Published in final edited form as:
J Cell Physiol. 2009 September ; 220(3): 569–573. doi:10.1002/jcp.21811.

DNA Interstrand Crosslink Repair in Mammalian Cells

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Abstract

DNA damage by agents crosslinking the strands presents a formidable challenge to the cell to repair for survival and to repair accurately for maintenance of genetic information. It appears that repair of DNA crosslinks occurs in a path involving double strand breaks in the DNA. Mammalian cells have multiple systems involved in the repair response to such damage, including the Fanconi anemia pathway that appears to be directly involved, although the mechanisms and site of action remain elusive. A particular finding relating to deficiency of the Fanconi anemia pathway is the observation of chromosomal radial formations. The basis of formation of such chromosomal aberrations is unknown although they appear secondarily to double strand breaks. Here we review the processes involved in response to DNA interstrand crosslinks which might lead to radial formation and the role of the nucleotide excision repair gene, ERCC1, which is required for a normal response, not just to DNA crosslinks, but also for double strand breaks at collapsed replication forks caused by substrate depletion.

Interstrand crosslinks (ICLs) are a potent form of DNA damage in which the strands are covalently linked by a bifunctional chemical. The result is a block of normal DNA replication and transcription. The most readily available template for repair, the opposing strand, is also involved with the damage, complicating error-free repair. Straight forward excision and gap filling, as with nucleotide excision repair (NER), seems to be precluded. The many steps involved in IC makes ICL- inducing agents attractive as chemotheraputic drugs (e.g. cisplatin). However, not all ICLs are products of exogenous chemicals; ICLs can also be created by byproducts of metabolism, including the lipid peroxidation product malondialdehyde (Minko et al., 2008). Thus, we find ICL repair mechanisms in organisms from bacteria through humans.

In *E. coli*, the ICL repair pathway utilizes both nucleotide excision repair (NER) and homologous recombination (HR) (Cole, 1973), apparently acting in a single pathway. UvrABC incises the DNA 5′- and 3′- of the ICL on one strand, and the 5′-exonuclease activity of DNA polymerase I creates a single stranded DNA (ssDNA) region required for RecA-mediated recombination. Strand invasion creates a structure on which UvrABC can act, removing the ICL-containing DNA fragment (Dronkert and Kanaar, 2001). The gap is filled by DNA polymerase I and covalently bonded by polynucleotide ligase.

In *Sacchromyces cerevisiae*, studies have shown many genes are involved in ICL repair. Genetic evidence indicates there are three distinct ICL repair pathways in *S. cerevisiae*, representing ‘NER’, post-replication repair, and HR represented by *SNM1*, *REV3*, and *RAD51* epistasis groups respectively (Grossmann et al., 2001). Apparently early in ICL repair

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double strand breaks (DSB) are formed as intermediates (Jachymczyk et al., 1981). Subsequently, a recombination-dependent step utilizing *RAD51* may act in completing ICL repair, through repair of the DSB (Jachymczyk et al., 1981). This occurs only when a homologous sequence is available.

Post-replication/translesion synthesis utilizing error-prone polymerases such as ζ (*REV3/Rev7*) or η might replicate past the ICL following DSB formation (Jachymczyk et al., 1981). While *rev3* mutants are sensitive to ICL damage, yeast DNA polymerase η mutants show normal sensitivity to ICL, suggesting no role for this bypass polymerase in repair (Grossmann et al., 2001). Less is known about this pathway than NER and HR repair; however, it appears that the pathway allows cells to bypass an ICL as opposed to actually repairing the lesion (Dronkert and Kanaar, 2001).

Yeast *snm1 Δ* mutants are specifically sensitive to ICL (Henriques et al., 1997) but display normal incision (Li and Moses, 2003). They do not, however, resolve the DSB and restore high molecular weight DNA after cross-links (Magana-Schwencke et al., 1982) (Li et al., 2005). Apparently the SNM1 protein, known to be a 5'-exonuclease (Li et al., 2005), acts to modify intermediates of DSB repair. Interestingly, *snm1* mutants show normal processing of mating type, indicating that the DSBs occurring in that pathway are processed normally. This leads to the conclusion that the DSBs arising during the ICL repair process have a specific structure, different from DSBs created in mating type switching, thus requiring different processing.

As noted, ICL repair appears to involve DSB intermediates; the processing of the DSB resulting from ICL repair requires specific activities peculiar to the process. Therefore, while RAD51, for example, is required for repair of DSBs after ionizing radiation or ICLs, SNM1 is required only for ICL DSB repair. Such a comparison illustrates that some components of ICL repair may act in a general DSB response whereas others act specifically. It appears that the DSBs occurring during normal DNA replication use many of the same components that ICL repair utilizes for genome stability and provide a substrate for HR (Ward et al., 2007).

The mammalian ICL repair pathway is more complex than *E. coli* or yeast. Like yeast, ICL repair operates through a DSB intermediate similar to those occurring in S-phase during replication [reviewed in (Patel and Joenje, 2007)]. While NER and HR pathways are implicated in ICL repair (De Silva et al., 2000; Zheng et al., 2003), other proteins, not found in *E. coli* or *S. cerevisiae* are involved. The Fanconi anemia (FA) pathway, as demonstrated by the extreme sensitivity of patients, model organisms, and cell lines to ICLs, is involved in mammalian ICL repair [reviewed in (Kennedy and D'Andrea, 2005; Patel and Joenje, 2007; Wang, 2007)]. Activation of the FA pathway occurs during normal S-phase, likely in response to DSBs or other events during replication, and also occurs with hydroxyurea (HU) treatment which results in stalled replication forks with no ICLs, leading to DSBs (Diffley et al., 2000; Fox, 1985). These observations suggest that DSBs caused by HU (substrate depletion) may be equivalent to those arising from ICL repair. FA patients also exhibit chromosome instability, leading to chromosomal radial formations (Figure 1), levels of which are increased as a result of ICL exposure. In fact, radials are not observed in normal cells without ICL damage at high levels. This implies an overload of the repair system in FA cells. The radial formations may occur as 'quadriradials' showing symmetry of four arms, or as adhesions of less than two complete sets of chromatids. The finding of radials is accompanied by apparent breaks in chromatids (Figure 1).

The participation of multiple repair pathways in ICL repair raises the question of whether there is a single monolithic repair response to ICLs in mammals, or if the pathways are independent, as has been unambiguously shown for yeast (Grossmann et al., 2001; McHugh et al., 2001). While models for a unitary path involving NER, HR and post-replication have been suggested

(Niedernhofer et al., 2005), results from mutant cell lines and siRNA depletion indicate there are multiple independent pathways in mammals (Hanlon Newell et al., 2008; Hemphill et al., 2008; Wang, 2007) (Jakobs, unpublished). In addition, it appears several bypass DNA polymerases, including Pol κ, may be involved (Minko et al., 2008), not just Pol ζ, further complicating interpretaion.

Stages of ICL repair: Recognition and Incision

The first step of ICL repair involves recognition of the damage and incision on DNA near the crosslink (Kumaresan et al., 2007) (Figure 2). Incision at the ICL could occur, before or after bypass, leaving a DSB subject to HR or non-homologous end joining (NHEJ) (Dronkert and Kanaar, 2001). Unlike yeast, where loss of any of several NER proteins leads to ICL sensitivity, only loss of either ERCC1 or XPF, which form a heterodimer, leads to exquisite ICL sensitivity in mammalian cells (Collins, 1993; De Silva et al., 2000). The ERCC1/XPF heterodimer can incise near a crosslink *in vitro* and *in vivo* (Fisher et al., 2008; Kumaresan et al., 2002). ERCC1/XPF also appears to incise crosslinked DNA as measured by the comet mobility assay (Rothfuss and Grompe, 2004). However, other work demonstrates that proteins other than ERCC1 and XPF may play a role in the initial recognition and incision of ICLs (Ahn et al., 2004; Hanada et al., 2006; Thoma et al., 2005). It appears that the Mus81-Eme1 complex is capable of incision at ICLs and cells deficient in the proteins do not create DSBs at stalled replication forks (Hanada et al., 2006). The question arises, if this is the case, why cell lines lacking ERCC1/XPF are so sensitive to ICLs. Possible explanations might rest on the action of ERCC1/XPF in incision in several independent pathways, or alternatively, that ERCC1/XPF has a post-incision function required for several pathways. That is, it is possible ERCC1/XPF may act in incision, but is not strictly required.

Stages of ICL Repair: DSB Formation, Resection, and Strand Invasion

Following recognition and incision, several possibilities exist for ICL repair, based on the pathways involved. The resulting DSBs, from the HR process, stalled replication forks, or activity of Mus81-Eme1 incision, would require resection to allow strand invasion. In addition to function in excision of bulky adducts in NER, ERCC1/XPF has also been shown to trim overhanging non-homologous single-stranded DNA (ssDNA) from HR intermediates to facilitate extension of the duplex DNA recombination intermediate, indicating a post-incision role for ERCC1 (Adair et al., 2000; Niedernhofer et al., 2001; Niedernhofer et al., 2004). Recent work from our group also supports a post-incision function for ERCC1, needed for normal activation of the FA pathway. Interestingly, ERCC1 is required in response to ICL or HU, a potent inducer of stalled replication forks, but with no ICLs (McCabe et al., 2008). This action occurs after γH2AX formation, an early indicator of DSB. These findings suggest a common DSB repair intermediate occurs in ICL repair and HU-caused stalled replication forks, both requiring the subsequent action of ERCC1/XPF for resolution (Figure 2).

The suggestion of a common intermediate arising from ICL or HU-induced stalled replication forks reinforces the view that, though the ICL has been incised, the intermediate remains impassible to replication forks, The model we present (Figure 2) requires bidirectional replication in order to lead to structures that could be intermediates in ICL repair. When a replication fork reaches the incised ICL, replication stalls and the replication fork collapses (Heller and Marians, 2006;Shrivastav et al., 2008), leading to a “chicken foot” formation, which is functionally a DSB. In that case, stalled replication forks would represent the common repair intermediate between ICL and HU damage. One strand of the collapsed fork could be resected, creating a substrate for strand invasion (Figure 2). This is a potential post-incision site for ERCC1 action. It is possible that resection of the arrested fork by ERCC1/XPF would alter

mobility in the gel-based comet assay and therefore appear as ‘incision’ reconciling the action with reported observations.

If extensive homology, perhaps in the form of a homologous chromosome or orthologous gene, is available as a recombination substrate, it may be that extension and bypass of the lesion occurs rapidly, utilizing HR repair mechanisms. If the homologue is damaged or not available, repair might result from strand invasion utilizing microhomology on non-homologous chromosomes (Figure 2). The notion of interaction between non-homologous chromosomes is supported by observations that radial formations after ICL damage are only observed between non-homologous chromosomes (Hanlon Newell et al., 2008). Non-allelic homologous recombination (NAHR) is a well-recognized mechanism utilizing short regions of 90% or greater homology for recombination in the mammalian genome (Gu et al., 2008;Lupski and Stankiewicz, 2005).

Bringing together the idea of fork stalling and the non-homologous chromosome radial formation, the recently described fork stalling, template-switching (FoSTeS) model (Gu et al., 2008; Lee et al., 2007) suggests a mechanism by which stalled replication fork restart might utilize non-homologous chromosomes, utilizing microhomology {Carvalho, 2009#194}. The concept of the FoSTeS mechanism acting in ICL and HU-induced stalled replication fork repair is attractive, but as yet has no supporting evidence for radial formation. RAD18 and RAD5 are known to be required for FoSTeS in yeast (Pages et al., 2008; Zhuang et al., 2008), so testing this model is feasible.

Stages of ICL Repair: End Trimming and Action of the FA Pathway

If the NAHR does not extend to the end of the strand, the resulting unpaired tail could prevent extension. The action of ERCC1/XPF in the trimming of overhanging non-homologous ssDNA from homologous recombination intermediates to facilitate resolution of the recombination intermediate might be required to continue the ICL repair process at this stage (Figure 2) (Adair et al., 2000;Niedernhofer et al., 2001). Such a role for ERCC1/XPF, well down-stream of the incision step, could also represent a basis in addition to resection, for sensitivity of ERCC1 and XPF mutants to ICL damage. Thus end trimming by ERCC1/XPF represents a second potential post-incision function for the ERCC1 protein. In addition, the observation of reduced radials with ERCC1 depletion (McCabe et al., 2008) might be explained by a failure to extend from the limited homology between these interacting non-homologous chromosomes; extension might stabilize this interaction between non-homologous chromosomes.

The inherited disease Fanconi anemia is characterized by several congenital abnormalities. While the phenotype is somewhat variable, patients typically exhibit short stature and other skeletal abnormalities, skin pigmentation abnormalities, bone marrow failure leading to anemia and leukemia, increased risk of solid tumors, and cellular sensitivity to ICL-inducing agents (Bagby and Alter, 2006). There are thirteen identified Fanconi genes: A, B, C, D1/BRCA2, D2, E, F, G, I, J, L, M and N [reviewed in (Patel and Joenje, 2007; Wang, 2007)].

Central to the FA pathway are FANCD2 and its paralog, FANCI, the ID complex (Smogorzewska et al., 2007). FANCD2 monoubiquitination is the marker of activation of the FA pathway and is required for nuclear focus formation, a necessity for normal ICL repair. FANCA, B, C, E, F, G, L and M form a core complex, which is required for monoubiquitination of FANCD2 at lysine 561 by the E3 ligase FANCL, in concert with the E2 subunit UBE2T (Machida et al., 2006). FANCI is monoubiquitinated, like FANCD2, in a core complex-dependent manner on lysine 523 in response to the cell cycle and DNA damage.

The FA pathway acts in maintenance of genome stability after ICL damage. In accord, the FA proteins interact with other DSB repair proteins. FANCD2 co-localizes with BRCA1 in response to DNA damage and at synaptonemal complexes (Garcia-Higuera et al., 2001). The identification of FANCD1 as BRCA2 directly linked the FA pathway and HR pathway (Hirsch et al., 2004). FANCD2 also has been shown to interact in a constitutive manner with FANCD1 (BRCA2) and co-localizes with RAD51 in nuclear foci (Hussain et al., 2004). Another link between DSB repair and FA was uncovered with the identification of FANCN as the partner and localizer of BRCA2 (PALB2) (Reid et al., 2007).

FANCD2 also directly interacts directly with the histone acetylase (HAT) Tip60 (Hejna et al., 2008). Tip60 is known to act in DNA DSB repair and to acetylate ATM prior to auto-phosphorylation of that protein (Ikura et al., 2000; Squatrito et al., 2006; Sun et al., 2005; Wong et al., 2006). Depletion of Tip60 makes cells sensitive to ICLs, as it does for ionizing radiation. Thus the function of FA in DSB repair may involve the remodeling protein, Tip60, acting as a protein acetylase in the FA pathway.

Formation and Suppression of Radials

Chromosome radials are routinely used in the diagnosis of FA, and deficiencies or depletions in FA genes lead to increased radials (Hanlon Newell et al., 2008). Radials are also observed after ICL formation in Bloom syndrome (BS) cells, so the structure is non-specific with regard to the FA pathway. The basis for formation of radials is not well understood although it does appear DSB are required (Hanlon Newell et al., 2008). Deficiencies or depletions for several genes acting in DNA repair, including BRCA1, BRCA2, and RAD51, as well as the NHEJ proteins Ku70, Lig4 and XRCC4, have also been shown to increase radial formation (2004, Bruun et al., 2003, Hanlon Newell et al., 2008). As Rad51 is required for HR, the finding that RAD51 depletion/loss, as well as RAD52 depletion, led to increased radial formation (Hanlon Newell et al., 2008) appears to exclude HR from the basis for radial formation.

Loss of ERCC1 has been shown to be associated with formation of radials (Niedernhofer et al., 2004) in mouse fibroblasts. Work from our group, however, indicates ERCC1 depletion reduces radial formation in normal human fibroblasts (McCabe et al., 2008), as well as ERCC1 co-depletion with FANCA or in FA-A cells, placing ERCC1 upstream of radial formation and FA action (Figure 2), leading to the suggestion that the action of ERCC1 may stabilize an intermediate of ICL repair, prior to the actions of the FA core complex, which would form radials (Figure 2) in the absence of FA function. In the scheme of ICL repair, it appears the FA pathway is a later actor than ERCC1, and deficiencies in FA proteins lead to radials, so the point of formation of radials is placed later than ERCC1 function, but depends on ERCC1 for normal levels (Figure 2).

The model presented indicates that DNA replication would be required for radial formation. This would be in agreement with observations that HU treatment, reducing replication, produces DSBs, but reduced radials (Johnstone et al., 1997). A tantalizing, but unexplained, finding regarding radials formed after ICL damage is that only autosomes are involved in radial formation, and radials do not form between homologous chromosomes (Newell et al., 2004); indeed radials can form between human and mouse chromatids {Hanlon Newell, 2008 #154}. This observation reflects a basic mechanism of radial formation that is not understood.

Conclusions

On the basis of the selected observations noted in this brief review we conclude that: **(A)**, ERCC1 plays a significant post-incision role in ICL repair. This is supported by observations that a normal response to HU, which introduces DSBs, but no crosslinks, requires ERCC1. Given the structure-specific nature of the nuclease function of the ERCC1/XPF heterodimer,

post-incision action might be at the stage of resection or trimming of overhanging DNA ends to allow propagation of HR strand invasion in NAHR; **(B)**, ERCC1 action is prior to the point of radial formation in the ICL repair pathway, and therefore, prior to action of the FA pathway; **(C)**, the molecular mechanisms of radial formation remain a mystery; however, notable specifics regarding radials do emerge: radials result from failure of steps in ICL repair at the point of action of the FA pathway, radials form between non-homologues, and radials are not seen following other types of DNA damage such as UV, but are seen following IR or HU, indicating DSBs are a basis for radial formation.

Acknowledgments

We thank Daniel Pauw and A. Hanlon Newell for skillful assistance. The work from the authors' laboratories was supported by NHLBI Program Project Grant 1PO1HL48546.

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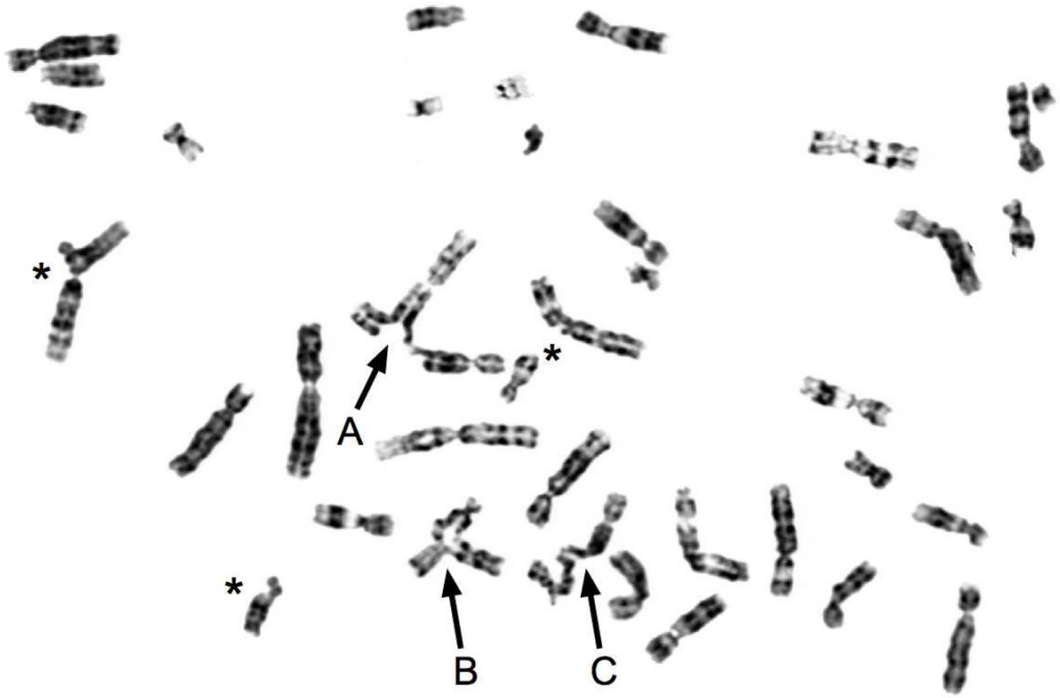

Figure 1.
Metaphase spread demonstrating chromosome radials.

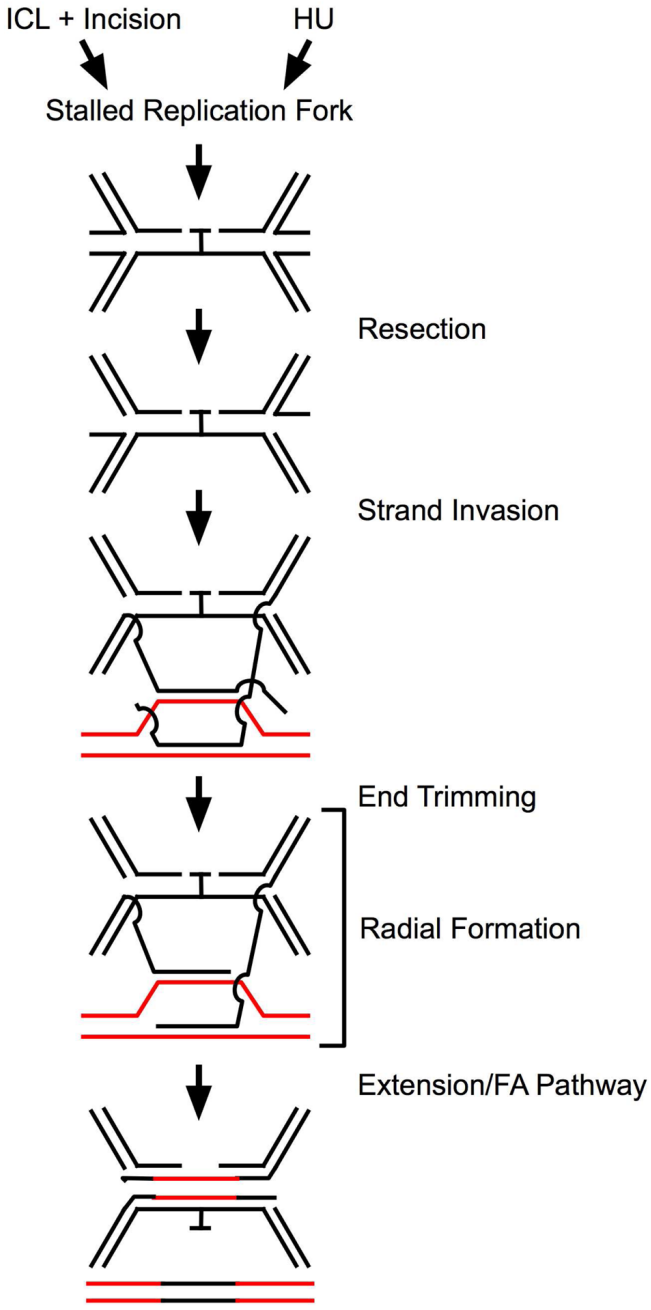

Figure 2. Mechanistic model for ICL repair

The model shows the ICL residue throughout the path, not specifying the point of final removal, and not affecting DSB formation or repair. Neither the ICL nor the accompanying incisions would be present after HU treatment.