THE ROLE OF THE HUMAN EXCISION REPAIR CROSS COMPLEMENTATION (GROUP 1) PROTEIN IN THE FANCONI ANEMIA PATHWAY

By Kevin McCabe

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CERTIFICATE OF APPROVAL

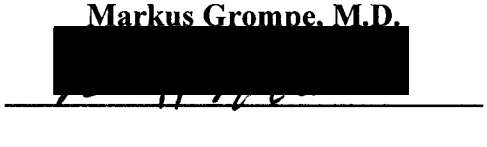
This is to certify that the Ph.D. dissertation of

Kevin McCabe

has been approved

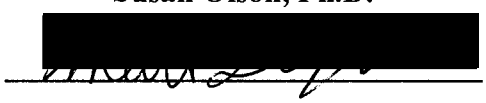
Peter Rotwein, M.D.

Markus Grompe, M.D.



Robb Moses, M.D.

Susan Olson, Ph.D.



Matt Thayer, Ph.D.

Table of Contents

List of Abbreviations:

αMEM	Alpha-modified minimum essential medium
ATM	Ataxia-telangectasia mutated gene
ATR	Ataxia-telangectasia mutated related gene
ATP	Adenosine triphosphate
BACH1	BRCA1-associated C-terminal helicase 1
BLM	Bloom syndrome helicase
BRAFT	Complex containing BLM, RPA, FANCA, C, E, F, and G, and Topo3A
BRCA1	Breast Cancer gene 1
BRCA2	Breast Cancer gene 2
BRG1	brahma-related gene 1
BRIP1	BRCA1 interacting protein C-terminal helicase 1 (aka BACH1 and FANCJ)
BSA	Bovine serum albumin
CHO	Chinese hamster ovary cell line
CO_2	Carbon dioxide
CypB	Cyclophilin B
°C	Degrees Celsius
DAPI	4',6-diamidino-2-phenylindole
DEB	Diepoxybutane
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DSB	Double strand break

dsDNA	Double strand DNA
dsRNA	Double strand RNA
ERCC1	Excision repair cross complimenting rodent repair deficiency, complementation group 1 (aka UV20)
ERCC4	Excision repair cross complimenting rodent repair deficiency, complementation group 4 (aka XPF)
FA	Fanconi anemia
FAAP	Fanconi anemia associated protein
FAAP24	Fanconi anemia associated protein of 24KDa
FAAP95	Fanconi anemia associated protein of 95KDa (aka FANCB)
FAAP250	Fanconi anemia associated protein of 250KDa (aka FANCM)
FANCA	Fanconi anemia protein group A
FANCB	Fanconi anemia protein group B (aka FAAP95)
FANCC	Fanconi anemia protein group C
FANCD1	Fanconi anemia protein group D1 (aka BRCA2)
FANCD2	Fanconi anemia protein group D2
FANCE	Fanconi anemia protein group E
FANCF	Fanconi anemia protein group F
FANCG	Fanconi anemia protein group G (aka XRCC9 and UV40)
FANCI	Fanconi anemia protein group I
FANCJ	Fanconi anemia protein group J (aka BACH1 and BRIP1)
FANCL	Fanconi anemia protein group L
FANCM	Fanconi anemia protein group M (aka FAAP250)

FANCN	Fanconi anemia protein group N (aka PALB2)
FBS	Fetal bovine serum
G_1	Gap 1 phase of the cell cycle
γH2AX	Phosphorylated form (γ) of histone 2A variant X
HR	Homologous recombination
hr	Hour
HRP	Horseradish peroxidase
HU	Hydroxyurea
ICL	Interstrand cross-link
IP	Immunoprecipitation
JPEG	Joint photographic experts group
KCl	Potassium (aka *kalium*) chloride
LDS	Laurel alcohol di-sulfate, lithium salt
µL	Microliter
µM	Micromolar
µm	Micrometer
min	Minute
mL	Milliliter
MLH1	MutL homologue 1
mM	Millimolar
MMC	Mitomycin C
MRE11	Meiotic Recombination 11
MRN	complex consisting of Mre11, Rad50, and Nbs1

mRNA	Messenger RNA
mutS	Mutator group S
NBS1	Nijmegen Breakage Syndrome 1
NER	Nucleotide excision repair
ng	Nanogram
PALB2	Partner and localizer of BRCA2 (aka FANCN)
PBS	Phosphate buffered saline
RAD50	Radiation sensitive mutant 50
RAD51	Radiation sensitive mutant 51
REV3	Reversionless group 3 (aka Polymerase zeta)
RIPA	Radio-immunoprecipitation assay
RISC	RNA-induced silencing complex
rpm	Rotations per minute
ROM	RNA one modulator protein
RNA	Ribonucleic acid
RPA	Replication protein A
SDS	Sodium dodecyl sulfate
sec	Second
shRNA	Short hairpin RNA
siRNA	Small inhibitory RNA
SNM1	Sensitive to Nitrogen Mustard 1 (aka PSO2)
S-phase	Synthesis-phase of the cell cycle
ssDNA	Single stranded DNA

SV-40	Simian virus 40
SWI/SNF	Switch genes involved in mating type switching/Sucrose non-fermenting genes, a complex of 10 proteins involved in chromatin remodeling
TBST	Tris buffered saline with Tween 20
Topo3A	Topoisomerase III A
UV20	Ultraviolet sensitive mutant 20 CHO line (aka ERCC1)
UV40	Ultraviolet sensitive mutant 40 CHO line (aka XRCC9 and FANCG)
UvrABC	Ultraviolet Repair (groups ABC)
V	Volt
XPF	Xeroderma pigmentosum, group F (aka ERCC4)
XRCC9	X-ray repair complementing defective, in Chinese hamster, 9 (aka UV40 and FANCG)

List of Tables:

List of Figures

Acknowledgements

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Table i Specific Acknowledgments	
Aaron Hemphill	Completed the depletions associated with the chromosome stability studies.
Cytogenetics Core: Yasimne Akkari, PhD	Treated and prepared the cells depleted by Aaron Hemphill for standard stain radial analysis, and completed said analysis.
Megan Holtorf	Assisted in blinding of nuclear foci studies.

Abstract

The Fanconi anemia pathway aids in maintaining genomic stability through coordination of several repair pathways in order to respond to DNA interstrand crosslinking (ICL). The hererodimer ERCC1/XPF has been implicated in various contrasting roles in the repair of ICL. Immunofluoresence and immunoblotting was carried out on lysates from ERCC1 siRNA depleted GM639 human immortalized fibroblast cells. We demonstrate hERCC1 is not required for the formation of double strand breaks in response to MMC, DEB, or HU, but is required for hFANCD2 monoubiquitination in response to these clastogens. Additionally, depletion of hERCC1 reduces the level of abnormal chromosome structures known as radials when co-depleted with hFANCA. We conclude hERCC1 acts downstream of H2AX phosphorylation, but upstream of the formation of radials and activation of the FA pathway, both in response to MMC, DEB and HU suggesting a common intermediate produced by these ICL inducing agents and HU induced stalled replication forks. Our work suggests the FA pathway acts not on double strand breaks, but rather, on an intermediate produced in the resolution of collapsed replication forks resulting from ICL inducing agents and stalled replication forks.

Chapter One: Introduction

1.1 Interstrand Crosslinks

Deoxyribonucleic acid (DNA) serves as the heritable blueprint for all functions of living organisms. Being composed of long anti-parallel polymers of nucleotides with a strong sugar-phosphate backbone, DNA is well suited for this role; the order of nucleotides encode the information, the structure is rugged, and the anti-parallel strands hold what essentially is a backup copy of each other. This anti-parallel structure permits faithful duplication of DNA because each strand represents the template for the opposite strand. In a similar fashion, if the information on one strand is damaged, the damage can be removed and the gap filled using the anti-parallel strand as the template.

There are certain chemicals, endogenous and exogenous, that covalently link both strands of a DNA molecule together. There are several classes of these interstrand crosslinking (ICL) agents. Each class of molecule acts on specific nucleotides to create a specific crosslink (see Table 1). These crosslinks typically begin as mono-adducts that then react with the anti-parallel strand. Some of these molecules also create intrastrand crosslinks, and other forms of DNA damage, though it is believed the critical damage produced by these agents is the ICL (Rothfuss and Grompe, 2004, Akkari and Olson, 2004). The specific crosslink created by each molecule creates a different distortion of the DNA double helix. It has been suggested that the distortion created by the ICL or the ensuing chromatin change could be one of the initial signals for repair (Dronkert and Kanaar, 2001).

With the creation of an ICL, no longer can the anti-parallel strands be used as template for one another. ICLs covalently link both strands of DNA together preventing strand separation required for transcription and replication, making ICL inducing agents

Table 1-Protperties of several classes of ICL inducing agents.

ICL Agent	Representative Compound	DNA Sequence Crosslinked	ICL as Percentage of Total Damage	DNA Distortion
Mitomycin C	Mitomycin C	5'-CG-3' / 3'-GC-5'	5-13	Minor
Epoxide	Diepoxybutane	3'-CNG-5' / 5'-GNC-3'	Unknown	Unknown
Psoralen	8-methoxypsoralen	5'-TA-3' \ 3'-AT-5'	30-40	Minor
Platinum Compounds	Cisplatin	5'-GC-3' \ 3'-CG-5'	5-8	Major
Nitrogen Mustards	Nitrogen Mustard	3'-CNG-5' / 5'-GNC-3'	1-5	Major
Nitrosureas	Carmustin	5'-G-3' | 3'-C-5'	<8	Unknown

Adapted from: Dronkert and Kanaar, 2001, Mutat Res, 486, p219

Table 1. A Summary of the properties of several classes of ICL agents, the sequence crosslinked with the location of that crosslink marked with a line between nucleotides, the approximate percentage of ICLs created by the crosslinking agent versus monoadducts and other adducts, and the relative helix distortion created by the different ICL classes.

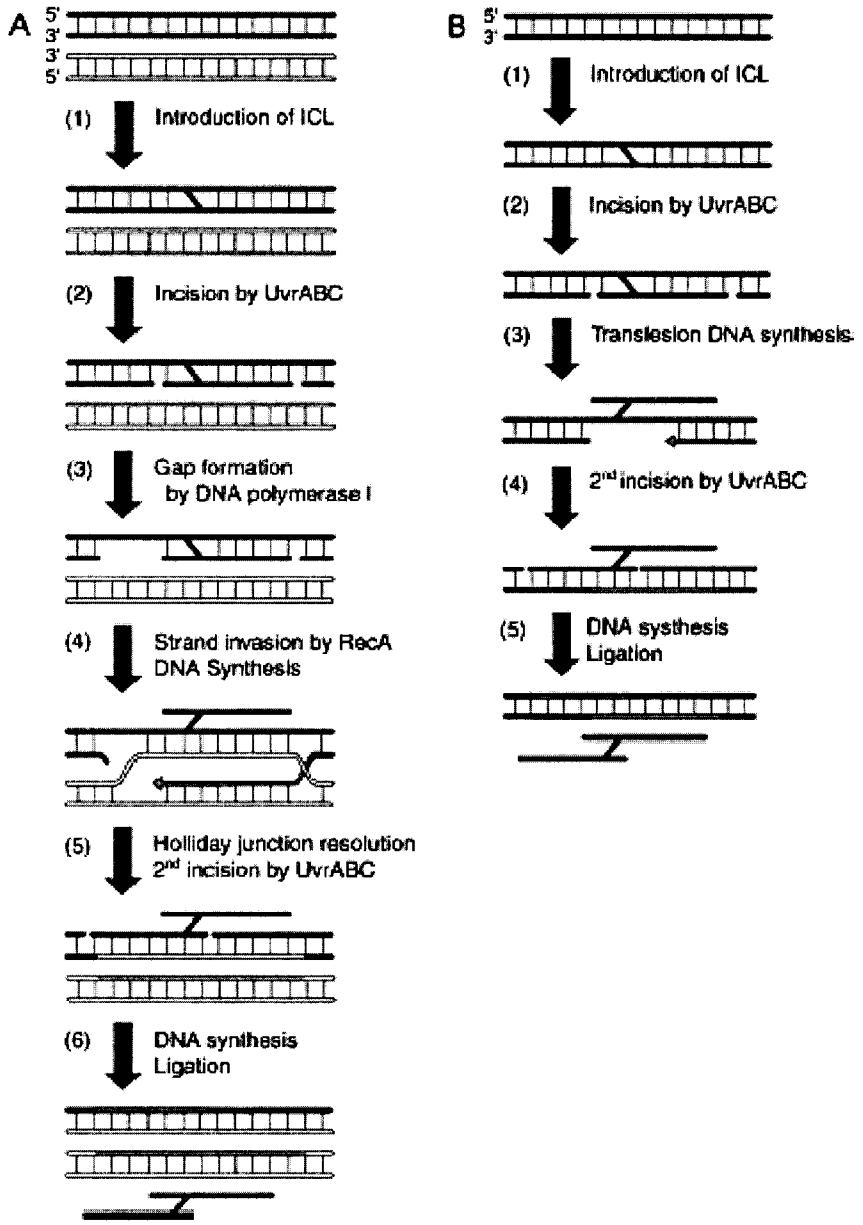
particularly potent killers of cycling cells (Dronkert and Kanaar, 2001). For this reason, many ICL inducing agents are used as chemotherapeutic agents in the treatment of cancer (Lawley and Phillips, 1996, Lord et al., 2002, Ryu et al., 2004).

Simple excision and gap filling, as with repair of monoadducts, is precluded because ICLs covalently join both strands, thus, a more complex method of repair must be utilized (Dronkert and Kanaar, 2001). In *E. coli* the major ICL repair pathway utilizes nucleotide excision repair (NER) followed by homologous recombination (HR) (Cole, 1973) (Figure 1 A). UvrABC incises the DNA 5' and 3' of the ICL on one strand (Figure 1 A2), and the exonuclease activity of DNA polymerase I creates a single stranded DNA (ssDNA) region (Figure 1 A3) required for RecA mediated recombination (Figure 1 A4). Strand invasion creates a structure upon which UvrABC can act again (Figure 1 A5), removing the ICL-containing DNA fragment (reviewed in (Dronkert and Kanaar, 2001). The gap is filled by DNA polymerase I and ligated (Figure 1 A6).

In the absence of a homologous sequence, DNA polymerase II can perform translesion synthesis across the gap created by the initial incision of UvrABC (Figure 1 B2-3). This creates a new substrate for UvrABC which can then remove the ICL (Figure 1 B4). The ensuing gap can then be filled by DNA polymerase I and ligated (Figure 1 B5). This translesion synthesis is mutagenic, as the bypass polymerase inserts bases across from the incised lesion without a proper template (reviewed in (Dronkert and Kanaar, 2001).

In *Sacchromyces cerevisiae*, genetic studies have revealed many of the genes involved in ICL repair in yeast. Genetic evidence indicates the existence of three ICL repair pathways in *S. cerevisiae,* representing NER, post-replication repair, and HR

Figure 1- ICL repair pathways in *E. coli.*



From: Dronkert and Kanaar, 2001, Mutat Res, 486, p222

Figure 1. 1A. *E.coli* ICL repair with homologous DNA. **A1** Introduction of ICL. **A2** Dual incisions on one strand by UvrABC. **A3** Gap formed by exonuclease activity of DNA polymerase I permits **A4** strand invasion by RecA and subsequent extension by DNA synthesis. **A5** Holliday junction is resolved and a second set of dual incisions by UvrABC, followed by **A6** DNA synthesis and ligation. **1B.** *E. coli* ICL repair without homologous DNA. **B1** Introduction of ICL. **B2** Dual incision on one strand by UvrABC. **B3** Translesion bypass DNA synthesis by DNA polymerase II. **B4** Second set of dual incisions by UvrABC followed by **B5** DNA synthesis and ligation.

represented by *SNM1, REV3,* and *RAD51* epistasis groups (Grossmann et al., 2001). In the *RAD51* group, biochemical evidence suggests that early in ICL repair, double strand breaks (DSB) are formed as intermediate following the action of *RAD3* (Jachymczyk et al., 1981). Subsequently, a recombination dependent step utilizing *RAD51* is involved in completing ICL repair, through repair of the DSB (Jachymczyk et al., 1981). This can only occur when a homologous sequence is available.

Post-replication/translesion synthesis utilizing error prone polymerases ζ (*REV3*) or η could replicate past the ICL following DSB formation (reviewed in (Dronkert and Kanaar, 2001). However, polymerase η mutants show normal sensitivity to ICL, demonstrating no role for this polymerase in repair (Grossmann et al., 2001). Little is known about this pathway compared to NER and HR repair; however, it is thought that this pathway helps the cells to bypass an ICL as opposed to actually repairing the lesion (Dronkert and Kanaar, 2001).

snm1Δ mutants are sensitive to ICL (Henriques et al., 1997), but display normal incision (Grossmann et al., 2000). They do not, however, resolve the DSB and restore high molecular weight DNA after cross-links (Magana-Schwencke et al., 1982). *SNM1* also requires MutS factors in the resolution of stalled replication forks in S-phase (Barber et al., 2005). This indicates *SNM1* functions in the later steps of a distinct pathway from HR or post-replication/translesion synthesis in the resolution of stalled replication forks.

The mammalian ICL repair pathway increases in complexity beyond that of *E. coli* and yeast. While NER and HR pathways are still clearly implicated in repair (De Silva et al., 2000), several other proteins, not found in *E. coli* and *S. cerevisiae* are involved. The Fanconi anemia (FA) pathway, as demonstrated by the extreme sensitivity

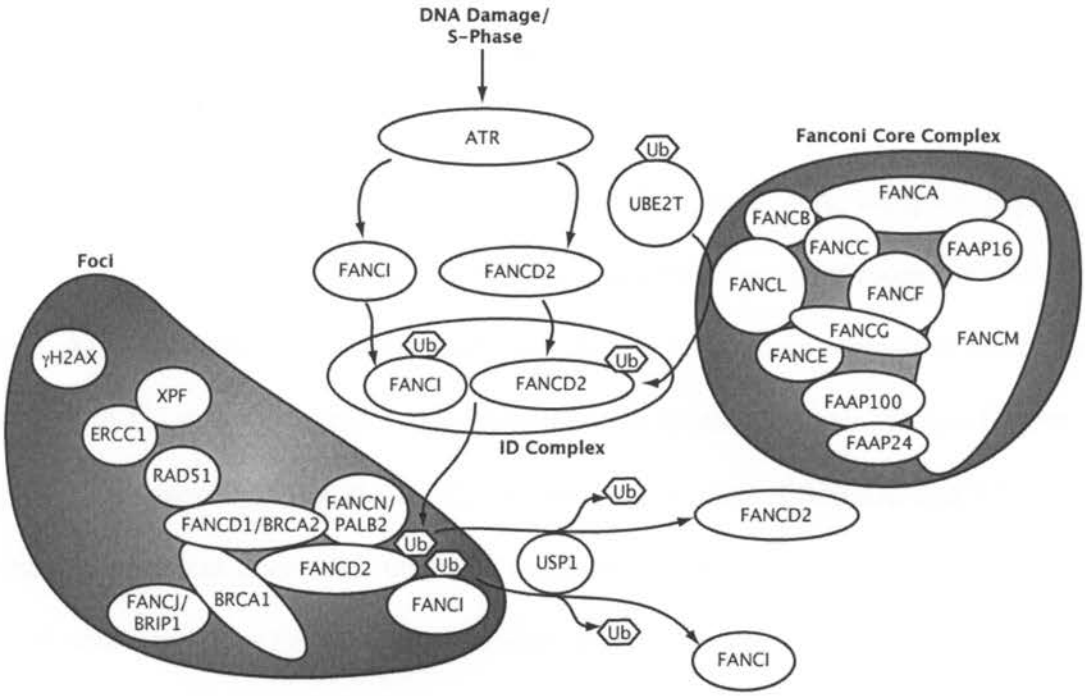
of patients, model organisms and cell lines to ICLs, is intimately involved in mammalian ICL repair (Bagby and Alter, 2006, Collis et al., 2006, Houghtaling et al., 2005, Kennedy and D'Andrea, 2005, Marek and Bale, 2006, Scata and El-Deiry, 2004).

1.2 Fanconi Anemia Pathway and Interstrand Crosslink Repair

Fanconi anemia (FA) is a rare genetic disorder with an incidence of approximately 3/1,000,000 (Tischkowitz and Dokal, 2004). First described by Guido Fanconi in 1927, FA 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 sensitivity to ICL inducing agents (Tischkowitz and Hodgson, 2003). There are a growing number of identified Fanconi genes, of which A, B, C, D1/BRCA2, D2, E, F, G, I, J, L, M and N have been cloned (Reid et al., 2007, Tamary and Alter, 2007, Tischkowitz et al., 2007).

Central to the FA pathway are FANCD2 and its recently characterized paralog FANCI, described recently as the ID complex (Grompe and van de Vrugt, 2007, Smogorzewska et al., 2007) (Figure 2). FANCI was identified in a screen for targets of ATR, whose kinase activity has been shown to be a key regulator of FA pathway activation (Smogorzewska et al., 2007). 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) (Figure 2). The exact role of the other FA core complex proteins in monoubiquitination of FANCD2 is unclear at this point. This post-translational modification occurs in a cell cycle dependant manner in S-phase and in response to DNA damage (Garcia-Higuera et al., 2001). FANCD2 monoubiquitination is traditionally looked upon as the marker of activation of the FA pathway (Machida et al., 2006, Seki et al., 2007).

Figure 2- Diagram of interactions in the Fanconi anemia pathway.



Adapted from: Grompe and van de Vrugt, 2007, Dev Cell, 12, p662

Figure 2. In response to DNA damage resulting in stalled replication forks and during S-phase, the FA pathway is activated leading to FANCD2 and FANCI monoubiquitination and localization to nuclear foci in a core complex dependent manner. USP1 regulates this activation through deubiquitination of FANCD2. (Details are discussed in greater detail throughout the text.)

Monoubiquitination is required for FANCD2's localization to chromatin and formation of nuclear foci, which will be discussed in greater detail later (Garcia-Higuera et al., 2001) (Figure 2).

FANCI is monoubiquitinated, like FANCD2, in a core complex dependent manner on lysine 523 in response to cell cycle and DNA damage (Smogorzewska et al., 2007). As with FANCD2, a lysine mutation at the site of monoubiquitination is extremely sensitive to ICLs and prevents FANCI's function in crosslink repair (Garcia-Higuera et al., 2001, Smogorzewska et al., 2007). Of particular interest, FANCD2 and FANCI are mutually dependent upon each other for monoubiquitination. Additionally, when USP1 (which deubiquitinates FANCD2) is inhibited, levels of both ubiquitinated FANCD2 and FANCI increase, pointing to a further level of coordinated regulation (Nijman et al., 2005, Smogorzewska et al., 2007) (Figure 2).

While no biochemical function has been ascribed to FANCD2, much is known about its interacting partners. FANCD2 colocalizes with BRCA1 in response to DNA damage and at synaptonemal complexes (Garcia-Higuera et al., 2001) (Figure 2). Additionally, FANCD2 has been shown to interact in a constitutive manner with FANCD1/BRCA2 and co-localizes with RAD51 in nuclear foci (Hussain et al., 2004) (Figure 2). FANCD2 and NBS1 interact in two distinct complexes to regulate the S-phase checkpoint and mitomycin-C sensitivity, FANCD2, NBS, ATM and MRE11, and FANCD2, NBS1, MRE11 and Rad50 respectively (Nakanishi et al., 2002). Cumulatively, these interactions suggest that the FA pathway acts in DNA repair pathways which most likely act through homologous recombination or recombination like intermediates.

Recent biochemical studies have identified large complexes including some of the FA proteins. Included in the BRAFT complex are five FA proteins (FANCA, C, E, F, and G), the Bloom syndrome helicase (BLM), replication protein A (RPA) and topoisomerase IIIa (Topo3a) (Meetei et al., 2003). This complex has a DNA duplex unwinding capability that required BLM, but not FANCA (Meetei et al., 2003). However, BLM is not required for FANCD2 monoubiquitination, demonstrating BLM functions in this pathway downstream of core complex signaling for activation of the FA pathway (Meetei et al., 2003). This complex is independent from another BLM containing complex, which includes MLH1, a protein implicated in mismatch repair (Meetei et al., 2003).

FANCA, in addition to being a core complex and BRAFT component (Meetei et al., 2003), has been shown to interact with several other proteins. Independently of the other FA proteins, FANCA interacts directly with BRCA1 without the requirement for DNA damage, showing this interaction is constitutive (Folias et al., 2002). Additionally, FANCA has been suggested to aid in the recruitment of the SWI/SNF complex subunit, brahma-related gene 1 (BRG1), and may be involved in chromatin remodeling at the site of action of the FA pathway (Otsuki et al., 2001).

FANCF is also found in several FA complexes including the core complex and BRAFT (Meetei et al., 2003). The homology of FANCF with the prokaryotic RNA binding protein ROM has led to the supposition it may bind RNA or DNA through this alpha-helical motif (de Winter et al., 2000); however, aside from its role as an adapter molecule in the complexes described above, no specific biochemical function has been defined for FANCF (Leveille et al., 2004).

FANCB was initially thought to be associated with BRCA2 mutations along with FANCD1 (Howlett et al., 2002), however, FANCB was subsequently identified as the Fanconi anemia associated protein of 95KDa (FAAP95) (Meetei et al., 2004). FANCB is the only FA gene cloned thus far that has X-linked inheritance, and is subject to X-inactivation, leaving only one active copy of the gene (Meetei et al., 2004). Immunoprecipitation (IP) studies demonstrate interactions between FANCB and FANCA, G and L (Meetei et al., 2004). Additionally, loss of FANCB leads to reduced FANCD2 monoubiquitination (Meetei et al., 2004), which, with the IP data, clearly demonstrates FANCB is also a component of the FA core complex (Figure 2).

FANCJ, was previously identified as BRCA1-associated C-terminal helicase 1 (BACH1) or BRCA1 interacting protein C-terminal helicase 1 (BRIP1) (Levitus et al., 2005, Levran et al., 2005). BRIP1 is a BRCA1 interacting protein that functions as an ATP dependent 5'-3' helicase. *In vitro* studies indicate that it can act on DNA:DNA and RNA:DNA substrates (Cantor et al., 2004, Cantor et al., 2001). Combined with the interaction of FA proteins with BLM, a 3'-5' helicase (Ellis et al., 1995), means FA complexes may be able to facilitate movement in both directions.

The discovery of FANCD1 as BRCA2 directly linked the FA pathway and HR pathway (Hirsch et al., 2004). BRCA2 is known to regulate RAD51 controlling the formation of the RAD51 single stranded DNA (ssDNA) nucleoprotein filament required for strand pairing during HR in DSB repair (Davies et al., 2001, Sharan et al., 1997). In addition, BRCA2 binds FANCD2 and G placing the core complex and FANCD2 at sites of homologous recombination repair (Hussain et al., 2004). There is also evidence that

identifies BRCA2 as a stabilizing element at stalled replication forks preventing breakage of the DNA (Lomonosov et al., 2003).

Additional studies show the FA proteins are involved in repair of replication or recombination intermediates. FANCM was initially identified as Fanconi anemia interacting protein of 250KDa (FAAP250) (Meetei et al., 2005). Ciccia *et al.* (2007) indicates that FANCM through its interaction with FA-associated polypeptide of 24KDa (FAAP24) might recruit the FA core complex to intermediates such as those acted upon by ERCC1/XPF, based upon the homology of FAAP24 to XPF (Ciccia et al., 2007). FANCM has also been shown to disassociate triplex DNA by translocating along duplex DNA (Meetei et al., 2005). These data demonstrate FANCM may function in moving the FA core complex along dsDNA to sites of damage recognized by FAAP24 (Ciccia et al., 2007).

Another biochemical study in *Xenopus* extracts adds support to this theory. The FA pathway was shown, in a core complex dependent manner, to monoubiquitinate FANCD2 in the presence of linear and branched double stranded DNA (Sobeck et al., 2007). In addition, FANCD2 monoubiquitination was observed with circular DNA showing that double strand DNA ends or double strand breaks (DSBs) are not the activators of the FA pathway (Sobeck et al., 2007).

Recently, another link between familial cancer and FA was uncovered with the identification of FANCN as the partner and localizer of BRCA2 (PALB2) (Reid et al., 2007, Tischkowitz et al., 2007, Xia et al., 2006). As its name suggests, PALB2 interacts with BRCA2 and is responsible for its localization to chromatin; thus, PALB2 is required for BRCA2's function in homologous recombination repair and cell cycle checkpoints

(Xia et al., 2007, Xia et al., 2006). With PALB2, like BRCA2, monoallelic mutation leads to increased risk of breast cancer (Rahman et al., 2007, Tischkowitz et al., 2007).

The cumulative data are consistent with the following hypothesis: the FA pathway is responsible for repair of DNA damage that has a stalled replication fork and resulting recombination as an intermediate. In the studies discussed herein, we will provide further support for this hypothesis. We demonstrate that the activation of the FA pathway, as demonstrated by FANCD2 monoubiquitination and focus formation, requires the action of ERCC1 in response to ICL and HU, a potent inducer of stalled replication forks.

1.3 Nucleotide Excision Repair Pathway in Interstrand Crosslink Repair

While ICL sensitivity is characteristic of FA, loss of any of several proteins in mammalian cells can also lead to ICL sensitivity (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 (Collins, 1993, De Silva et al., 2000). In addition to their function in excision of bulky adducts in NER, ERCC1/XPF has also been shown to trim overhanging non-homologous single-stranded DNA (ssDNA) from homologous recombination intermediates to facilitate extension of the duplexed DNA recombination intermediate (Adair et al., 2000, De Silva et al., 2000, McHugh et al., 2001, Niedernhofer et al., 2001, Niedernhofer et al., 2004, Tsodikov et al., 2005). The sensitivity of ERCC1 and XPF cell lines to ICL agents and their action in both NER and recombination, demonstrates an important role specifically for ERCC1/XPF in ICL repair. This dual role has lead to much interest in using hERCC1 status in tumor samples for addressing issues of outcome and tailoring treatment of tumors with or without hERCC1 (Ceppi et al., 2006, Lee et al., 2005, Lord et al., 2002, Ryu et al., 2004).

Opinions differ as to whether the ERCC1/XPF heterodimer is required for formation of DSBs in response to ICLs. Rothfuss and Grompe showed ERCC1 is required for initial incision as observed by restoration of a longer tail moment by comet assay following treatment with psoralen-UVA (Rothfuss and Grompe, 2004). In addition, they observed reduced formation of γH2AX foci in S-phase in an ERCC1-/- CHO line as compared to a parental control line as calculated by decreased fluorescence with γH2AX immunofluoresence (Rothfuss and Grompe, 2004). They propose the model shown in

Figure 3 in which the ERCC1/XPF is responsible for the initial incision and unhooking of the ICL, leading to DSB formation, activation of the FA pathway and ultimately homologous recombination repair of the DSB.

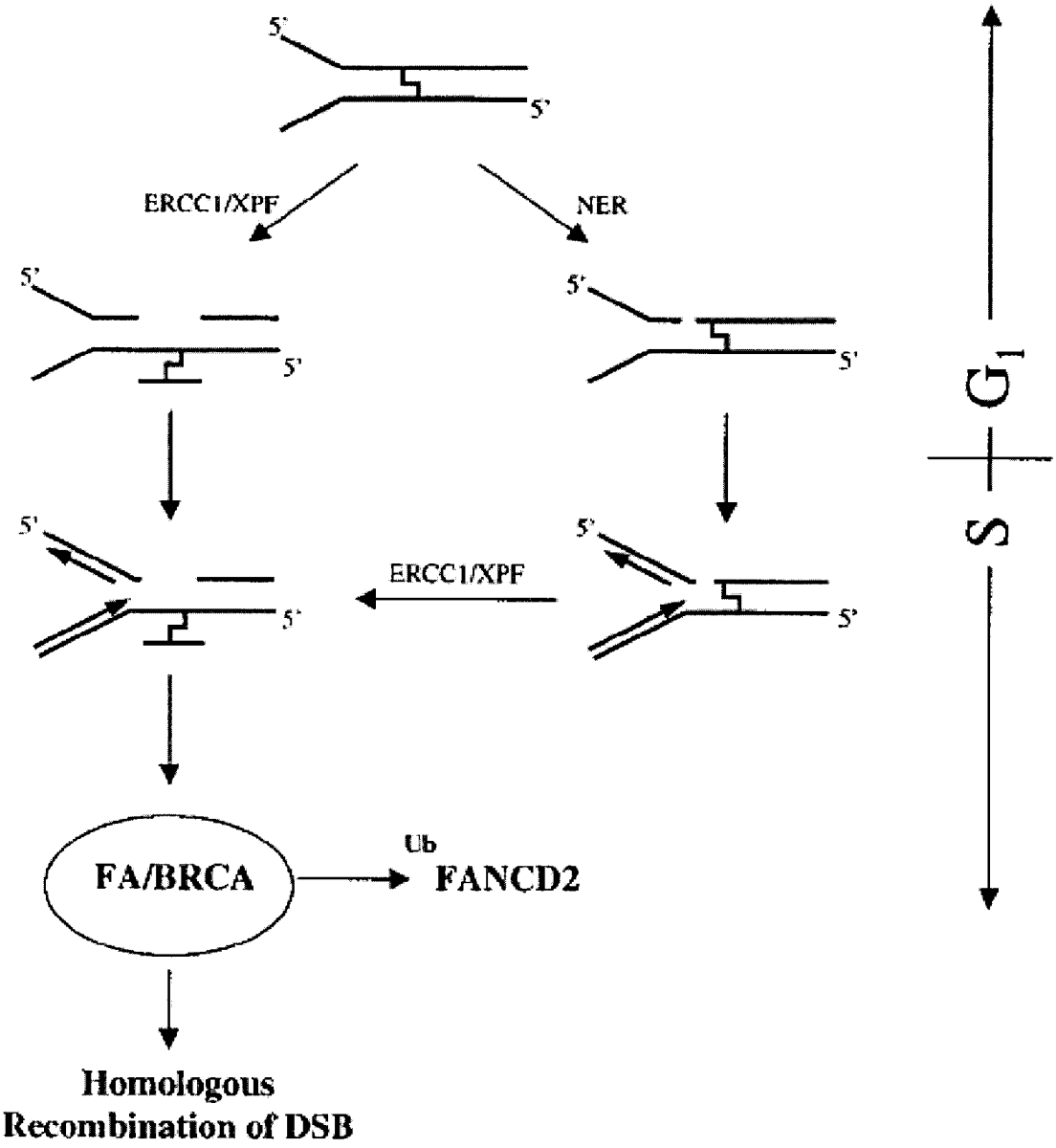
The work of Niedernhofer *et al.* (2004) indicates there is no significant difference in γH2AX focus formation between ERCC1 mutant and control cells as observed by counting foci per cell. Instead, they observe persistence of γH2AX foci in mutant cells, showing ERCC1 is required for the repair of DSBs in response to ICL treatment. These data are suggestive of a model in which the actions of ERCC1 are required after an initial incision event and downstream of DSB formation. They suggest that ERCC1 may be responsible for resolving a recombination intermediate (Niedernhofer et al., 2004), possibly a structure similar to that shown in Figure 4. Perhaps resolving this structure permits ICL repair to proceed leading to repair of the DSBs.

The differing results in these papers may come from several sources. While both papers induce ICLs, they use different drugs, which produce not only different amounts of DNA distortion, they produce differing levels of ICLs versus mono-adducts. They utilize different cell lines, with very different properties; the CHO lines used by Rothfuss and Grompe are hemizygous and prone to loss of additional genes as some of my preliminary work with these same CHO lines has suggested, as they can be corrected for ICL sensitivity, but not FA pathway function by the addition of recombinant human ERCC1 expression constructs (K. McCabe, unpublished). Additionally, the timescale at which they looked at H2AX phosphorylation, especially relative to replication time, is quite different. Finally, the methods used to analyze H2AX phosphorylation differ; while Rothfuss and Grompe used a new method examining the relative fluorescence of nuclei

immunolabeled for γH2AX to yield a relative fluorescence index, Niedernhofer utilized the counting of γH2AX foci.

The studies herein address these differing results. We have found that γH2AX phosphorylation and focus formation shows only a marginal difference between control and hERCC1 depleted GM639 cells with MMC, DEB and HU treatments, indicating hERCC1 is not required for DSB formation in response to ICL or stalled replication forks. Rather, hERCC1 is likely involved in processing of a recombination intermediate. In the absence of hERCC1, in response to both ICL and HU induced stalled replication forks, the FA pathway is not activated as assayed by hFANCD2 monoubiquitination and focus formation. This does not rule out a role for hERCC1 in the initial excision of ICL, but points to its vital role downstream of DSB formation.

Figure 3- Model for the role of ERCC1 in ICL repair proposed by Rothfuss and Grompe, 2004.



From: Rothfuss and Grompe, 2004, Mol Cell Biol, 24, p132

Figure 3. Model for the role of ERCC1 in ICL proposed by Rothfuss and Grompe (2004). The model proposes that ERCC1 is required for a rapid incision of ICLs in G1 that leads to DSB formation in S-phase when a replication fork encounters the incision. This DSB then leads to activation of the FA pathway and homologous recombination repair of the DSB.

Figure 4- Possible substrate for ERCC1/XPF downstream of DSB formation in ICL repair.

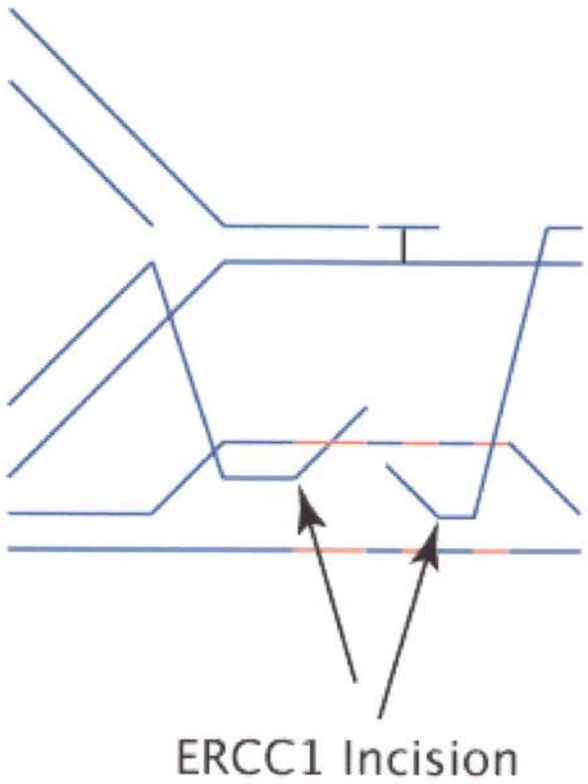


Figure 4. In the above diagram is depicted a strand invasion following a formation of a double strand break as an intermediate step in ICL repair. Lines represent single strands of DNA with the colors denoting homology; those strands with the same color share homology, those with differing colors represent heterologous sequences. ERCC1/XPF has been shown to be required for trimming heterologous tails from homologous recombination intermediates (marked with the arrow) to allow extension and the completion of recombination.

1.4 DNA Damage Induced Nuclear Foci

Many proteins from DNA repair pathways have been found to form foci by immunofluoresence. These punctate patterns of protein localization and co-localization have various components at different times after DNA damage or during S-phase, demonstrating an order of action for the various components (Tembe and Henderson, 2007). In addition, different types of damage (*eg.* IR, UVC, ICL, monoadducts, etc.) lead to different mutli-protein complexes colocalizing in foci (Tembe and Henderson, 2007).

One of the earliest proteins to form foci in response to several types of DNA damage that create DSBs or act through DSB intermediates is the ser-139 phosphorylated form of the histone H2AX, commonly referred to as γH2AX. ATM, ATR, or DNA dependent protein kinases (DNA-PKs) can phosphorylate γH2AX in response to double strand breaks in megabase regions surrounding the site of damage (Rogakou et al., 1999). This large region of γH2AX then recruits the needed DNA repair factors and these collections of multi-protein complexes are then visible by immunofluoresence as DNA damage induced nuclear foci (Tembe and Henderson, 2007).

In the case of ICL, proteins involved in NER, HR, and FA pathways localize to nuclear foci in a damage dependent manner. The signaling kinases responsible for ICL response, in addition to phosphorylating H2AX, also act on several other proteins leading to their recruitment to γH2AX foci, including BRCA1, BRCA2/FANCD1, FANCD2, Rad51, and the MRN complex consisting of MRE11, Rad50 and NBS1 (reviewed in (Dronkert and Kanaar, 2001). FANCI, FANCJ and FANCN also localize to DNA damage induced nuclear foci (reviewed in (Grompe and van de Vrugt, 2007). In addition, ERCC1 has been shown to colocalize with RAD51 in nuclear foci (Volker et al., 2001).

1.5 Current Studies

The experiments described herein were undertaken to better understand the role of hERCC1 in ICL repair. As mentioned throughout this introduction, it has been shown that incision of ICLs in G_1 by ERCC1/XPF is required for DSB formation, specifically in S-phase (Mogi and Oh, 2006, Rothfuss and Grompe, 2004). However, other data indicate ERCC1/XPF is not required for DSB formation, but rather required for their resolution (Niedernhofer et al., 2004). In order to examine these differing observations in greater detail, we have depleted hERCC1 with siRNA and examined a detailed time-course of γH2AX phosphorylation and focus formation. Additionally, FANCD2 monoubiqutination and focus formation were examined in parallel in an effort to better establish the roles of γH2AX phosphorylation, ERCC1/XPF and FANCD2 monoubiquitination in the repair of ICLs.

In addition, there remains debate within the field regarding the specific type of damage upon which the FA pathway acts. While some suggest the substrate or signal for activation is a DSB (Lyakhovich and Surralles, 2007, Rothfuss and Grompe, 2004), others suggest the substrate is a stalled replication fork (Hussain et al., 2004, Thompson et al., 2005) or a homologous recombination substrate (Yamamoto et al., 2003). The actions of ERCC1 within the early steps of FA pathway, particularly its role in DSB formation or resolution, place it at a critical point in pathway activation. Clarification of the role of ERCC1 may shed light on the activation of the FA pathway. Determination of the substrate for activation of the FA pathway or upon which the pathway acts would provide much needed insight into the role of the FA proteins in DNA repair.

Our studies demonstrate γH2AX phsophorylation and focus formation in response to ICL and hydroxyurea (HU) treatment is only slightly reduced when ERCC1 is depleted. FANCD2 monobiquitination and focus formation, however, is greatly reduced in response to ICL and HU treatments. This further demonstrates ERCC1 is not required for DSB formation, and also demonstrates the requirement for ERCC1 to process an intermediate down-stream of DSB formation in order to activate the FA pathway as observed by FANCD2 monoubiquitination and focus formation. Additionally, this role for ERCC1 is not limited to ICL formation, but is also required to resolve stalled replication forks resulting from HU treatment, indicating that the intermediate structure is shared between the pathways responsible for ICL repair and the resolution of stalled replication forks.

From the cumulative evidence, we conclude that hERCC1 acts downstream of DSB formation in the FA pathway, while still requiring its action on an intermediate formed by both the early stages of ICL repair and the resolution of stalled replication forks resulting from HU treatment. The function of hERCC1 at this point in the FA pathway likely serves to trim 3' ssDNA overhangs adjacent to duplexed DNA at the sites of strand exchange. It has been suggested that such overhangs prevent extension from the site of strand exchange, and result from a non-homologous tail failing to pair with nearby stretches of homology that are forming the duplex (Figure 4) (Adair et al., 2000, De Silva et al., 2000, McHugh et al., 2001, Niedernhofer et al., 2001, Niedernhofer et al., 2004, Tsodikov et al., 2005). While this role for ERCC1 in recombination has been previously described and this role in ICL repair has been suggested, its place within the FA pathway has not.

Chapter Two: Methods

2.1 Cell Culture

GM639, an SV-40 immortalized human fibroblast cell line, was provided by the lab of Dr. Robb Moses. All cells were cultured in αMEM (Mediatech, Herndon, VA) supplemented with 10% FBS (Hyclone, Logan, UT) at 37°C with 5% CO_2. Cultures were serially passaged two to three times weekly with frozen stocks freshly thawed at least monthly to keep passage numbers low.

hERCC1 was depleted using an siGENOME siRNA smartpool (Dharmacon, Lafayette, CO). siRNA utilizes what is thought to be a defense mechanism against viruses to reduce the expression of genes. siRNAs are typically 19-21bp in length and are designed to have sequence identity with the gene to be knocked down. Longer double stranded RNA (dsRNA) and small hairpin RNA (shRNA) are processed by the protein dicer into siRNAs. These short double stranded RNAs are bound by the RNA-induced silencing complex (RISC), which utilizes only one of the strands to identify the complementary mRNA. RNA-dependent RNA polymerase extends from the homologous stretch. The dsRNA that is created gets cut up by dicer creating additional siRNA. This is how siRNA appears to act catalytically (reviewed in (Hutvagner and Zamore, 2002).

CypB siCONTROL siRNA (Dharmacon, Lafayette, CO) was used as a control. T-25 flasks (Falcon/Becton Dickinson, Franklin Lakes, NJ) were seeded with $5x10^4$ cells 12-16 hours prior to siRNA transfection. 7.5μL 20μM siRNA was mixed with 166.5μL OptiMEM (Invitrogen-GIBCO Carlsbad, CA) and 3μL oligofectamine (Invitrogen, Carlsbad, CA) was mixed with 12μl OptiMEM for each flask to be transfected. These mixes were incubated at room temperature for 5 minutes, mixed and incubated for an additional 10 minutes. The media from each T-25 flask was aspirated

and washed with OptiMEM. 0.8mL of OptiMEM was added to each flask, followed by the addition, drop-wise, of the transfection mixture. Following a 4hr incubation at 37° with 5% CO_2, 0.5mL αMEM containing 30% FBS was added. Approximately 16hr later, the media was replaced with αMEM containing 10% FBS.

2.2 Immunoblotting

48hr after transfection, hERCC1 and control knock-down flasks were treated with a one hour pulse of 500ng/mL MMC (Sigma-Aldrich, St. Louis, MO), 300ng/mL DEB (Sigma-Aldrich, St. Louis, MO), or 3mM HU (Sigma-Aldrich, St. Louis, MO), or harvested by trypsinization (Invitrogen-Gibco, Carlsbad, CA) as untreated controls. Treated cells were washed once with PBS and αMEM with 10% FBS was added. Flasks were harvested by trypsinization at 1,2,4,6,and 8hr, and these cell pellets were frozen at -80°C until used.

Lysates were prepared by resuspending the cell pellets with cold RIPA lysis buffer. Samples were then sonicated on ice for 10sec using a sonicator (Heat Systems-Ultrasonics, now know as Misonix, Inc. Farmingdale, NY) set at the microprobe maximum. Protein levels were quantitated using BioRad Protein Assay (BioRad, Hercules, CA) and equalized for loading using the RIPA lysis buffer. 4X LDS sample buffer (Invitrogen, Carlsbad, CA) and 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) were added to 1X and 5% respectively. Samples were then placed in boiling water for 5min and quickly centrifuged.

Samples for hFANCD2 immunoblotting were loaded on a 10 well Novex 3-8% Tris-acetate gel (Invitrogen, Carlsbad, CA) that had been pre-run for 7min at 100V. These gels were run for 8-9hr at 110V in Tris-acetate running buffer (Invitrogen, Carlsbad, CA). Transfer to a 0.2μm pore supported nitrocellulose Optitran membrane (Whatman, Brentford, Middlesex, UK) was performed in Towbin buffer (50mM Tris (Fisher Bioreagents, Fair Lawn NJ), 380mM glycine (MP Biomedicals, Solon, OH)) at 25V overnight at 4°C. All subsequent steps were performed on an orbital shaker at

100rpm. Membranes were blocked for 1hr with TBST with 0.2% tween-20 and 5% powdered, non-fat milk, probed with mouse anti-FANCD2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 in block for 1hr, washed 3X5min in TBST containing 0.2% tween-20, probed with goat anti-mouse HRP conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 in block, and the washes were then repeated. Blots were then immersed in Imobilon Western HRP Substrate (Millipore, Billerica, MA) for 5min and placed against film.

When preparing the membranes for being read by the Li-Cor Odyssey (Li-Cor Biosciences, Lincoln, Nebraska), instead of HRP/luminal and film, the membrane was blocked in Odyssey Buffer (Li-Cor Biosciences, Lincoln, Nebraska) for 1hr. Membranes were then probed with the Novus rabbit anti-FANCD2 at 1:1200 (Novus Biologicals, Littleton, CO) in 1:1 TBST (0.5% Tween-20):Odyssey Buffer (Li-Cor Biosciences, Lincoln, Nebraska) for 1hr, washed 3X5min in TBST (0.5% Tween-20), probed with Molecular Probes Alexa Fluor 680 goat anti-rabbit secondary (Invitrogen-Molecular Probes Eugene, OR) in 1:1 TBST (0.5% Tween):Odyssey Buffer (Li-Cor Biosciences, Lincoln, Nebraska). The washes were then repeated and the blot was scanned on a Licor Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, Nebraska).

Samples for γH2AX immunoblotting were loaded onto a 15% acrylamide:bis 29:1 (BioRad, Hercules, CA) with a 4% stacking gel and run at 110V for approximately 2hr in Towbin buffer containing 0.5% SDS. Transfer to a 0.2μm pore supported nitrocellulose Optitran membrane was performed in Towbin buffer at 70V for 1hr at 4°C. All subsequent steps were performed on an orbital shaker at 100rpm. Membranes were blocked for 1hr in TBST with 0.2% tween-20 and 5% BSA, probed with rabbit anti-

γH2AX antibody (Novus Biologicals, Littleton, CO) at 1:1000 in block for 1hr, washed 3x5min in block, probed with goat anti-rabbit HRP conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 in block for 1hr, and the washes were then repeated. Blots were then immersed in Imobilon Western HRP Substrate for 5min and placed against film.

Samples for hERCC1 immunoblotting were loaded onto a 10% acrylamide:bis with a 4% stacking gel and run at 110V for approximately 2hr in Towbin buffer containing 0.5% SDS. Transfer to a 0.2μm pore supported nitrocellulose Optitran membrane was performed in Towbin buffer at 70V for 1hr at 4°C. All subsequent steps were performed on an orbital shaker at 100rpm. Membranes were blocked for 1hr with TBST with 0.2% tween-20 and 5% powdered, non-fat milk, probed with mouse anti-ERCC1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1000 in block for 1hr, washed 3X5min TBST with 0.2% tween-20, probed with goat anti-mouse HRP conjugated secondary antibody at 1:1000 in block, and the washes were then repeated. Blots were then immersed in Imobilon Western HRP Substrate for 5min and placed against film.

2.3 Immunofluoresence

24hr after transfection flasks were trypsinized and the cells were transferred to 4-well CultureSlides (BD Falcon, San Jose, CA) adding 5,000 cells per well in 500μL. 24hr later the chambers are treated as the flasks were for immunoblotting with untreated, 500ng/mL MMC, 300ng/mL, and 3mM HU treated chambers for hERCC1 and control knock-down cells. 4hr post-treatment, the cells were fixed with 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS for 15min on a laboratory tipper as with all subsequent steps. Cells were then permeabalized with 0.5% Triton-X 100 (Sigma-Aldrich, St. Louis, MO) in PBS for 15 minutes, followed by blocking with 15%FBS in PBS for 1hr. Primary antibody was then added for 1hr at room temperature. For -γH2AX foci, rabbit anti-γH2AX was added at 1:2500 in block, and for the hFANCD2 foci, rabbit anti-FANCD2 (Novus Biologicals, Littleton, CO) was added at 1:400 in block. Slides were then washed 3x5 minutes with 0.2% tween-20 in PBS. Alexa fluor 594 Donkey anti-rabbit (Invitrogen-Molecular Probes Eugene, OR) was then added 1:750 in block for 1hr at room temperature. Washes were repeated, followed by addition of ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) and application of a coverslip. The following day after curing, the coverslips were fixed to the slide and the samples were examined by fluorescent microscopy.

2.4 Densitometry

Immunoblot films were scanned on a Canon Pixma^{tm} MP170 (Canon USA, Lake Success, NY) into JPEG format. Files were uploaded to ImageJ (Version 1.36b, NIH) and 3 parallel lines were drawn across the bands to be quantified. A Plot Profile was applied to each line, each of the subsequent plots was then traced using the freehand drawing tool, and area under the curve was measured. The triplicate areas were then averaged and standard deviations calculated.

2.5 Kill Curves

48hr post-transfection, cells were trypsinized and 300 cells were transferred to each 100mm dish (Falcon/Becton Dickinson, Franklin Lakes, NJ) containing αMEM with 10% FBS. MMC and DEB were added at doses of 0, 5, 10, 20 and 40 ng/mL and 0, 2, 4, 6, 8 μM respectively. Each concentration was tested in triplicate. After 10 days, plates were stained with new methylene blue (Sigma-Aldrich, St. Louis, MO) in 70% ethanol. Colonies were counted and percentages of untreated colonies were calculated.

2.6 Chromosome Stability

24 hours after siRNA transfection with control scrambled, FANCA or ERCC1 siRNA, flasks were left as untreated controls or treated with the appropriate dose of MMC or DEB. The dose was varied according to sensitivities created by gene deficiency or depletion, such that an increase in radial formation might be detected. After 24-28 hours, colcemid (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 150ng/mL. Approximately 3hr later, cells were trypsinized, and fixed with 3:1methanol:acetic acid and 75mM KCl. Slides were stained with Wright's stain (Fisher Scientific, Pittsburgh, PA). Nuclei were examined qualitatively for mitotic index to confirm there was no significant difference between the variously treated samples. 50 metaphase nuclei per sample were then examined using a Nikon E800^{tm} fluorescence microscope and captured using CytoVision software (Applied Imaging, San Jose, CA).

Chapter Three: Results

3.1 ERCC1 Depletion

To study the role of hERCC1 in the FA pathway, we adopted the strategy of siRNA depletion. In order to demonstrate depletions of hERCC1, immunoblotting of depleted and control lysates was carried out. The immunoblot demonstrated a significant depletion of hERCC1 (Figure 5A). Quantitation of the hERCC1 compared to control revealed a depletion of 87.7% +/- 1.6%.

While this level of depletion was quite substantial, we tested the effect on ICL repair. As sensitivity to inter-strand cross-linking agents is a hallmark of ERCC1 mutation or absence (Collins, 1993, De Silva et al., 2000), sensitivity of the depleted lines was compared to control using a colony forming assay with increasing doses of MMC and DEB. The MMC LD50 of the control CypB depletion was approximately two-fold greater than the hERCC1 depletion (Figure 5B). DEB sensitivity was similarly increased with hERCC1 depletion (data not shown). This increased sensitivity indicated that the hERCC1 depletion is significant and affecting hERCC1's function within the Fanconi pathway.

Another aspect of hERCC1 depletion to be addressed was whether there occurred a significant change in the cell cycle of depleted cells. Changes to the percentage of cells in S-phase could exaggerate or mask changes to γH2AX phosphorylation and hFANCD2 monoubiquitnation levels as a result of depletion of hERCC1. Depleted and CypB depleted control cultures were fixed, stained with propidium iodide (PI), and analyzed by fluorescence-activated cell-sorting (FACS). The hERCC1 depleted culture contained 23.65% +/- 3.39% cells in the M2 fraction and the control CypB depleted culture

contained 27.06% +/- 1.04% cells in the M2 fraction, demonstrating there was no significant change in the proportion of cells in S-phase (Figure 5C).

Figure 5- hERCC1 depletion, demonstration of effective knockdown and, cell cycle analysis

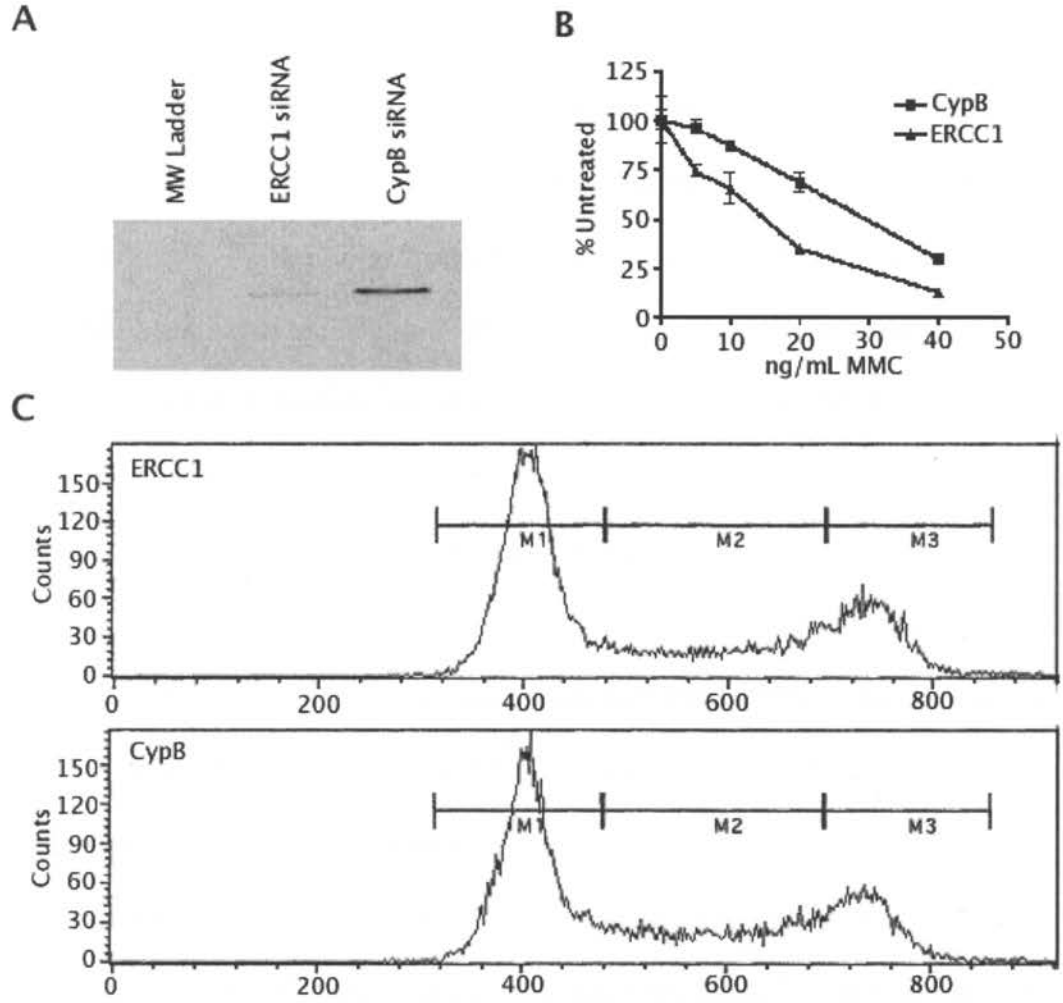


Figure 5. 5A. hERCC1 immunoblot demonstrating depletion of hERCC1 by 87.7% +/- 1.6%. **5B.** MMC kill curve demonstrating increased ICL sensitivity of hERCC1 depleted GM639 cells over control CypB depleted Gm639 cells, indicating the depletion is effective. **5C.** Representative cell cycle analysis of hERCC1 depleted versus control CypB depleted GM639 show similar profiles. The hERCC1 depleted cultures contained 23.65% +/- 3.39% cells in the M2 fraction and the control CypB depleted culture contained 27.06% +/- 1.04% cells in the M2 fraction, demonstrating there was no significant change in the proportion of cells in S-phase.

3.2 Chromosome Stability

One of the hallmarks of Fanconi anemia is chromosome instability, and radials, historically, are diagnostic of FA (Newell et al., 2004). Deficiencies or depletions for several other genes related to DNA repair, including BRCA1 and BRCA2, have also been shown to induce radials (Kohzaki et al., 2007, McPherson et al., 2004, Bruun et al., 2003). Previously, loss of ERCC1 has been shown to be associated with formation of radials (Niedernhofer et al., 2004). To examine the role of ERCC1 in the FA pathway, as it relates to radial formation, we depleted hERCC1 and hFANCA individually and together in GM639 cells.

The level of radials in untreated cells was low, with virtually no radials in either the untransfected control or hERCC1 depleted lines. When hFANCA was depleted, there was a slight increase in spontaneous radials; approximately 15% of the cells had at least one radial. When both hFANCA and hERCC1 were depleted, the level of spontaneous radials dropped to below 5% (Figure 6).

When treated with MMC, the levels of radials increased. The control transfected cells had radial levels of approximately 18% while the hERCC1 depleted line was even lower and still under 10%. As expected, the hFANCA depleted line showed a marked increase in radials such that over 65% of the cells had at least one radial. When both hERCC1 and hFANCA were depleted, we expected to find, at the very least, the level of radials associated with the loss of hFANCA; however, when both hFANCA and hERCC1 were depleted, the percentage of cells with radials was reduced more than two-fold (Figure 6). These results place hERCC1 in the FA pathway, and more specifically,

upstream of radial formation. These results indicate radials form from an intermediate of the FA pathway downstream of the involvement of hERCC1.

Figure 6- Epistasis analysis of hERCC1 and hFANCA by siRNA depletion in GM639 cells by chromosome stability.

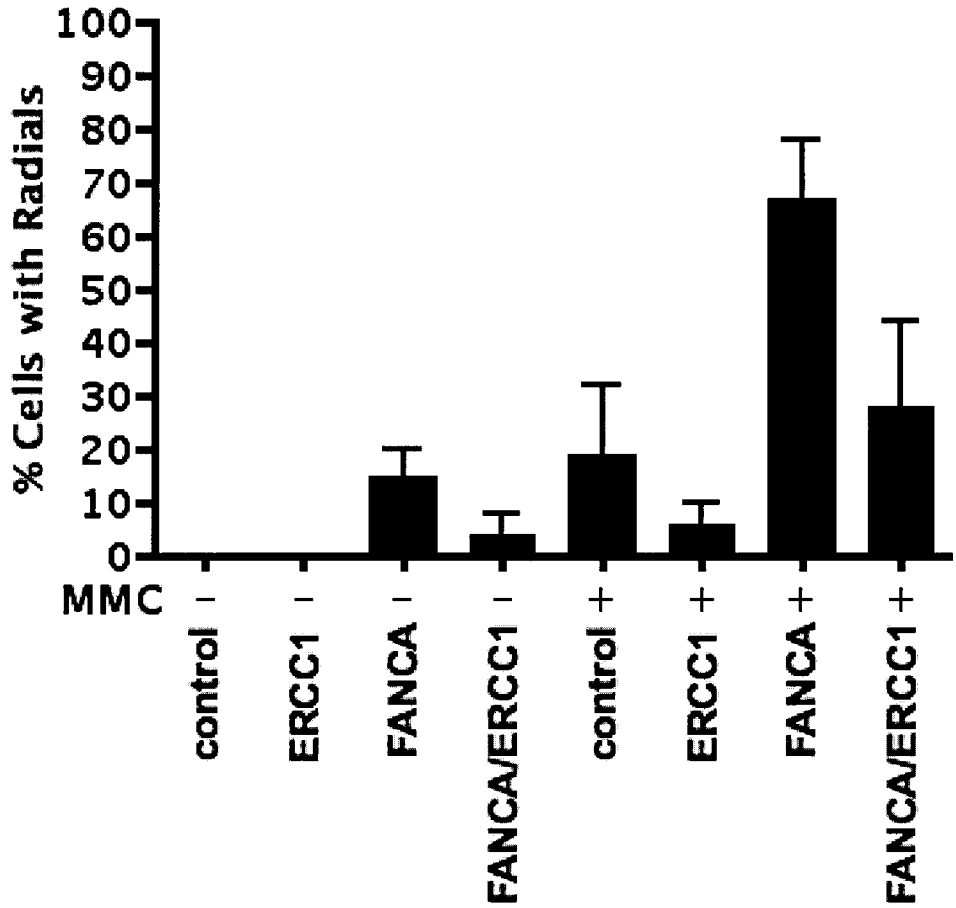


Figure 6. Graph of percent radials observed in GM639 cells depleted with ERCC1 siRNA, FANCA siRNA, or dual depletion with ERCC1 and FANCA siRNAs. Spontaneous radials were not observed in control siRNA transfected or ERCC1 siRNA transfected cells. With FANCA siRNA transfection, spontaneous radials increased modestly, but decreased slightly with dual ERCC1 and FANCA siRNA transfection. With MMC treatment, levels of radials increased as expected. Control siRNA transfected radials increased to near 20%. ERCC1 siRNA transfection reduced levels of radial formation below control transfected. As expected FANCA siRNA transfection dramatically increased levels of radials. Dual transfection with MMC treatment reduced levels of radials below FANCA siRNA transfection alone, as with the untreated dual transfection. Bars represent the mean with error bars representing the range.

3.3 Foci

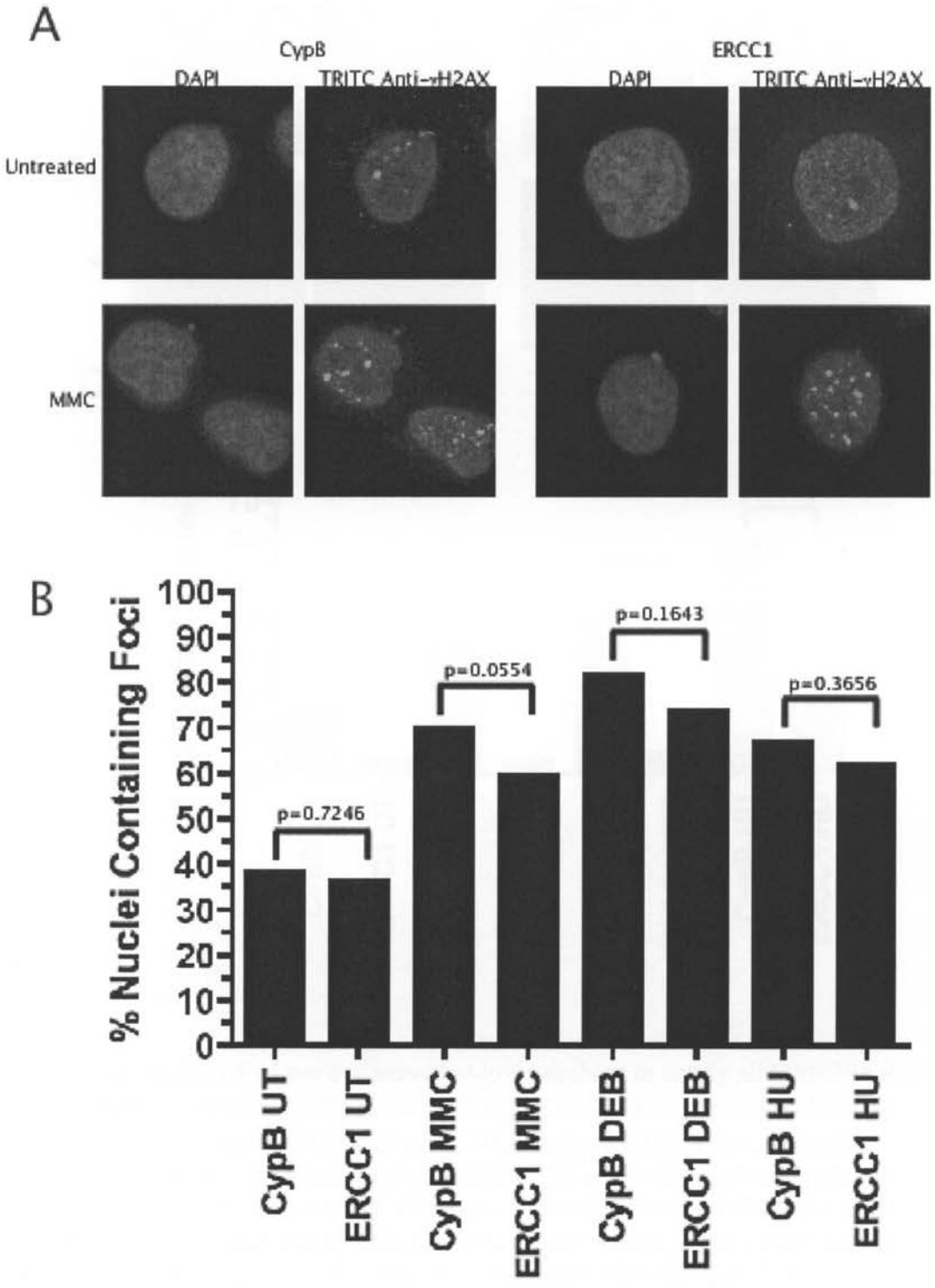
Control and hERCC1-depleted cells were cultured briefly in chamber slides and then either untreated, or treated with MMC, DEB, or HU. Slides were processed for immunofluoresence of γH2AX or hFANCD2 foci. γH2AX foci mark sites of DSB (Rogakou et al., 1999), while hFANCD2 foci are believed to be markers of activation of the FA pathway, and thus monoubiquitination of FANCD2 (Bagby and Alter, 2006). A recent study functionally links γH2AX with BRCA1 dependant recruitment of FANCD2 (Bogliolo et al., 2007); however, our data show there are additional intervening steps.

With the γH2AX immunofluoresence, greater than 10 foci were observed in an increased number of cells with all clastogen treatments (Figure 7A and B). There was no statistically significant difference between control and hERCC1 depleted lines (Figure 7A and B). This means hERCC1 was not required for formation of the majority of DSBs in response to MMC, DEB, or HU. There was a trend toward a decrease in γH2AX foci observed in ERCC1 knock-down cells. While this decrease might indicate a role for hERCC1 in the initial incision of an ICL as observed by Rothfuss and Grompe (Rothfuss and Grompe, 2004), this difference in γH2AX focus formation was mirrored in the HU sample, which contained no crosslinks. Therefore, we can conclude that hERCC1 is not required for formation of DSB in response to ICL and stalled replication forks. Our data do not directly contradict Rothfuss and Grompe (Rothfuss and Grompe, 2004), as these studies did not address initial incision of ICLs.

The hFANCD2 immunofluoresence provided different results from the γH2AX immunofluoresence. The control cells demonstrated an increase in hFANCD2 foci in response to MMC, DEB and HU treatment (Figure 7C and D). The hERCC1 depleted

cells, however, showed a decrease in hFANCD2 focus formation (Figure 7C and D). Only the hERCC1 depleted HU treated cells reached the level of control untreated cells (Figure 7D). This demonstrates a requirement for hERCC1 in the formation of hFANCD2 foci in response to MMC, DEB and HU, whereas no such requirement exists for formation of γH2AX foci. These results place hERCC1 downstream of γH2AX focus formation, but upstream of hFANCD2 focus formation.

Figure 7- γH2AX and hFANCD2 foci formation



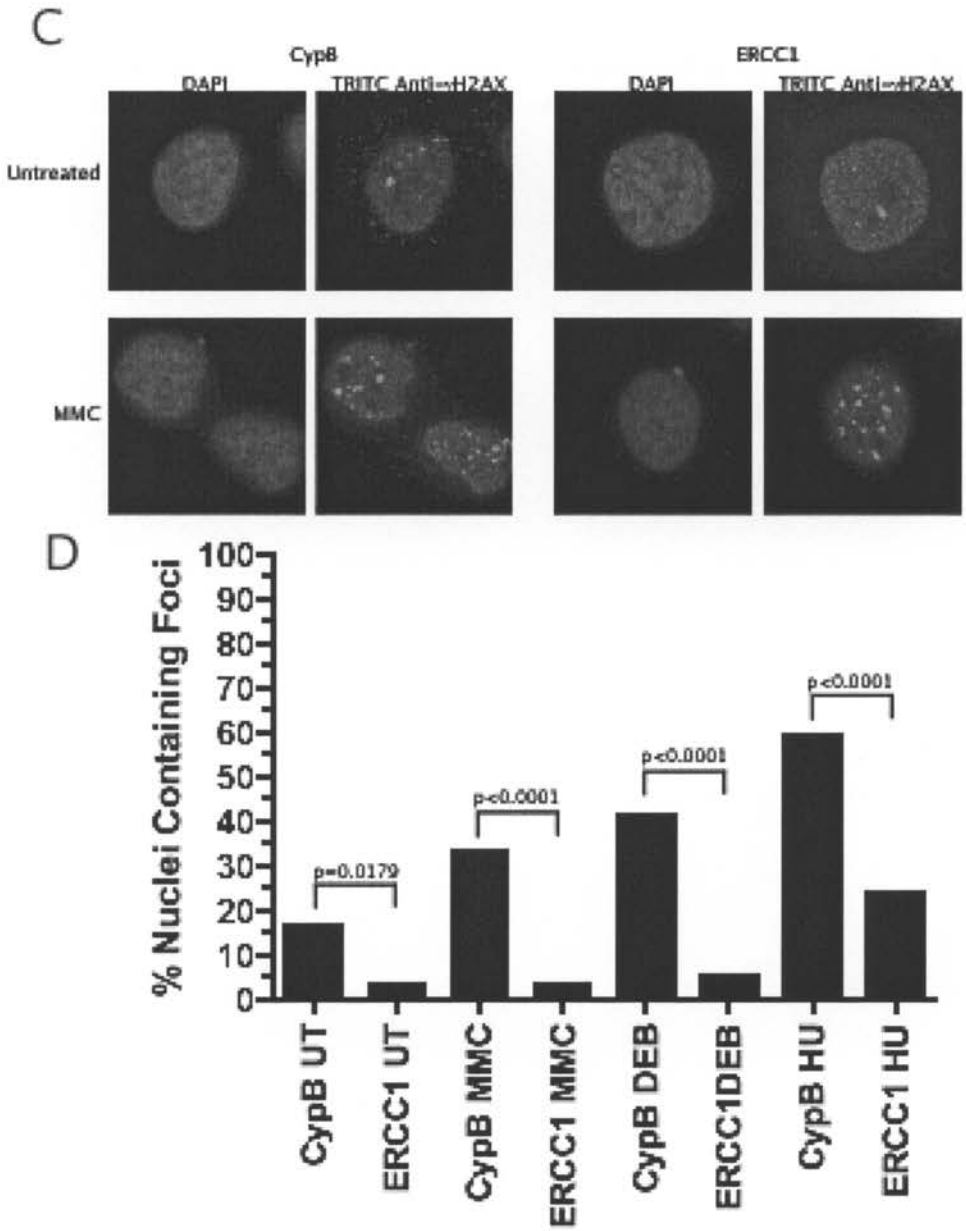


Figure 7. 7A. γH2AX foci were observed at low numbers in nearly all GM639 cells. MMC induced increases in γH2AX above the cutoff of 10 foci per cell, in both control CypB and hERCC1 depleted GM639 cells. **7B.** Graph of γH2AX foci formation observed in untreated and MMC, DEB and HU treated GM639 cells with control CypB versus hERCC1 depletion (n=~200 nuclei). Clastogen treatment increased the level of γH2AX foci in both control CypB and hERCC1 depleted cells, with a slight trend toward fewer foci in the hERCC1 depleted cells than in control depleted cells treated with the same drug. (Mann Whitney Test) **7C.** FANCD2 foci were observed at significant levels only with MMC treatment in control depleted GM639 cells. **7D.** Graph of FANCD2 foci

formation observed in untreated and MMC, DEB and HU treated GM639 cells with control CypB versus hERCC1 depletion (n=~200 nuclei). hERCC1 depletion significantly reduces the levels of hFANCD2foci compared to CypB control (Mann Whitney Test, $p<0.0001$ is a Gausian approximation)

3.4 Immunoblotting

Immunoblotting against γH2AX and hFANCD2 was carried out to confirm the immunofluoresence data and determine if the failure to form hFANCD2 foci was the result of failure of localization or monoubiquitination. To confirm the depletion of hERCC1 throughout the experiments, hERCC1 western blots were performed on all samples, with β-tubulin as a loading control. In all ERCC1 siRNA treated samples, the depletion of hERCC1 was similar to or greater than that presented in Figure 5A, indicating a functional knockdown throughout the time-points examined. hERCC1 depleted GM639 cells were treated with MMC, DEB, or HU for one hour and harvested at four hours from the initiation of clastogen treatment, or were left untreated as a control.

With the hFANCD2 immunoblots, the ratio of the upper monoubiquitinated form of hFANCD2 (Ub-hFANCD2) to the lower unmodified form (hFANCD2) is indicative of the level of activation of the FA pathway (Smogorzewska et al., 2007). In the control lysates, treatment with MMC and HU dramatically increased the ratio of Ub-hFANCD2 to the unmodified hFANCD2 band (Figure 9A and B). This was not the case with the hERCC1 knock-down lysates; there was no significant increase in the ratio of the upper band to the lower band in the clastogen treated versus untreated control (Figure 8A and 9A). In this particular immunoblot (Figure 8A), DEB did not induce an increase in Ub-D2. We have observed an increase in Ub-D2 with DEB in other blots (Figure 9B), however. The induction by DEB is somewhat inconsistent experiment to experiment. These results support the hFANCD2 immunofluoresence, indicating hERCC1 was required for activation of the FA pathway and thus monoubiquitination of hFANCD2 in

response to the crosslinking agents MMC and DEB and HU, which produces stalled replication forks.

A time-course of MMC treatment was then carried out to better order the phosphorylation of γH2AX, with the actions of hERCC1 and hFANCD2 mono-ubiquitination in the FA pathway. γH2AX immunoblotting confirmed the results of the γH2AX foci formation. Both control and hERCC1 depleted cells phosphorylated H2AX at similar levels at 1, 2, and 4hr in response to MMC (Figure 8B and 9). At 8hr, while γH2AX levels were elevated above control in both samples, there was a 28% reduction in the control from the 4hr time point to the 8hr time point while the hERCC1 depleted samples maintained constant levels of γH2AX (Figure 8B and 9). This may be indicative of repair through the FA pathway in the control cells, and is consistent with the observations of a prolonged increase in γH2AX in ERCC1 -/- cells of Niedernhofer, *et al.* (Niedernhofer et al., 2004).

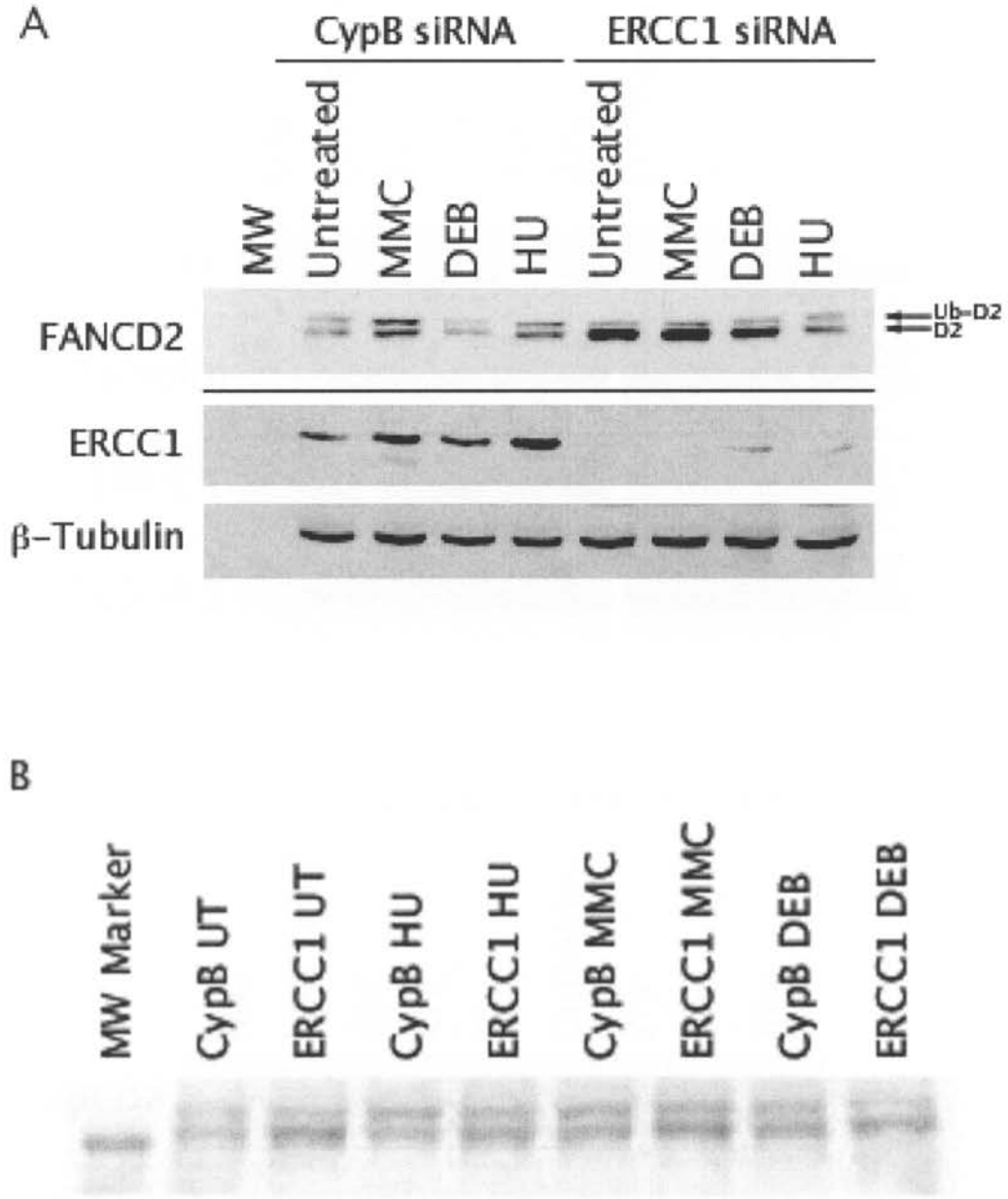
hFANCD2 immunoblots of hERCC1 depleted lysates harvested at 2hr and 4hr post treatment with MMC did not have any increase in Ub-hFANCD2 over the untreated control, with only a small increase in the 8hr sample (Figure 8C). 8hr post-treatment, the increase in Ub-hFANCD2 above untreated control is still below the CypB control transfected untreated sample. This time course confirmed that the actions of hERCC1 are essential to activation of the FA pathway and monoubiquitination of hFANCD2. With the first visible increase in Ub-hFANCD2 in hERCC1 depleted cells observed at 8hr, it seems that the action of hERCC1 is not readily bypassed.

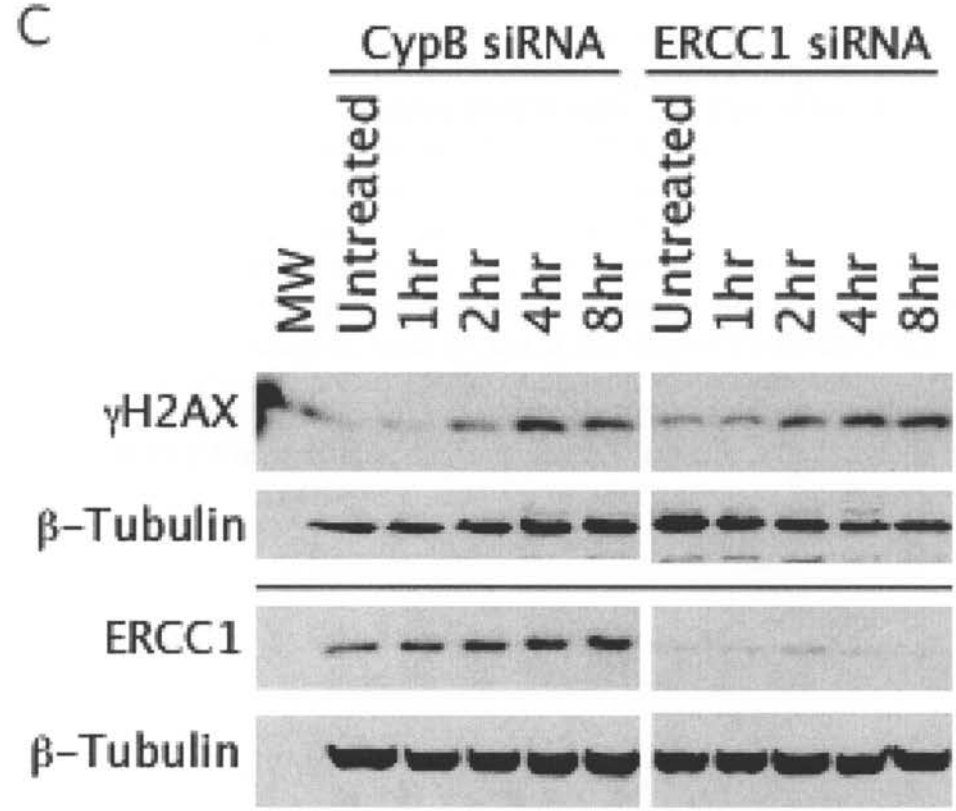
These results demonstrate hERCC1 is not required for DSB formation and H2AX phosphorylation in response to ICL or HU treatment. This shows DSBs formed as a result

of ICLs can occur in the absence of hERCC1, consistent with Niedernhofer *et. al.* (Niedernhofer et al., 2004). Additionally, the resultant DSBs formed in S-phase are not activating the FA pathway directly. Furthermore, the immunoblotting for hFANCD2 confirms the hFANCD2 immunofluoresence result is not merely a localization defect, but is a result of hERCC1 depleted cell's inability to monoubiquitinate hFANCD2.

These data separate the formation of the DSB from activation of the FA pathway as observed by hFANCD2 focus formation and monoubiquitination. Further, by demonstrating the intermediate upon which the FA pathway acts is common between ICL repair and resolution of stalled replication forks indicates that the intermediate is either the stalled replication fork, or a late recombination intermediate. If, in fact, the action of hERCC1 that is required for activation of the FA pathway lies in incision, or in the processing of a non-homologous tail of a recombination intermediate, it must be this processed recombination intermediate or some subsequent intermediate that is acted upon by the FA pathway.

Figure 8- Immunoblotting of FANCD2 and γH2AX




C
CypB siRNA
ERCC1 siRNA
MW
Untreated
1hr
2hr
4hr
8hr
Untreated
1hr
2hr
4hr
8hr
γH2AX
β-Tubulin
ERCC1
β-Tubulin

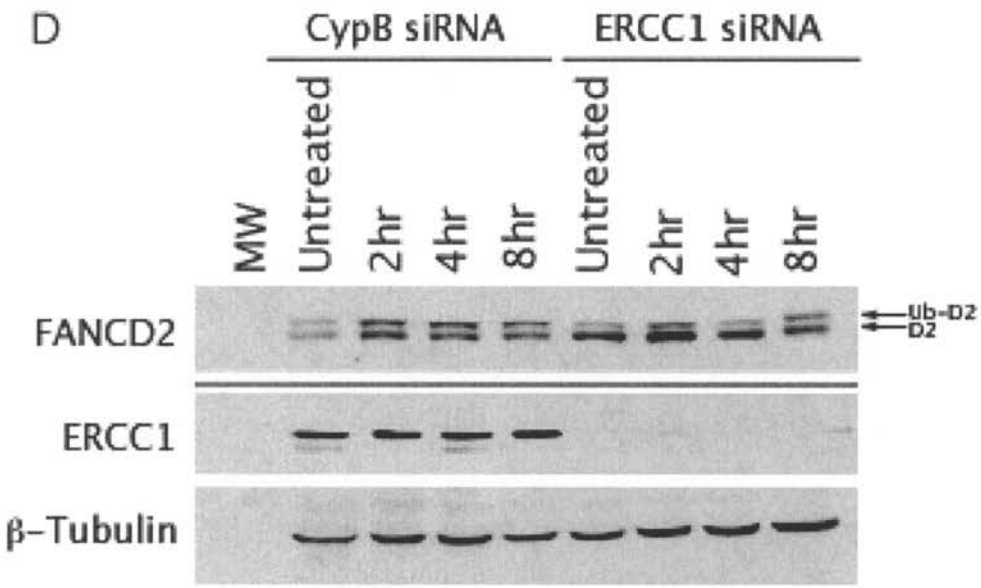
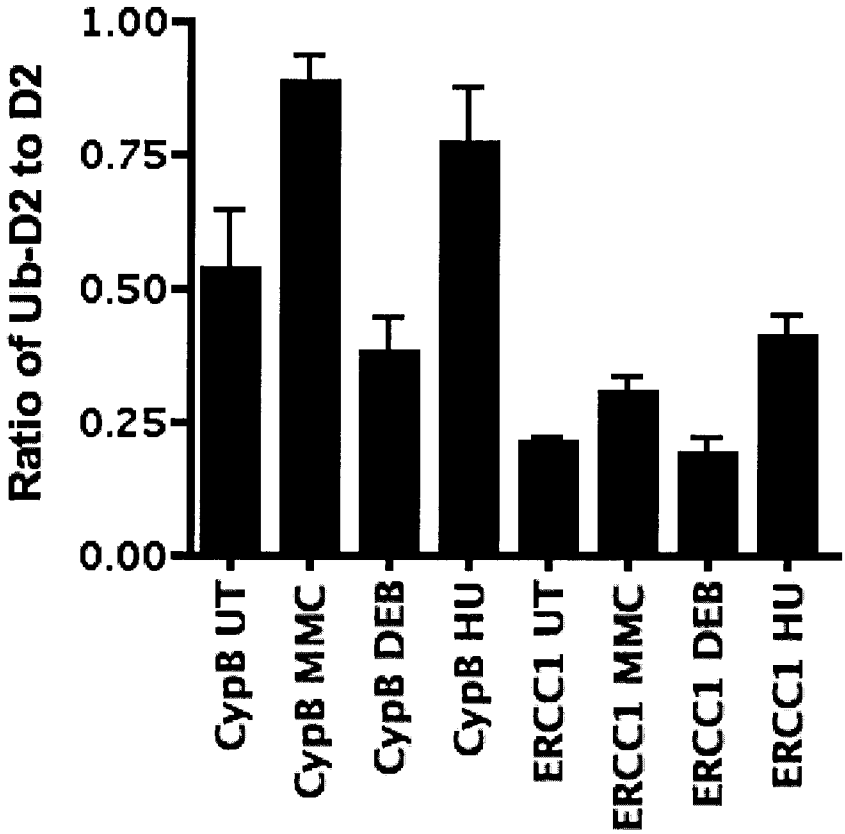

D
CypB siRNA
ERCC1 siRNA
MW
Untreated
2hr
4hr
8hr
Untreated
2hr
4hr
8hr
FANCD2
Ub-D2
D2
ERCC1
β-Tubulin

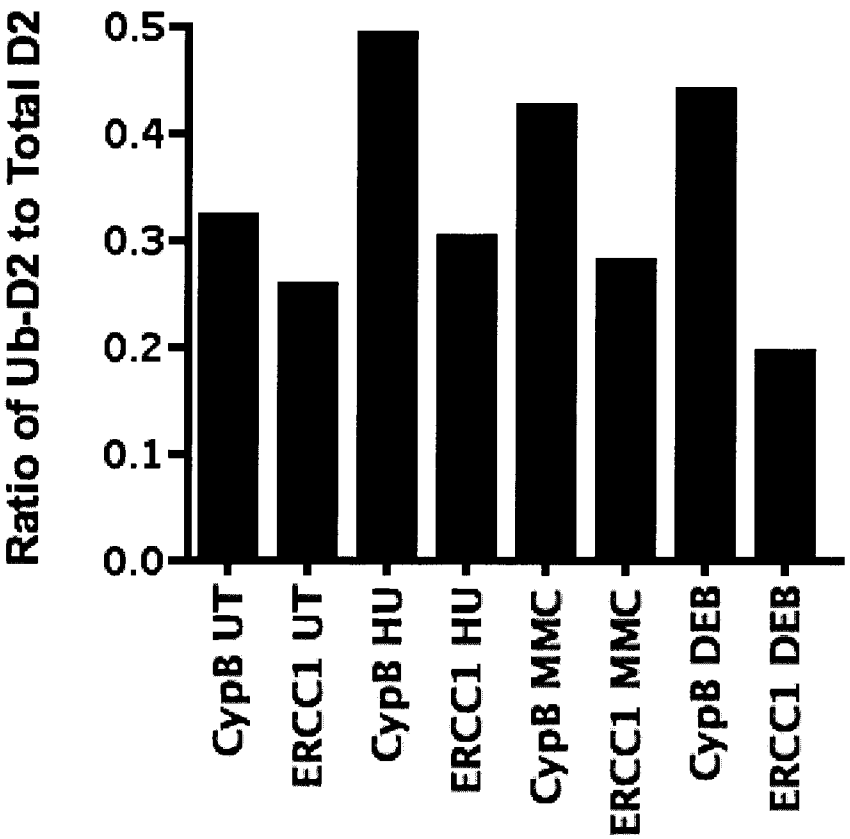
Figure 8 In all panels, space between blots denotes different sections of the same membrane. A line between blots, indicates different membranes. **8A.** hFANCD2 immunoblot with control CypB and hERCC1 depletion of GM639 cells. Clastogen treatment increased the ratio of the Ub-hFANCD2 upper band of the doublet to the lower band, whereas in the hERCC1 depleted GM639 cells, very little of the Ub-hFANCD2 was observed. hERCC1 immunoblotting demonstrated depletion with β-tubulin as a loading control. **8C.** γH2AX immunoblot of MMC time course as in 4B. Levels of γH2AX were similar at respective time points between control CypB and hERCC1 depleted lines with β-tubulin as a loading control. hERCC1 immunoblotting demonstrated depletion with β-tubulin as a loading control. **8D.** hFANCD2 immunoblot time course of MMC treatment. As in 8A and B, the ratio of Ub-hFANCD2 to hFANCD2 increased in control CypB depleted GM639 cells with MMC treatment at all time points. hERCC1 depletion led to lower ratio of Ub-hFANCD2 to hFANCD2 at all respective time points. hERCC1 immunoblotting demonstrated depletion with β-tubulin as a loading control.

Figure 9- Densitometry of Immunoblots from Figure 8

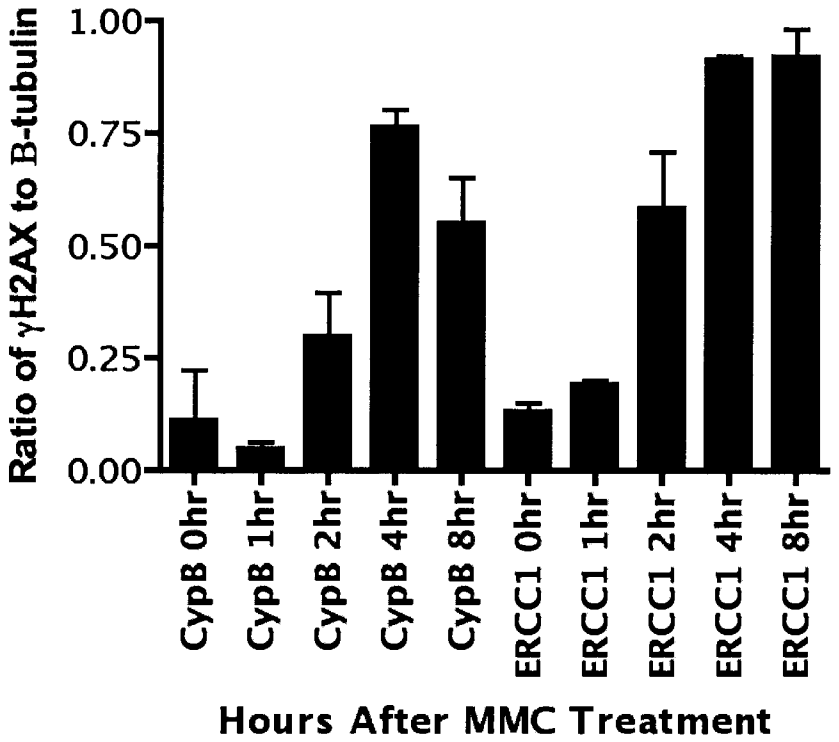
A



B


Ratio of Ub-D2 to Total D2
0.5
0.4
0.3
0.2
0.1
0.0
CypB UT
ERCC1 UT
CypB HU
ERCC1 HU
CypB MMC
ERCC1 MMC
CypB DEB
ERCC1 DEB

C


Ratio of γH2AX to B-tubulin
1.00
0.75
0.50
0.25
0.00
CypB 0hr
CypB 1hr
CypB 2hr
CypB 4hr
CypB 8hr
ERCC1 0hr
ERCC1 1hr
ERCC1 2hr
ERCC1 4hr
ERCC1 8hr
Hours After MMC Treatment

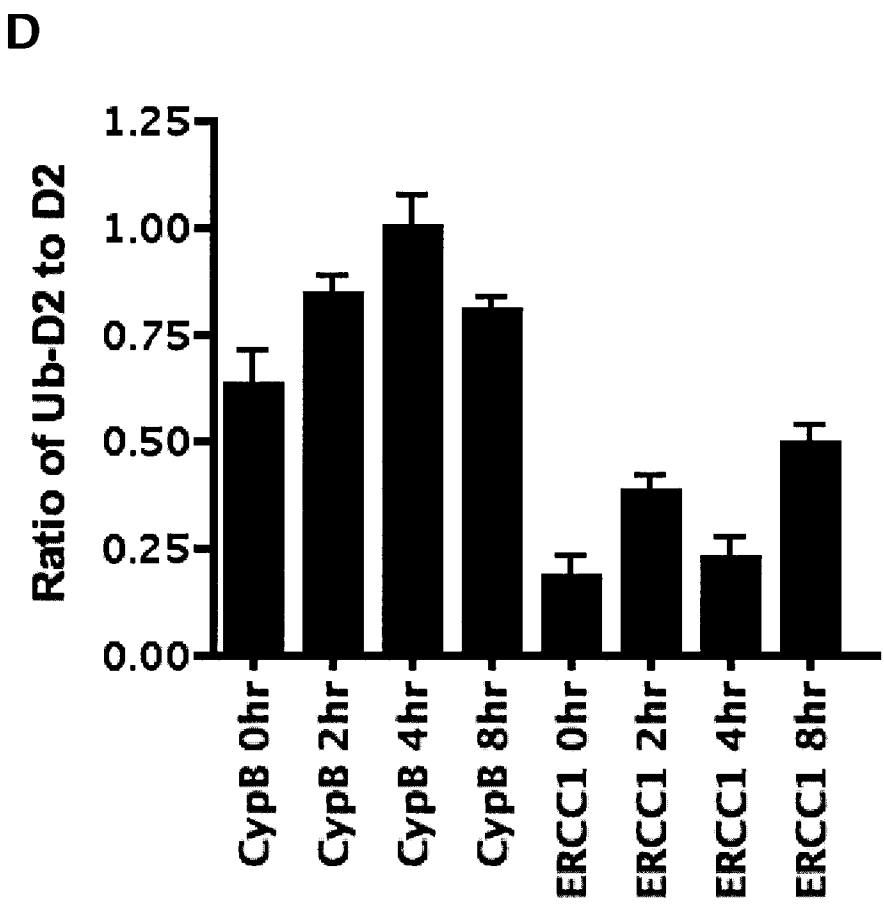


Figure 9 9A A graph representing densitometry of the immunoblot in Figure 8A. The ratio of Ub-D2 to D2 is shown. Densitometry measurements were performed in triplicate and the error bars represent the standard deviation of these measurements. **9B** A graph representing densitometry of the immunoblot in Figure 8B. The ratio of Ub-D2 to total D2 is shown. Densitometry measurements were performed using the Licor Odyssey software. **9C** A graph representing densitometry of the immunoblot in Figure 8C. The ratio of intensity of the γH2AX bands to the loading control β-tubulin bands is shown. Densitometry measurements were performed in triplicate and the error bars represent the standard deviation of these measurements. **9D** A graph representing densitometry of the immunoblot in Figure 8D. Densitometry measurements were performed in triplicate and the error bars represent the standard deviation of these measurements.

Chapter Four: Discussion and Conclusions

4.1 Introduction

The Fanconi anemia pathway serves to maintain genomic stability of complex organisms through coordination of several repair pathways that faithfully repair ICL damage. The exact manner in which the FA pathway, in concert with HR and NER, achieves this is still being uncovered. In these studies, we elucidated the role of hERCC1 in the repair of ICLs in the human immortal fibroblast cell line, GM639. More specifically, we tested the following hypothesis: the FA pathway is responsible for repair of DNA damage resulting from MMC, DEB and HU, indicating a common repair intermediate likely to be a recombination intermediate resulting from a stalled replication fork. We showed that FA pathway activation requires the action of hERCC1, and that hERCC1 action is in response to MMC and DEB and the potent inducer of stalled replication forks, HU. Therefore, we confirmed the hypothesis and showed how ERCC1 is involved in the FA pathway.

4.2 Functional knock-down of hERCC1 in GM639 cells

Sensitivity of ERCC1 and XPF cell lines to inter-strand crosslinks has led to the study of their role in the FA pathway. Using siRNA inhibition of hERCC1 in the human immortalized fibroblast cell line GM639, we demonstrated a substantial reduction in hERCC1 protein levels by western blot. In addition, we showed the knock-down is functional by demonstrating induced MMC sensitivity. This knock-down of hERCC1 did not affect cell cycle distribution, which is consistent with previously published reports.

4.3 ERCC1 functions in the FA pathway upstream of radial formation

Niedernhofer *et al.* (2004) showed ERCC1 -/- ES and CHO cells form radials at low levels spontaneously, and high levels with ICL inducing agents. In GM639 cells with depleted hERCC1, we observed results that are inconsistent with their findings. We observed low levels of radials both with and without MMC. As expected, these same GM639 cells when depleted of hFANCA showed low levels of spontaneous radials and high levels of ICL induced radials. When both hERCC1 and hFANCA were depleted, one would typically expect to find the level of radials associated with the loss of hFANCA, however, we observed a greater than 50% reduction in the formation of radials.

This result indicates that the MMC repair intermediate that is observed as a radial in chromosome spreads is produced downstream of the action of hERCC1 in the FA pathway. If the function of hERCC1 at this point in the FA pathway serves to trim 3' ssDNA overhangs adjacent to duplexed DNA at the sites of strand exchange (Figure 10), this observation can be more readily explained. It has been suggested that such overhangs prevent extension from the site of strand exchange, and result from a non-homologous tail failing to pair with nearby stretches of homology that are forming the duplex (Adair et al., 2000, De Silva et al., 2000, McHugh et al., 2001, Niedernhofer et al., 2001, Niedernhofer et al., 2004, Tsodikov et al., 2005). This is consistent with a previous study indicating that all of the radials observed in FA cells are between non-homologous chromosomes (Newell et al., 2004). Recombination between such short stretches of homology flanked by non-homologous regions would therefore be expected to form with recombination between non-homologous chromosomes, and would only be further

stabilized by extension at the site of strand exchange (Figure 10). If this extension can only occur after the actions of hERCC1 removing the non-homologous tail, then it follows that in the absence of hERCC1, there are fewer radials than in hFANCA cells, presumably whose defect in ICL repair lies downstream.

4.4 hERCC1 is not required for formation, but repair of DSB in response to ICL

Previous studies have indicated differing roles for ERCC1 in ICL repair. Rothfuss and Grompe (2004) described a role for ERCC1 in the initial incision of psoralen-UVA crosslinks and maintained that ERCC1 was required for the formation of DSB in S-phase. The work of Niedernhofer *et al.* (2004) indicated that ERCC1 was not required for formation of DSB in S-phase, but rather was required for the repair of DSB formed after MMC and cisplatin treatment. The studies herein clear up some of this confusion.

In our system, with hERCC1 knocked-down in an immortalized human fibroblast line, we observed only a slight decrease in the γH2AX focus formation in the hERCC1 knock-down cells as compared to control cells. While this slight decrease may be suggestive of a role for hERCC1 in the initial incision, this slight difference in γH2AX focus formation was mirrored in the HU sample, which contains no crosslinks. Therefore from this experiment, we can only conclude that hERCC1 is not absolutely required for formation of DSB in response to MMC and DEB and stalled replication forks, however it may play a role in early incision that our current studies do not directly address.

In order to examine the phosphorylation of H2AX in more detail, a time-course of MMC treatment was conducted. At 1, 2, and 4 hr after MMC treatment, γH2AX levels increased to similar levels in both control and ERCC1 siRNA treated cells, supporting our earlier data from the immunofluoresence that hERCC1 was not required for DSB formation in response to MMC or DEB. At the 8hr time point in the MMC time-course there was a decrease in the γH2AX levels in the control cells as compared to the hERCC1 knock down. This observation is consistent with the observation of Niedernhofer *et al.*

(2004) that ERCC1 is required for the repair of DSB formed as the result of interstrand crosslinking agent induced damage.

4.5 hERCC1 is required for activation of the FA pathway in response to ICL

A leading hypothesis in the FA field has put forth that the FA pathway is activated by DSB (Lyakhovich and Surralles, 2007, Rothfuss and Grompe, 2004). Our studies support a competing hypothesis (Hussain et al., 2004, Thompson et al., 2005, Yamamoto et al., 2003), that the FA pathway is activated by recombination intermediates. While we show hERCC1 was not required for formation of DSB in response to MMC, DEB or HU treatment, we demonstrated the requirement of hERCC1 for monoubiquitination of hFANCD2 not only in response to MMC and DEB, but also HU, which does not form ICL.

Immunofluoresence of hFANCD2 in control and hERCC1 depleted cells indicated that hFANCD2 focus formation was nearly eliminated when there was no hERCC1 present. In untreated control cells and in MMC and DEB treated hERCC1 depleted cells, the levels of hFANCD2 foci were lower than in the untreated control in the control knock-down cells. Only in the HU treated hERCC1 depleted cells, did the hFANCD2 focus levels approach the untreated controls, which was still 3-fold reduced from HU treated control depleted cells.

Immunoblotting for FANCD2 confirms this result was not merely a localization defect, but was a result of hERCC1 depleted cell's inability to monoubiquitinate hFANCD2. In untreated, MMC, DEB, and HU treated ERCC1 depleted cells, the ratio of Ub-hFANCD2 to hFAND2 was lower than the control depleted cells. In addition, the MMC time course indicated that the inability to monoubiquitinate hFANCD2 held beyond 8hr and was not readily bypassed by an alternate pathway.

4.6 Discussion

From the cumulative evidence, we conclude that hERCC1 acts downstream of DSB formation in the FA pathway, while still requiring its action on an intermediate formed by both the early stages of MMC and DEB repair and the resolution of stalled replication forks resulting from HU treatment. This intermediate will likely be a product of mitotic recombination between non-homologous chromosomes, in which an unpaired stretch of non-homologous DNA neighboring a stretch of paired more highly conserved DNA must be cleaved for extension and stabilization of this intermediate (Figure 10). A lack of hERCC1 results in a less stable intermediate, due to only the short stretch of homology base-pairing, reducing the observed radial formations. In addition, it seems the action of the FA pathway is involved in resolution of such an intermediate. With the recently defined FANCJ having a 5'-3' helicase and FANCM/FAAP24 dimer thought to have translocase activity, it may follow that these downstream FA proteins may resolve such a mitotic recombination between non-homologous chromosomes (Adair et al., 2000, Cantor et al., 2004, Cantor et al., 2001, Ciccia et al., 2007, Levitus et al., 2005, Levran et al., 2005).

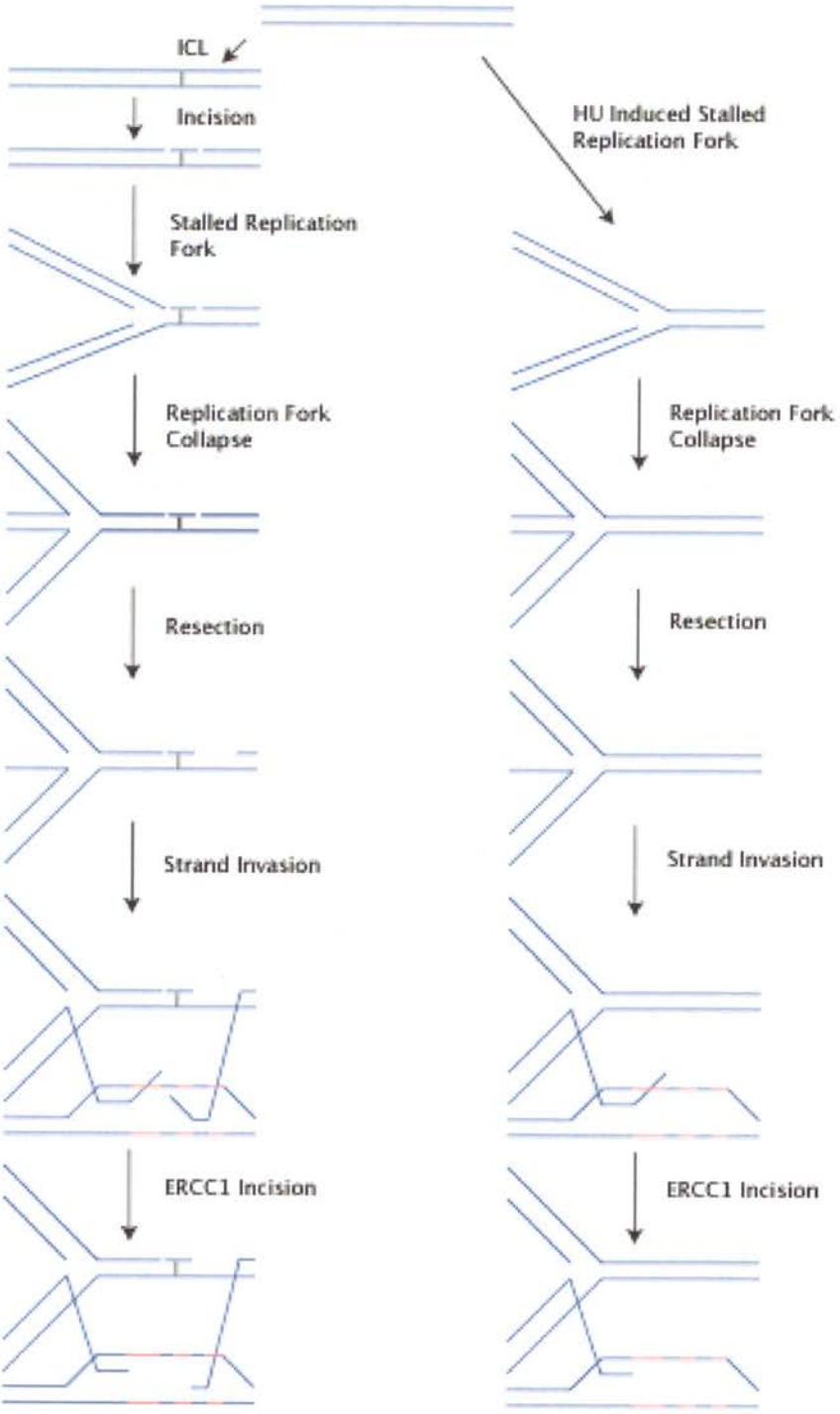
Data from the dissertation of A.H. Newell, however, indicates radials may not be the result of recombination. Depletion of BRCA2 and RAD51, thought to be required for recombination, increases radials. Perhaps the requirement of ERCC1 for activation of the FA pathway in response to ICL inducing agents and HU stems from its incision activity as shown in an alternate model in Figure 11. The structure resulting from replication forks meeting at an incised versus an un-incised molecule would be quite different. It is also possible for ERCC1 to incise the stalled replication forks themselves as shown in the

case of HU induced stalled replication forks (Figure 11). The role of ERCC1 in reducing radials in this case seems less clear. However, given how little we know regarding the molecular structure of radials, study of the role of ERCC1 in the repair of stalled replication forks resulting from ICLs and HU, and its capacity to reduce radials may yet provide great insight into what these radials represent, and how loss of FA proteins leads to their formation.

Recent publications demonstrate that additional NER proteins aside from ERCC1 and XPF play a role in the initial recognition and incision of ICLs (Ahn et al., 2004, Thoma et al., 2005). Thoma *et al.* (2005) showed XPC-hHR23R and XPA-RPA complexes bound to psoralen ICLs together, demonstrating that the global genome repair sub-pathway of NER may be involved in the recognition of psoralen ICLs. If this is the case, why are cell lines lacking these proteins not sensitive to ICLs?

Our findings together with those of Niedernhofer *et al.* (2004) show that the important role of ERCC1 is in resolution of recombination DSB-containing intermediates by the FA pathway created at later steps of ICL repair downstream of the initial incision steps. It may be that the other NER proteins are involved in ICL repair, as in yeast, at the initial steps of recognition and repair, but that in mammals, sufficient alternate mechanisms for creation of intermediates upon which downstream proteins can act, that loss of these other NER proteins is not as severe a deficit as in yeast. The sensitivity of ERCC1 and XPF deficient lines may lie, not in their action in the initial incision as suggested by Rothfuss and Grompe (2004) and many others, but rather in their action on replication or recombination intermediates as observed in these and other studies.

Figure 10- Model of ICL and Stalled Replication Fork Repair



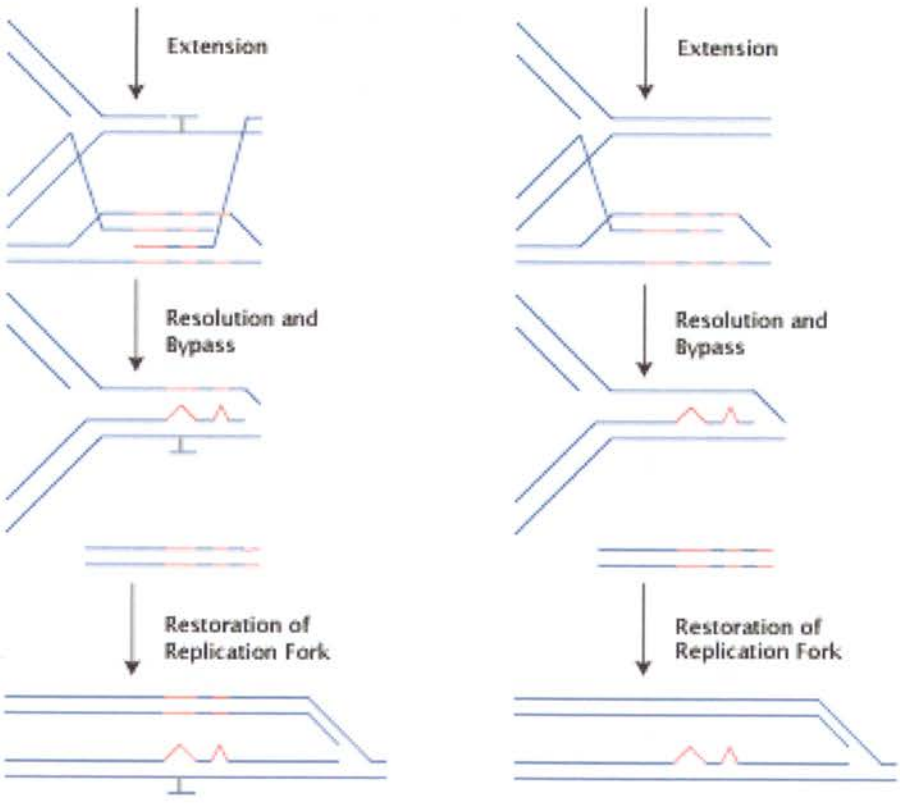


Figure 10 Model of ICL and stalled replication fork repair with a shared intermediate upon which ERCC1 could act, involving recombination between non-homologous chromosomes. Action of ERCC1 upon this substrate permits extension along the non-homologous chromosome, stabilizing this intermediate. It may be this intermediate that is observed in chromosome spreads as radials. Following extension, the ICL can be bypassed ,and, ultimately, the replication fork can be restored. Lines represent single strands of DNA with the colors denoting homology; those strands with the same color share homology, those with differing colors represent heterologous sequences.

Figure 11- Alternate Model of the Role of ERCC1 in ICL and Stalled Replication Fork Repair

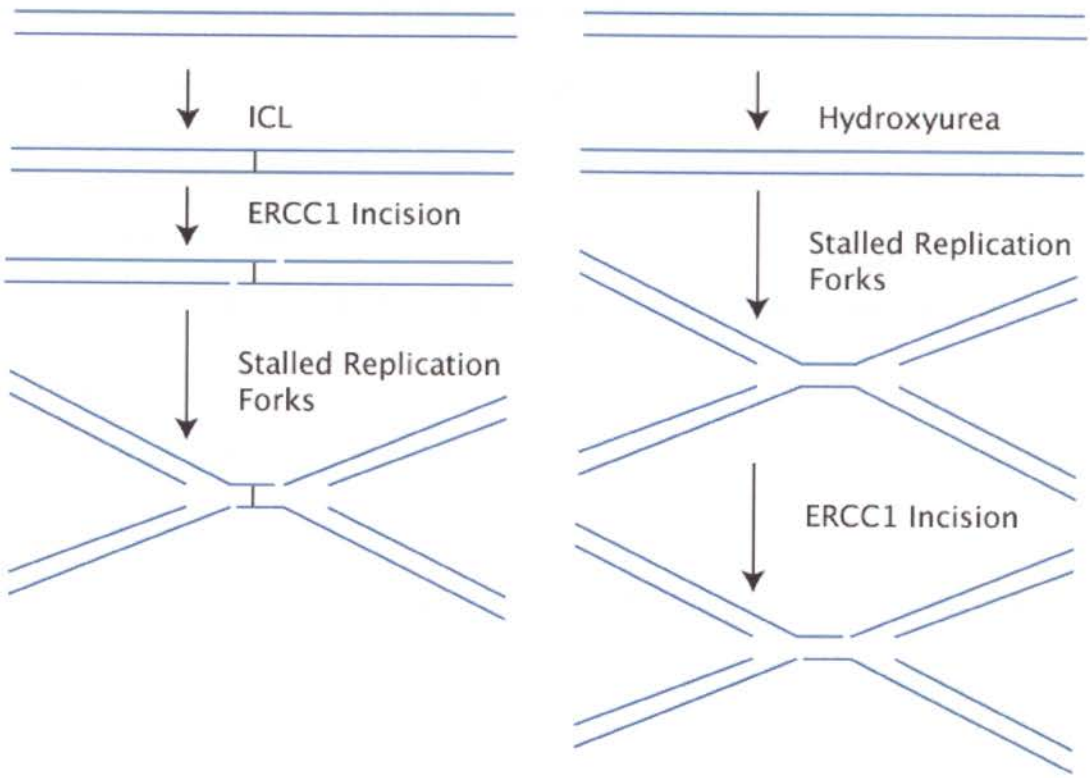


Figure 11 An alternate model for the role of ERCC1 in ICL and stalled replication fork repair. ERCC1s role in the initial incision could lead to a different structure in S-phase as replication forks reach an ICL. Additionally, ERCC1 could play a similar role in resolution of stalled replication forks resulting from HU treatment.

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