly supported by studies in *Xenopus* egg extracts (see below) (Räschle et al. 2008). In vertebrates, REV1 and Pol ζ are also required for bypass of a psoralen, MMC or cisplatin site-specific ICL in a *nonreplicating* plasmid-based reporter assay (Shen et al. 2006; Enoiu et al. 2012).

The importance of other TLS polymerases for ICL repair is less clear-cut. Human cells deficient in Pol η (XP-V patient cells) are hypersensitive to cross-linking agents such as cisplatin or psoralen (Misra and Vos 1993; Raha et al. 1996; Albertella et al. 2005; Chen et al. 2006; Mogi et al. 2008). Human Pol η can bypass various structurally distinct unhooked ICLs (Ho et al. 2011). Pol η was shown to be involved, but not essential in the repair of plasmid-borne MMC and psoralen ICLs in replication-independent ICL repair (Wang et al. 2001; Zheng et al. 2003). These studies suggest that Pol η has a role in the repair of certain ICLs, but that this role may be at least partially redundant.

Pol κ -deficient cells are hypersensitive to cross-linking agents, in particular the minor groove ICL forming agent MMC, suggesting a role of Pol κ in bypassing minor groove lesions (Minko et al. 2008a; Williams et al. 2012). Consistent with this observation, Pol κ can bypass N^2 - N^2 guanine ICLs model substrates efficiently (Minko et al. 2008b), whereas reduced activity is observed using cisplatin and nitrogen mustard substrates, which are major groove ICLs (Ho et al. 2011). Pol κ is therefore likely to have an important role in the repair of a subset of ICL lesions.

Pol ν -knockdown cells are hypersensitive to MMC (Zietlow et al. 2009; Moldovan et al. 2010). Pol ν interacts with FANCD2-FANCI as well as RAD51, suggesting a role in replication-dependent ICL repair (Moldovan et al. 2010). In vitro, Pol ν efficiently bypasses major groove ICLs and, with very low efficiency, psoralen ICLs (Zietlow et al. 2009; Yamanaka et al.

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2010). The context in which Pol ν might operate in ICL repair remains to be determined.

In summary, our current understanding of TLS polymerases is that REV1 and Pol ζ have essential roles in ICL repair and that other enzymes have minor roles and may contribute to the repair of ICLs with particular structures or in specific pathways or situations.

Homologous Recombination

The importance of HR in the repair of ICLs was established some time ago in lower organisms (*E. coli*, *S. cerevisiae*) (Sinden and Cole 1978a,b; Jachymczyk et al. 1981). More recently, several key mammalian HR proteins were identified as part of the FA pathway, including FANCD1/BRCA2, FANCN/PALB2, FANCI/BRIP1/BACH1, and FANCO/RAD51C. Accordingly, depletion of key proteins required for HR in human fibroblasts causes hypersensitivity to cross-linking agents and the formation of radial structures when exposed to mitomycin C (Hanlon Newell et al. 2008), a diagnostic criterion of FA. Interestingly, the same is true if key proteins required for nonhomologous end-joining of DSBs are depleted (Hanlon Newell et al. 2008).

Once FANCM-FAAP24-MHF unwinds DNA behind a stalled replication fork, ssDNA is exposed, RPA is bound, ATR is activated followed by FA pathway activation, and the HR machinery is recruited to the stalled fork. CtIP coordinates the recognition and resection of DSBs by MRE11-RAD50-NBS1 to create a 3' single-strand overhang amenable to HR (Sartori et al. 2007) and is required for resistance to cross-link damage (Duquette et al. 2012). CtIP is ubiquitinated by BRCA1 and has been shown to accumulate at sites of locally induced ICLs downstream from FANCM (Duquette et al. 2012). It then promotes accumulation of RPA, ATR, and FANCD2 at damage sites.

BRCA2/FANCD1 loads RAD51 onto stalled forks and this process is independent of FA proteins (Kitao et al. 2006; Long et al. 2011). Studies in *Xenopus* reveal that RAD51 is loaded onto single-stranded regions of the stalled fork even before nucleolytic incisions occur (Long

et al. 2011). MCM8 and MCM9 are two replicative helicase-related Mcm family members that form a complex that is required for resistance to cross-linking agents (Nishimura et al. 2012). The proteins form nuclear foci that colocalize with RAD51 after cross-link damage and are required for HR repair of ICL-induced replication-dependent DSBs.

Once incisions are made, the RAD51 nucleofilament promotes strand invasion of the broken end into the intact sister chromatid for HR-mediated repair of the DSB. RAD51 colocalizes with FANCD2 after cross-link damage; however, recruitment of either protein to the chromatin does not depend on the presence of the other (Kitao et al. 2006). RAD51C, encoding a RAD51 paralog, was identified as a Fanconi anemia complementation group (FANCO) (Vaz et al. 2010). FANCO/RAD51C also functions downstream of FANCD2 monoubiquitination and DSB formation, but is essential for HR-mediated repair of ICL-induced replication-dependent DSBs (Somyajit et al. 2012). Additional RAD51 paralogs, in particular XRCC2 and XRCC3, also contribute to the HR step in ICL repair (Liu et al. 1998).

FANCD1/BRCA2 is a single-strand DNA-binding protein that promotes RAD51-dependent strand invasion. FANCN/PALB2 binds BRCA2 to promote strand invasion. FANCI/BACH1/BRIP1 is a 5' to 3' helicase that binds BRCA1. All three of these FA proteins are enriched in the chromatin fraction of cells after cross-link damage during the S/G₂ phase of the cell cycle and chromatin recruitment is independent of the FA core complex (Shen et al. 2009b). BRCA1 also plays a role in DNA damage signaling and nonhomologous end-joining of DSBs. Hence, BRCA1 may facilitate ICL repair through other mechanisms that are independent of HR (Bunting et al. 2012). Restoration of replication forks stalled by ICLs is a slow process requiring several hours (Vare et al. 2012).

MODEL FOR REPLICATION-DEPENDENT ICL REPAIR

Although the cell biology and genetic studies discussed above have provided a list of players

involved in ICL repair, they have not provided a mechanistic basis for the individual steps. In S phase, ICL repair is triggered by the complete blockage of the advancement of the replication fork at an ICL. As a consequence, ICLs show their greatest toxicity during S phase making their repair at that stage critical. Biochemical studies of replication-dependent ICL repair have been challenging, but the use of a biochemical system using plasmids with site-specific ICLs and a defined replication system in *Xenopus* egg extract have provided a basis for a biochemical model (Räschle et al. 2008; Knipscheer et al. 2009; Long et al. 2011) of replication-dependent ICL repair (Fig. 2).

As the leading strand of a replication fork approaches the ICL, it stalls approximately 20–40 nucleotides from the ICL, consistent with a blockage of the replicative helicase, the MCM complex. The 5' ends of the lagging strands also stalls at variable distances from the lesion. This is believed to lead to the disassembly of the replicative helicase and enabling further approach of the replication fork toward the ICL. In the *Xenopus* system and possibly in human cells, two replication forks may converge at the ICL. The basic steps discussed below are likely to be similar whether triggered by one or two replication forks. This close encounter of the replication fork with the ICLs coincides with the activation of the FA pathway as evidenced the ubiquitination of FANCD2/FANCI complex. FA pathway activation is required for further steps in ICL repair in the *Xenopus* system (Knipscheer et al. 2009).

The first step is likely the unhooking of the ICL from the lagging strand template by two incisions on either side of the ICL. This unhooking step is thought to be mediated by two different structure-specific endonucleases with opposite polarities. Although the identity of these enzymes remains to be shown, it is likely that XPF-ERCC1 makes the incision 3' to the ICL on the lagging strand template (Fig. 2). The rationale for this is that ICLs are converted to DSBs in the absence of XPF-ERCC1, suggesting the 5' cut still occurs. In the absence of MUS81-EME1, ICLs are not converted to DSBs, which could be interpreted as a role in

unhooking ICLs. However MUS81-EME1 has the same polarity as XPF-ERCC1, which is incompatible for making the 5' incision on the lagging strand template. Current thinking is that MUS81-EME1 incises stalled or regressed replication forks in a different context, such as converging forks or persistent DNA damage (Wang et al. 2011). Hence, the identity of the nuclease making the incision 5' to the ICL on the lagging strand template remains unclear. FAN1 has the correct polarity but is likely to act at a later step in ICL repair. SLX1 is another candidate, but it is currently unclear how important its role in ICL repair is, because mutating the SLX1-binding site of scaffold protein SLX4 does not render cells hypersensitive to cross-linking agents. Another possibility is that a single incision by XPF-ERCC1 could be followed by an exonucleolytic degradation of the lagging strand around the ICL by SNM1A (Wang et al. 2011).

These incision reactions lead to the formation of a DSB in the lagging strand and an unhooked ICL in the leading strand. The unhooked ICL then provides a template for translesion synthesis. This step likely requires multiple TLS polymerases for DNA synthesis up to the lesion, bypassing the lesion and extending from a potentially mismatched primer template. Based on genetics and depletion studies in the *Xenopus* system, REV1 and Pol ζ are the likely key players in this step, although the identity of the TLS polymerase inserting a dNTP opposite the ICLs and extending past the incision step may be dependent on the structure of the ICL (Minko et al. 2008a; Räschle et al. 2008). After translesion synthesis, the leading strand will be extended (likely by Pol ζ) and ligated to the first downstream Okazaki fragment (Räschle et al. 2008). This yields an intact sister chromatid that can serve as a target for initiation of HR-mediated DSB repair.

Strand invasion by the 3' overhang of the lagging strand template occurs to capture sequence lost at the DSB created during the unhooking of the ICL. RAD51 was shown to have the expected key role in this step using the *Xenopus* system (Long et al. 2011). Interest-

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ingly, RAD51 appears to interact with ICL-containing sites before activation of FANCD2-FANCI, pointing to a tight coordination of all the steps involved. Completion of the recombination step and resolution of HR intermediates is dependent on FANCD2-FANCI. How other FA and HR proteins contribute to the regeneration of an intact replication fork remains to be determined. This process formally generates a fully replicated DNA molecule that still contains an unhooked ICL. This unhooked ICL does not present an obstacle for the completion of S phase and may eventually be removed by NER.

MODEL FOR REPLICATION-INDEPENDENT ICL REPAIR PATHWAY

There is also strong evidence that ICLs are repaired in the absence of DNA replication. Such repair events were initially observed using site-specifically cross-linked reporter plasmids that have no replication origins necessary to allow replication-dependent repair to occur (Wang et al. 2001; Zheng et al. 2003; Shen et al. 2006; Hlavin et al. 2010; Enoiu et al. 2012). Psoralen, MMC, cisplatin, and alkyl ICLs are repaired in this context, which depends on NER proteins and TLS polymerases, in particular REV1 and Pol ζ . Whereas most ICLs are recognized by both global genome NER and transcription-coupled NER, the removal of cisplatin ICL was absolutely dependent on the transcription-coupled NER protein CSB (Enoiu et al. 2012). Based on these observations, it has been suggested that this repair pathway involves two rounds of unhooking the ICL from one strand plus gap-filling DNA synthesis (Fig. 3).

Biochemical studies aimed at determining how the NER machinery and possibly other proteins recognize and incise ICLs have not yet yielded many insights. Also, because in NER the two DNA strands are unwound around the lesion, it is not obvious that NER proteins would be able to make an incision on both sides of an ICL. Indeed, in *in vitro* studies with model ICL substrates and cell-free extracts, NER proteins did not incise cisplatin ICLs (Zamble et al.

1996), whereas with psoralen or alkyl ICL substrates dual incisions are observed only on one side (5' to the ICL) (Bessho et al. 1997; Mu et al. 2000; Smeaton et al. 2008). Whether such intermediates are further processed by exonucleases to facilitate removal, or if additional factors are required to facilitate dual incisions around ICLs, remains to be determined. Interestingly, certain ICLs are incised in an NER-independent manner, but the factors responsible for this alternative incision remain to be identified (Smeaton et al. 2008). It is further possible that additional proteins, for example the mismatch repair complex MutS β , could bind ICLs and modulate or facilitate repair processes (Zhao et al. 2009).

Involvement of NER proteins in replication-independent ICL repair is further supported by studies using locally induced psoralen damage, which clearly showed that the psoralen lesions are repaired in G₁ cells and that the NER proteins XPC, XPB, XPA, and XPF are recruited to damaged sites (Muniandy et al. 2009). Genetic studies revealed that simultaneous depletion of CSB and FANCD2 causes additive sensitivity to cross-linking agents, suggesting that replication-independent ICL repair is critical for reducing the cellular burden of cross-link damage (Enoiu et al. 2012). Similar repair pathways have been described in *S. cerevisiae* and *E. coli*, suggesting that it is evolutionarily conserved (Berardini et al. 1997, 1999; Sarkar et al. 2006).

In summary, there has been an explosion of new information about ICL damage and repair in the last decade. This includes the identification of endogenous lesions and new repair factors. Challenges for the future include detecting and quantifying endogenous ICLs and precisely defining the sequential steps of ICL repair. This likely will require further advances in the analytical tools used to measure both DNA damage and repair. ICL damage is implicated in causing cancer and driving organism aging. ICL repair is implicated in genetic predispositions to cancer as well as resistance to cancer therapy. Hence, continued advances in this field are anticipated to have a significant impact on predicting cancer risk and therapeutic outcomes as well as identifying behavior aspects (diet,

DNA Interstrand Cross-Link Repair

Figure 3. Current model for replication-independent ICL repair. If an ICL occurs in nonreplicating cells, it may be recognized by interfering with transcription or because it induces helix distortion, which is recognized by the nucleotide excision repair pathway. This could trigger recruitment of downstream NER factors including XPF-ERCC1. Incisions around the lesion on one strand of DNA unhook the lesion from that strand. Translesion polymerases can bypass the ICL and fill the gap with new DNA synthesis. This is sufficient to restore double-strand DNA that is free of interstrand cross-links.

exposures, drugs) that promote cancer and aging.

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