DOCTOR OF PHILOSOPHY

The role of nucleotide excision repair in providing resistance to the nucleoside analogue gemcitabine.

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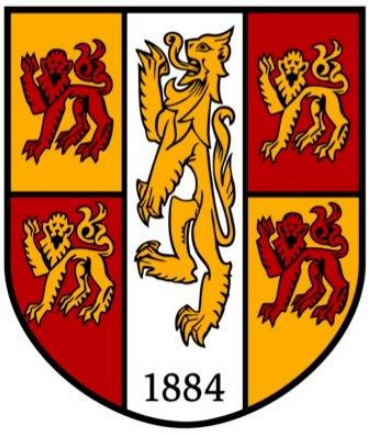
Award date:
2017

Awarding institution:
Bangor University

THE ROLE OF NUCLEOTIDE EXCISION REPAIR IN PROVIDING RESISTANCE TO THE NUCLEOSIDE ANALOGUE GEMCITABINE



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This dissertation is submitted for the degree of Doctor of Philosophy

January 2018

ABSTRACT

Gemcitabine is a clinically important chemotherapy drug, used to treat a variety of solid tumours. It is a cytidine analogue and when inserted into DNA in place of cytidine serves to inhibit further chain extension, causing replication stress and ultimately cell death. The nucleotide excision repair (NER) pathway is known to repair a variety of bulky DNA lesions but is not known to have a role mitigating replication stress. However, it was found in a *S. pombe* screen that mutants lacking homologues of *xpa*, *xpc*, *xpf* and *ercc1* showed sensitivity to gemcitabine. Further to this, patient fibroblasts mutated in NER genes were shown to be sensitive to gemcitabine when compared to NER proficient fibroblasts.

The sensitivity of these mutants to gemcitabine is not explained by the current understanding of NER and this project set out to unearth a new role for NER factors in gemcitabine resistance in human cells. However, data presented here show large differences in sensitivity between two NER proficient fibroblast lines MRC-5 and GM637, which confounded the previous work showing NER contributed to gemcitabine resistance in human cells. Different experimental strategies which enabled the use of controls with the same genetic background were then employed to circumvent this problem. Two ERCC1 knockout cell lines, generated via CRISPR-Cas9 in MRC-5 and HEK293 backgrounds, were characterised as part of this project. ERCC1 was shown to have a role in gemcitabine resistance in MRC-5 cells, but not HEK293 cells. The role of XPA was investigated by using a human lymphoblast (TK6) knockout cell line and complementation of an XPA deficient fibroblast line with a functional copy of the gene and neither approach showed any effect of XPA on gemcitabine resistance. XPC and XPG were also investigated by complementation of deficient fibroblasts which resulted in a small rescue of gemcitabine sensitivity and a small sensitisation respectively.

A role for ERCC1 in gemcitabine resistance in human cells has not been previously reported and may have both clinical implications and implications for understanding the processes which occur at stalled replication forks. It is unclear from these results whether this role is related to NER or the NER-independent functions of this gene, but there is a suggestion that a second NER factor, XPC may also be involved. The cell line dependence of the role of ERCC1 also suggests that the DNA repair response to gemcitabine and the role of NER factors in this process differs between cell lines, and this should be an important consideration for future work on this topic.

ACKNOWLEDGEMENTS

I would like to thank Tenovus Cancer Care for funding this project. Thank you to my supervisor Dr. Edgar Hartsuiker for his guidance and encouragement. I would also like to thank all the members of D2 and D7 labs, particularly Dr. Rick Beardmore, Dr. Karim Garrido, Dr. Ellen Vernon and Dr. Jana Jezkova off of whom I have frequently stolen reagents and learnt a great deal. I am indebted to Dr. Rolf Kraehenbuehl who has spent hours in a basement coaxing results from a temperamental mass spectrometer. I am very grateful to my wonderful partner Jim McCormack and my family and friends for their unwavering support.

I received excellent training in DNA fibres from Dr. Eva Petermann, Dr. Rebecca Jones and Dr. Panos Kotsantis at Birmingham University and excellent training in microscopy from Prof. Cath Green at Oxford University.

Thank you to Kayla Friedman and Malcolm Morgan for producing the Microsoft Word thesis template used to produce this document and the courageous Alexandra Elbakyan without whom access to articles would have been a major problem. Thank you also to the indomitable people of Sierra Leone and the inspirational Jade 'Jariatu' Richards.

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LIST OF ABBREVIATIONS AND ACRONYMS

AML	Acute myelogenous leukaemia
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3 related
BER	Base excision repair
BIR	Break induced replication
BLM	Bloom syndrome helicase
BRCA	Breast cancer
CHO	Chinese hamster ovary
CldU	5-chloro-2'-deoxyuridine
CPD	Cyclobutane pyrimidine dimers
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CS	Cockayne syndrome
CSA	Cockayne syndrome A
CSB	Cockayne syndrome B
CSK	Cytoskeletal buffer
dCK	Deoxycytidine kinase
dHJ	Double Holliday junction
DNA2	DNA replication ATP dependent helicase 2
dNTP	Deoxynucleotide
DPBS	Dulbecco's phosphate buffered saline
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DSB	Double strand break
EC_{50}	Effective concentration
EdU	5-ethynyl-2'-deoxyuridine
ERCC1	Excision repair cross-complementing group 1
ES cells	Embryonic stem cells
FA	Fanconi anaemia
FBS	Fetal bovine serum
Gemcitabine-TP	Gemcitabine triphosphate
GG-NER	Global genome NER
hCNT	Human concentrative nucleoside transporter
hENT	Human equilibrative nucleoside transporter

HJ	Holliday junction
HR	Homologous recombination
HRP	Horse radish peroxidase
HU	Hydroxyurea
ICL	Interstrand crosslink
IdU	5-iodo-2'-deoxyuridine
iPOND	Isolation of proteins on nascent DNA
IR	Ionising radiation
LC/MS/MS	Liquid chromatography tandem mass spectrometry
MCM	Mini chromosome maintenance helicase
MEFs	Mouse embryonic fibroblasts
MMC	Mitomycin C
MMR	Mismatch repair
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
ORC	Origin recognition complex
PARP	Poly(ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
Pol	Polymerase
6-4PP	6-4 photoproduct
RFC	Replication factor C
RNR	Ribonucleotide reductase
RPA	Replication protein A
RPMI	Roswell park memorial institute medium
RT	Room temperature
S. pombe	*Schizosaccharomyces pombe*
SDSA	Synthesis dependent strand annealing
SSA	Single strand annealing
SSB	Single strand break
ssDNA	Single stranded DNA
TC-NER	Transcription coupled NER
TFIIH	Transcription factor II H
TLS	Translesion synthesis
TTD	Trichothiodystrophy
TopBP1	Topoisomerase binding protein 1
UDS	Unscheduled DNA synthesis

UV-DDB	UV-damage binding protein
XP	Xeroderma pigmentosum

1 INTRODUCTION

1.1 DNA repair and the hallmarks of cancer

Cancer is initiated by the stepwise accumulation of characteristics which allow it to escape normal regulatory mechanisms and proliferate out of control (Vogelstein and Kinzler, 1993). Hanahan and Weinberg (2000) proposed 6 distinct abilities, briefly described below, that must be acquired by cancer cells in order to become tumorigenic. They include the ability to sustain proliferative signalling, evade growth suppressors, activate invasion and metastasis, achieve replicative immortality, induce angiogenesis and resist cell death. Cells can respond to growth promoting signals by way of cell surface receptors that, once bound to their ligand, activate signalling cascades which alter gene expression to allow entry into and progression through the cell cycle. Gain-of function mutations, amplifications or rearrangements of proto-oncogenes can allow for the sustained activation of this system in a number of ways, such as enabling the secretion of growth factors for autocrine signalling, or constitutive activation of a receptor or downstream elements of the pathway (Lemmon and Schlessinger, 2010; Polsky and Cordon-Cardo, 2003). Several pathways act to negatively regulate cell growth and the genes important for this function are known as tumour suppressors, of which RB and P53 are important examples. Loss of activity of tumour suppressor genes by mutation or epigenetic silencing permit the sustained proliferation of cancer cells. The ability of cancer cells to migrate and invade other tissues can be triggered by the loss of proteins that govern adherence to neighbouring cells and extracellular matrix such as E-cadherin (Berx and van Roy, 2009). Downregulation of cell to cell adherence proteins can occur as part of a wider transcriptional programme which facilitates metastasis known as the epithelial to mesenchymal transition

(Polyak and Weinberg, 2009). Chromosome ends are protected by noncoding stretches of repetitive DNA known as telomeres, which are eroded at every cell division. Complete erosion of the telomeres leads to fusions between chromosomes, resulting in senescence and cell death and therefore cells are limited in their replicative potential by the length of their telomeres (Blasco, 2005). Cancer cells commonly circumvent this limitation and achieve replicative immortality by overexpressing telomerase or maintaining their telomere length by a recombination dependent mechanism (termed alternative lengthening of telomeres, Henson et al., 2002). Tumours induce the formation of new blood vessels to encourage a supply of oxygen and nutrients with which to support their proliferation. Cancer cells are known to secrete growth factors, for example VEGF-A, which bind to cell surface receptors on vascular endothelial cells and stimulate blood vessel branching (Potente et al., 2011). Programmed cell death, or apoptosis normally ensures the destruction of cells which are at risk of becoming tumorigenic. It can be initiated by extracellular signals, such as FAS and tumour necrosis factor, or intracellular signals such as the accumulation of DNA damage which is transduced by P53. The end result is the activation of proteases known as caspases which cleave specific substrates leading to the destruction of the cell. In order to resist cell death cancer cells can acquire various alterations in this pathway. They may become insensitive to pro-apoptotic signals, often through the loss of P53, or alternatively suppress the activity of the apoptotic caspases or upregulate anti-apoptotic signals such as BCL-2 (Fernald and Kurokawa, 2013).

More recently two additional hallmarks have been proposed; the reprogramming of cancer cell energy metabolism from aerobic respiration to glycolysis and mechanisms for avoiding detection by the immune system. The hallmarks are commonly acquired by mutation and for this reason genomic instability is considered an important cancer enabling characteristic as it allows mutations to accumulate at a faster rate (Hanahan and Weinberg, 2011). Genomic instability is promoted by defects in DNA repair and the experience of replication stress (which is itself exacerbated by defects in DNA repair and the activation of oncogenes, Gaillard et al., 2015). Genomic instability is also promoted by exposure to mutagens, including mutagenic cancer treatments, which means that while these treatments have the ability to kill cancer cells, they can also enable the acquisition of hallmark capabilities.

Thousands of DNA lesions will occur each day in every cell and in order to deal with this threat cells have a number of DNA repair pathways that are specialised to deal with different kinds of lesions and guard against the acquisition of mutations (Fig. 1.1, reviewed in Ciccia and Elledge, 2010).

Deficiencies in DNA repair, which can be acquired by a somatic mutation or inherited via a germline mutation, hasten this process as unrepaired lesions give rise to mutations. Consistent with this model, many sporadic cancers harbour DNA repair abnormalities and many cancer-prone syndromes such as Xeroderma pigmentosum, Lynch Syndrome and Fanconi Anaemia result from the inheritance of a defective DNA repair gene. The genomic instability associated with deficient or aberrant DNA repair can then be harnessed by the tumour to drive tumour progression and evade responses to therapy (Birkbak et al., 2011; Sansregret et al., 2017).

As described above, disruption of DNA repair can play an important role in the initiation and progression of cancer, but it can also affect how the cancer cells respond to drug treatment. Most chemotherapy drugs are DNA damaging agents and a certain threshold of damage must be achieved for the treatment to be effective. Therefore in the context of chemotherapy DNA repair can be counterproductive and lead to drug resistance. Many cancers display defective DNA repair in comparison to surrounding healthy tissue (Nik-Zainal et al., 2012; Vogelstein et al., 2013), and abnormalities in DNA repair can be targeted to provide selective tumour cell kill (reviewed in Gavande et al., 2016). Many chemotherapy drugs cause a specific type of lesion. If a tumour is deficient in its ability to repair a specific lesion and the healthy tissue is proficient, treating with an agent that causes that specific lesion will maximize the cytotoxic effect in the tumour cells relative to healthy tissue.

This synthetic lethality approach is exploited successfully in the treatment of cancers without functional copies of breast cancer (BRCA) 1 or 2 genes with poly (ADP-ribose) polymerase (PARP) inhibitors. PARP is involved in the repair of single strand breaks, to which it binds and recruits other repair proteins. Inhibiting PARP results in these lesions being converted to double strand breaks which are repaired by homologous recombination. The presence of this redundant pathway means that this is not a problem for normal cells however, BRCA deficient cells are defective in homologous recombination making them over 1000 times more sensitive to PARP inhibition (Bryant et al., 2005; Farmer et al., 2005; Lord et al., 2015). Incidentally, it has since been found that PARP inhibitors have a greater sensitising effect on cells than just deleting PARP. This has been explained by phenomenon termed PARP trapping, where instead of the inhibitors preventing PARP from binding to DNA ends, they prevent its dissociation from DNA. These PARP-DNA complexes are more cytotoxic and difficult to repair than breaks lacking any PARP (Murai et al., 2012).

Likewise, low expression of the repair factor excision repair cross-complementing 1 (ERCC1), which is central to the repair of intra- and interstrand crosslinks is a promising biomarker for

sensitivity to the crosslinking agent cisplatin (Bepler et al., 2011; Olaussen et al., 2006). Full exploitation of this approach is hampered by gaps in our knowledge of the interaction between different DNA repair factors and chemotherapy drugs. This project aims to contribute to this area by investigating the interaction between a DNA repair pathway, nucleotide excision repair (NER) and a chemotherapy drug, gemcitabine, in mammalian cells. This introduction will first give a detailed description of nucleotide excision repair and outline the other DNA repair pathways operating in eukaryotic cells, then go on to describe gemcitabine and its mechanism of action and finally discuss what is known about gemcitabine resistance and the ways in which NER factors may contribute to it.

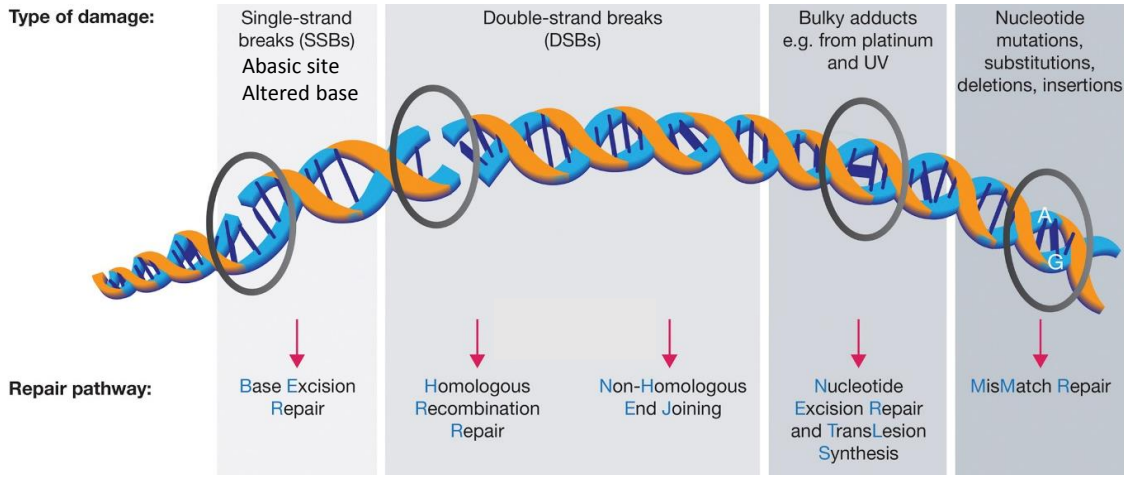


Figure 1.1 Illustration of different types of DNA damage and the specialised repair pathways which respond to them. Adapted from O'Connor (2015)

1.2 DNA repair pathways

The integrity of DNA is under threat from many different endogenous and exogenous sources of damage which create distinct lesions requiring repair by specialised pathways. Endogenous sources of damage include: the mispairing of bases, which can occur during normal replication; reactive oxygen species generated by metabolic processes which can cause oxidation of bases or breaks; and the alteration or loss of bases which can occur spontaneously. Base mispairs are repaired by a combination of polymerase proofreading activity and mismatch repair (MMR), while chemically altered or absent bases are repaired by either direct reversal or base excision repair (BER). UV light and ionising radiation (IR) are exogenous sources of damage. UV causes the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), in which adjacent bases bind causing a distortion of DNA structure. These and other

helix distorting lesions are repaired by nucleotide excision repair (NER). Ionising radiation can cause double strand breaks, as well as single strand breaks and oxidation of bases. Double strand breaks are considered the most cytotoxic lesion as a single DSB can be sufficient to cause cell death, and are mostly repaired by either homologous recombination (HR) or non-homologous end joining (NHEJ, Bennett et al., 1993). Environmental mutagens and chemotherapy drugs are also exogenous sources of DNA damage of various kinds.

1.2.1 Nucleotide excision repair

Nucleotide excision repair is a versatile DNA repair process, conserved between prokaryotes and eukaryotes (Fig. 1.2). It removes bulky, helix-distorting lesions from DNA, such as the UV photoproducts cyclobutane-pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs), intrastrand crosslinks caused by cisplatin and lesions formed by several different kinds of environmental mutagens (Gillet and Schärer, 2006). Deficiency or loss of core NER factors in humans can give rise to the genetic disorder Xeroderma pigmentosum (XP). XP is characterised by extreme photosensitivity, which manifests as sunburn reaction and changes in pigmentation and elevated risk of developing skin cancer (Lehmann et al., 2011). These symptoms stem from an inability to efficiently repair UV photoproducts and the main clinical test for XP measures the amount of DNA synthesis in non-replicating cells after UV exposure, termed unscheduled DNA synthesis (UDS). Complementation analysis of cells from XP patients revealed 7 complementation groups, which correspond to 7 XP genes (A-G) involved in the core NER reaction. There is one other complementation group (XP-V) which is proficient in NER but deficient in a separate process, translesion synthesis, in which the damaged bases are traversed by a low fidelity polymerase (pol η) during replication and is also important for survival after UV irradiation (reviewed in Lehmann et al., 2011). The core NER reaction can be reconstituted in cell free extracts with the factors XPA, XPC, the TFIIH complex (containing XPB and XPD), XPF, ERCC1, XPG, replication protein A (RPA), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), DNA polymerase ε or δ and DNA ligase I (Aboussekhra et al., 1995; Araújo et al., 2000) .The basic steps of NER involve lesion recognition, dual incisions made 5’ and 3’ of the damage site which releases an oligonucleotide containing the damage, repair synthesis where the resulting gap is filled and finally ligation of the synthesised DNA. The role of these factors and the steps of NER will be discussed in detail below.

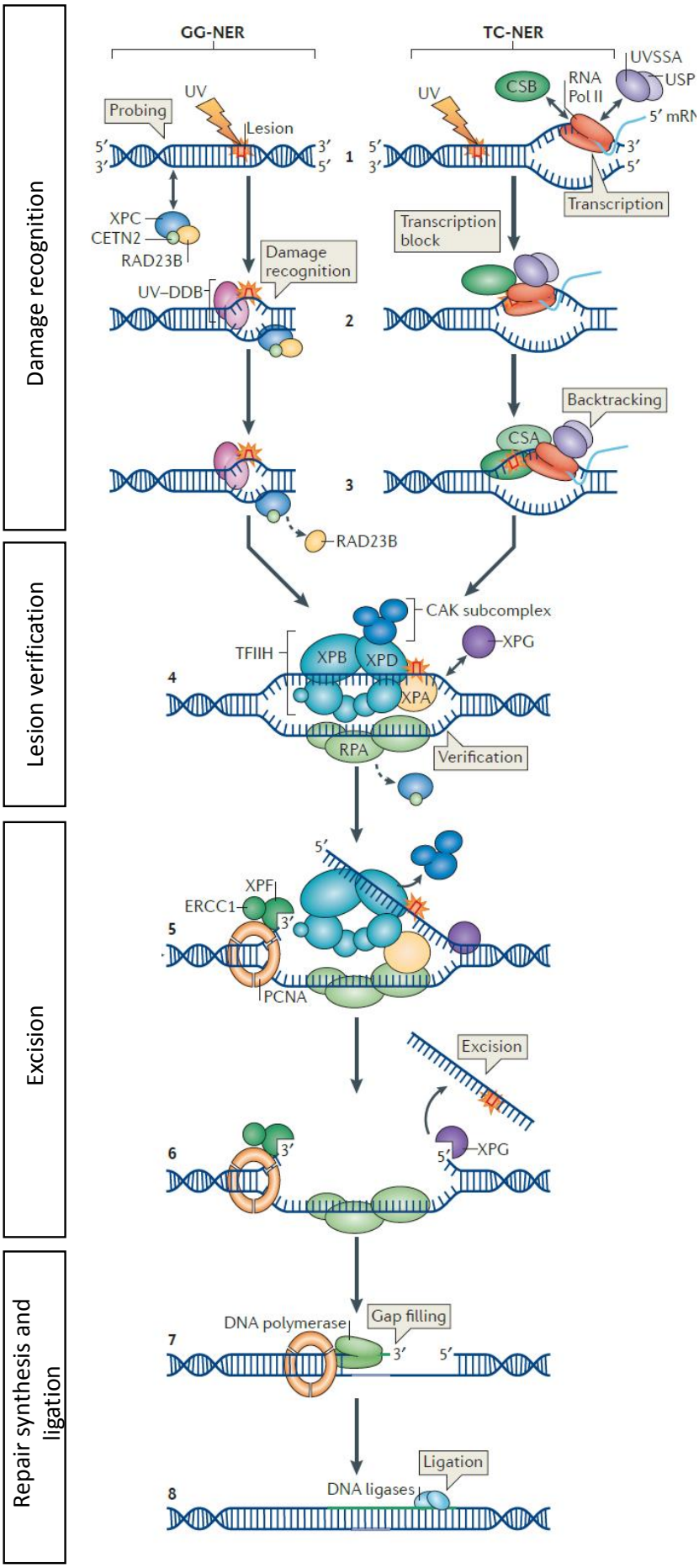

Damage recognition
Lesion verification
Excision
Repair synthesis and ligation
GG-NER
TC-NER
UV
Probing
Lesion
XPC
CETN2
RAD23B
Damage recognition
UV–DDB
UV
CSB
RNA Pol II
UVSSA
USP
5′ mRN
Transcription
Transcription block
Backtracking
CSA
RAD23B
CAK subcomplex
XPG
TFIIH
XPB
XPD
XPA
RPA
Verification
XPF
ERCC1
PCNA
Excision
XPG
DNA polymerase
Gap filling
DNA ligases
Ligation

Figure 1.2 Diagram to show the steps of the NER pathway and the roles of core NER factors (Marteijn et al., 2014). Lesions are recognised as NER substrates either by XPC in the case of global genome NER (GG-NER) or RNA polymerase II in the case of transcription coupled NER (TC-NER). The transcription factor II H (TFIIH) complex is recruited and the combined actions of XPB and XPD open a repair bubble around the lesion. XPA interacts with several factors and ensures correct positioning. The XPG nuclease is recruited which allows the ERCC1-XPF heterodimer to make an incision 3' of the lesion. XPG then cuts 5' of the lesion and releases a damage-containing oligonucleotide. DNA polymerase and PCNA synthesize across the gap and the remaining nick is ligated by DNA ligase I.

1.2.1.1 Lesion recognition

There are two ways in which NER can be initiated (Fig. 1.2). Transcription coupled NER (TC-NER) is initiated when RNA polymerase II (RNA Pol II) stalls at a lesion during transcription. This mechanism prioritises the repair of the template strand of actively transcribed genes. In contrast Global genome repair (GG-NER) involves recognition of a lesion by XPC, which probes the entire genome for damage and is transcription independent.

Recruitment of XPC to a lesion is required for the recruitment of the other NER factors (Volker et al., 2001). XPC has been shown to bind small DNA bubbles even in the absence of damaged bases, such as those caused by mismatches, and this is thought to be the basis of its ability to recognise diverse lesions. XPC does not recognise the lesion itself, rather the distorted DNA opposite (Schärer, 2013; Sugasawa et al., 2001). It is therefore not well suited to recognising lesions which do not much distort the helix, as is the case for CPDs, and for these lesions it requires some molecular signposting from UV-damage binding protein (UV-DDB). This protein binds to photoproducts with greater affinity and facilitates their discovery by XPC (Scrima et al., 2008; Sugasawa et al., 2005). XPC binding is not sufficient for the incision reaction to take place and therefore another factor must be responsible for verifying the presence of a lesion (Sugasawa et al., 2001).

TC-NER does not require XPC or UV-DDB but does require all the downstream NER factors. RNA Pol II stalls when it comes into contact with lesions such as CPDs, which is thought be the initial event in TC-NER (Selby et al., 1997). The large RNA Pol II molecule prevents access to the lesion so it must first be moved or degraded (Tornaletti et al., 1999). Current evidence suggests in repair proficient cells RNA Pol II reverses along the DNA strand until the lesion is exposed (Sigurdsson et al., 2010). The proteins Cockayne Syndrome A (CSA) and Cockayne

Syndrome B (CSB) are essential for TC-NER and although their roles are incompletely understood, they are necessary for recruitment of the NER machinery and may have a role in the backtracking of the polymerase (Citterio et al., 2000; Fousteri et al., 2006).

1.2.1.2 Lesion verification and dual incision

Following lesion recognition by one of the mechanisms described above the transcription and NER factor complex, transcription factor II H (TFIIH), is recruited to the lesion. TFIIH is composed of 10 subunits including the helicases XPB and XPD (Compe and Egly, 2012). The ATPase activity of XPB is required for NER but not the helicase activity and its structure indicates XPB has a role in opening the DNA structure around the lesion (Coin et al., 2007; Fan et al., 2006). The helicase activity of XPD is required for NER and several lines of evidence suggest XPD translocates along the DNA but is blocked by the presence of a lesion, thereby providing a mechanism for damage verification (Coin et al., 2007; Naegeli et al., 1992; Sugasawa et al., 2009).

XPA, RPA and XPG can then be recruited to the complex. XPA is an essential NER factor, however its role is poorly defined. XPA interacts with a large number of factors within the complex including RPA, ERCC1-XPF, PCNA, TFIIH, XPC and DDB2 (reviewed in Schärer, 2013), suggesting a role in co-ordination of the excision reaction. RPA binds the single stranded DNA (ssDNA) opposite the lesion and interacts with XPA (De Laat et al., 1998a; Li et al., 1995). RPA binding is important in ensuring excision is followed by repair synthesis to avoid the accumulation of harmful single strand break (SSB) intermediates (Overmeer et al., 2011). XPG and XPF are the two endonucleases that enable excision of the damaged oligo. XPG is the 5’ flap endonuclease and recruited before XPF (Volker et al., 2001). It has a structural role separate to its nuclease activity, as XPG without its catalytic activity is sufficient for completion of the pre-incision complex by recruitment of XPF (Wakasugi et al., 1997). XPF is a 3’ flap endonuclease and exists as a mutually stabilising heterodimer with ERCC1 (Enzlin and Schärer, 2002). ERCC1-XPF is the final part of the complex to arrive at the lesion and its recruitment is dependent on the presence of XPA and XPG (Orelli et al., 2010; Volker et al., 2001). Conversely, XPF makes the first incision 5’ of the lesion and XPG the second incision 3’ of the lesion. It is proposed that this sequence of events guards against the nucleases making incisions in the absence of factors necessary for the completion of repair (Staresincic et al., 2009).

1.2.1.3 Repair synthesis and ligation

Repair synthesis is carried out by DNA polymerases ε, κ, δ in association with PCNA, RFC and RPA (Ogi and Lehmann, 2006; Shivji et al., 1995). It can begin immediately following the incision by ERCC1-XPF which leaves a 3' hydroxyl group, and in so doing minimises the presence of the gap (Staresincic et al., 2009). When synthesis reaches the end of the excised patch a nick remains that is sealed by DNA ligase I or IIIα (Moser et al., 2007; Shivji et al., 1995).

1.2.2 Base excision repair and direct reversal

Base excision repair (BER) allows repair of abasic sites and chemically altered bases that are largely the result of endogenous sources of damage or spontaneous decay of DNA. A damaged base is recognised by one of several DNA glycosylases that are specialised to bind different base modifications. The glycosylase cleaves the bond between the base and deoxyribose leaving an abasic site. The endonuclease APE1 then cleaves the abasic site to create an SSB. The damaged ends are processed by APE1, DNA polymerase (Pol) β, polynucleotide kinase 3′-phosphatase and aprataxin coordinated by XRCC1. The missing nucleotide can then be added by Pol β and the remaining nick sealed by DNA Ligase IIIα. Described is the predominant 'short-patch' BER pathway, where only 1 nucleotide is excised and replaced. 'Long patch' BER involves the removal and replacement of 2- 12 nucleotides and involves some additional factors. BER can also be initiated by SSBs that are not BER intermediates which are detected by poly(ADP-ribose) polymerase 1 (PARP1) (reviewed in Caldecott, 2008).

Alkylation of bases can also be directly reversed by proteins such as O^6-meG-DNA methyltransferase (MGMT) and ABH2/3, which repair O^6-methylguanine and 1-methyladenine and 3-methylcytosine lesions respectively without any incision of the DNA backbone (reviewed in Eker et al., 2009).

1.2.3 Double strand break repair

Double strand breaks (DSBs), such as those formed by ionising radiation, are the most cytotoxic form of DNA lesion as one break can be sufficient to cause cell death (Bennett et al., 1993). There are two broad mechanisms of DSB repair, homologous recombination (HR) and non-homologous end joining (NHEJ). Homologous recombination is a largely error free process which is restricted to S and G2 phases of the cell cycle and requires the use of a

template, which is most usually a sister chromatid (Fig. 1.3; Haber, 2000; Mao et al., 2008). In this pathway the ends of the DSB are resected by the action of several nucleases and helicases including Bloom syndrome helicase (BLM), MRN (a complex comprising of Mre11, Rad50 and Nbs1), CtBP interacting protein (CtIP), Exonuclease 1 (Exo1) and DNA replication ATP dependent helicase (DNA2), to generate single stranded 3' overhangs. The single stranded overhangs are then coated by RPA which is displaced by RAD51. The loading of RAD51is facilitated by Breast Cancer (BRCA) 1 and 2. The Rad51 filaments can then invade the sister chromatid and anneal to the homologous sequence, which it uses as a template for synthesis across the break site, forming a structure known as a D loop. HR can then proceed in a number of ways; the invading strand can be displaced from the D loop and anneal to the other end of the DSB which is termed synthesis dependent strand annealing (SDSA). Alternatively the second end of the DSB can be captured to form an intermediate known as a double Holliday junction (dHJ). This structure is then either resolved by nucleolytic cleavage or dissolved without cleavage in a process dependent on the BLM helicase (Wechsler et al., 2011). SDSA and the dissolution of dHJs always results in non-crossover products, whereas the resolution of dHJs by nucleases can result in either crossover or non-crossover products. The role of HR factors and variations of HR that occur at stalled replication forks will be discussed later in this introduction.

By contrast to HR NHEJ is an error prone process that directly joins the broken ends. The ends of the break are bound by a KU70-80 heterodimer followed by DNA-dependent protein kinase catalytic subunit. End processing factors are then recruited if required, followed by ligation of the ends by XRCC4-XLF-DNA ligase 4 complex. For a more extensive review of these pathways see (Chang et al., 2017; Jasin and Rothstein, 2013; Panier and Boulton, 2014).

There are also two other known mechanisms for repairing DSBs, alternative end-joining (A-EJ) and single strand annealing (SSA). Both of these pathways involve resection to uncover sections of homology flanking a break, which are then annealed resulting in the loss of sequence between the patches of homology. A-EJ most commonly anneals at micro-homologies 4-6bp in length whereas SSA requires greater resection and larger sections of homology over 20bp in length (Chang et al., 2017). Both of these pathways are Ku and ligase 4 independent, but SSA is dependent on Rad52 and A-EJ requires PARP and polymerase theta. Through the creation of large deletions and through joining homologous sections on different chromosomes, upregulation of these pathways are thought to facilitate genomic rearrangements (Bhargava et al., 2016).

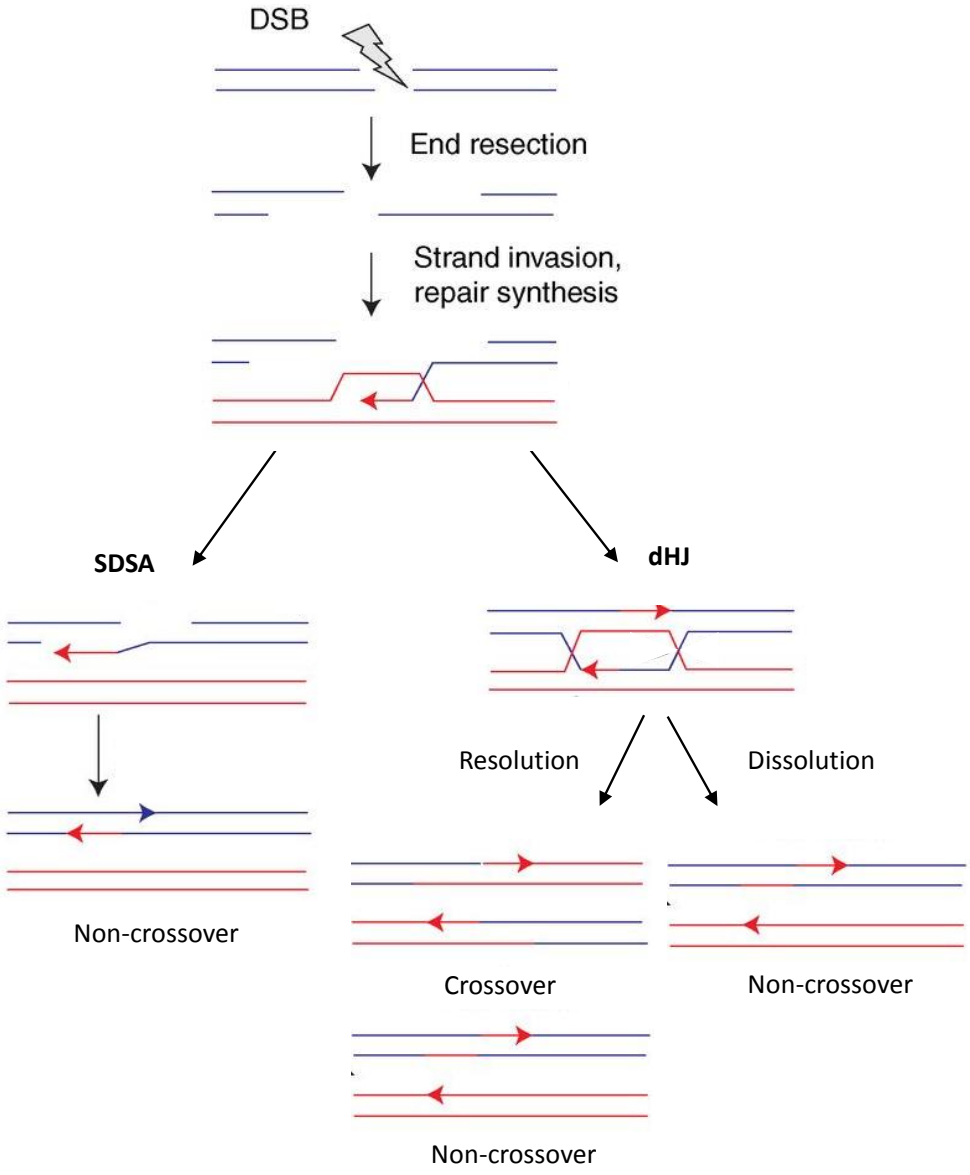


Figure 1.3 Homologous recombination repair of double strand breaks. End resection and invasion of the template DNA are common to all HR. The repair can be completed by synthesis dependent strand annealing (SDSA) or by double Holliday junction (dHJ) formation followed by resolution by nucleases or dissolution by BLM. Adapted from Jasin and Rothstein, (2013).

1.2.4 Mismatch repair

During replication bases can be mispaired or the strands can slip with respect to each other creating an insertion/ deletion loop. If these are not corrected by the proofreading activity of the polymerases they can be removed and replaced by the mismatch repair pathway (MMR). The mismatch or insertion/ deletion loop is recognised by either MutSα (heterodimer of MSH2 and 6) or MutSβ (heterodimer of MSH2 and 3). The MSH heterodimers undergo a

conformational change upon binding and this allows the recruitment of MutLα (comprised of Mlh1 and Pms2) which is activated by PCNA and creates nicks in the nascent strand. These nicks enable loading of EXO1 which digests past the replication error in a 5' to 3' direction. Lastly, the DNA is resynthesized by Pol ε or δ. A more detailed description of MMR can be found in reviews by Jiricny (2013) and Kunkel and Erie (2015).

1.2.5 Fanconi anaemia

The Fanconi anaemia (FA) pathway has evolved in higher eukaryotes and facilitates the repair of interstrand crosslinks (ICLs; reviewed in Ceccaldi et al., 2016a). Defects in this pathway in humans often give rise to bone marrow failure and an increased risk of cancers. This pathway has only been elucidated relatively recently and is the subject of much ongoing investigation. ICLs are primarily repaired during replication where they cause stalling, as the two strands cannot separate (Räschle et al., 2008). ICLs can be recognised by FANCM after phosphorylation by ataxia telangiectasia and RAD3-related (ATR) kinase. The FA core complex which is composed of 14 proteins is recruited by FANCM. The core complex acts as a ubiquitin ligase for the FANCD2-FANCI heterodimer, which controls the subsequent nucleolytic incisions (Knipscheer et al., 2009). *In vitro* studies using *Xenopus* egg extracts have elucidated a mechanism of ICL repair that requires replication forks to converge on the crosslink (Fig. 1.4). The converging forks pause 30-40 nucleotides away. The helicase is unloaded by BRCA1 allowing one fork to advance to within 1 nucleotide of the crosslink at which point dual incisions, termed unhooking, are made either side of the crosslink. ERCC1-XPF is responsible for one or both of the unhooking incisions (Klein Douwel et al., 2014). The leading strand of the advanced fork can then be extended and the resulting gap on the opposite strand can be repaired by homologous recombination. This is the most well characterised role of the pathway, however it is likely it has a role in mitigating replication stress even in the absence of ICLs, as the pathway is activated after hydroxyurea (HU) and aphidicolin treatment, which are both replication inhibitors that do not cause ICLs (Howlett et al., 2005).

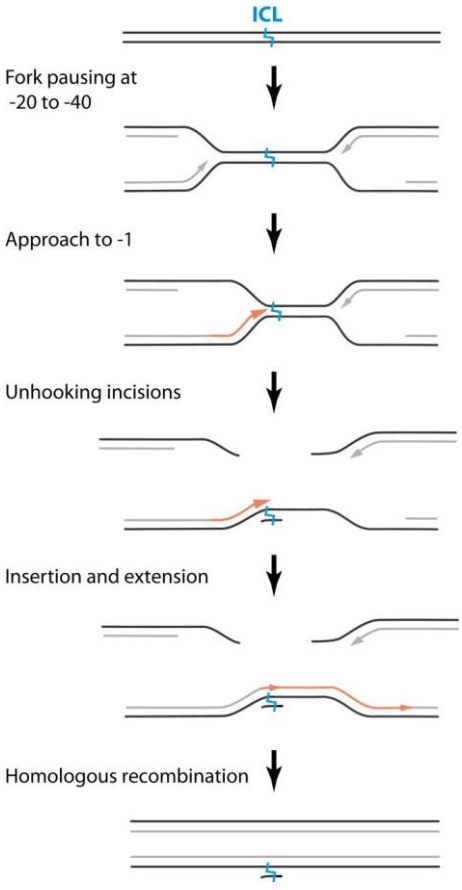


Figure 1.4 The steps of replication coupled ICL repair by the Fanconi Anaemia pathway. (Klein Douwel et al., 2017)

1.2.6 DNA damage signalling

DNA repair can form part of a wider DNA damage response (DDR) co-ordinated by the ataxia telangiectasia mutated (ATM) and ATM and Rad3 related (ATR) kinases (Fig. 1.5). These kinases are activated by damage via factors acting as sensors and go on to phosphorylate a large number of target molecules which facilitate DNA repair, cell cycle arrest and in some cases senescence or apoptotic cell death (Fig. 1.5). Cell cycle arrest serves to promote cell viability and genome stability after DNA damage by allowing more time for repair, whereas senescence and apoptosis prevent the expansion of cells carrying badly damaged DNA that are at high risk of oncogenic transformation. ATM is activated in response to DSBs through recruitment by the MRN complex which converts ATM from an inactive homodimer to an active monomer form. It has also been shown to initiate signalling in response to oxidative stress (Paull, 2015). ATM is estimated to be able to phosphorylate over 1000 substrates, including several DNA

repair proteins, the checkpoint kinase Chk2 and the tumour suppressor p53 (Shiloh and Ziv, 2013).

ATR, in complex with its mutually stabilising binding partner ATRIP, is activated in response to the formation of ssDNA, which occurs at stalled replication forks and is a feature of many repair intermediates (Cortez et al., 2001). RPA binds and stabilises ssDNA and is required for ATR recruitment. ATR activation is stimulated by topoisomerase binding protein 1 (TopBP1), which is thought to be stabilised at the ssDNA by the (Rad9-Rad1-Hus1) 9-1-1 complex (Delacroix et al., 2007; Kumagai et al., 2006). Like ATM, ATR can phosphorylate a vast number of substrates including DNA repair proteins, components of the replisome and the checkpoint kinase Chk1 (Cimprich and Cortez, 2008). Chk1 is essential for viability in mammals and activation of Chk1 has an important role in regulating replication origin firing and replication fork speed in both unperturbed S phases and under conditions of replication stress (Petermann et al., 2010).

Activation of the checkpoint kinases Chk1 and Chk2 leads to the phosphorylation of targets which induce cell cycle arrest by inhibiting CDK activity. Cell cycle arrest in G1, which is mediated by ATM and Chk2, prevents damaged DNA being carried into S phase where it may hinder replication. By contrast checkpoint activation during S phase inhibits the firing of new origins causing an S phase delay and allowing more time to complete replication (Kastan and Bartek, 2004). G2 arrest, which can also result from activation of either ATM or ATR prevents the cell entering mitosis with damaged or under-replicated DNA which may lead to mis-segregation or breakage of chromosomes, resulting in genomic instability or cell death or senescence through mitotic catastrophe (Shaltiel et al., 2015; Vitale et al., 2011). The tumour suppressor p53 is also a downstream target of ATM and ATR and activation of this factor can drive the cell towards either G1 arrest or apoptosis (Carvajal and Manfredi, 2013). DNA damage signalling can therefore promote genomic stability and prevent oncogenic transformation, but also can promote damage tolerance and resistance to DNA damaging drugs (Sherr and Bartek, 2017).

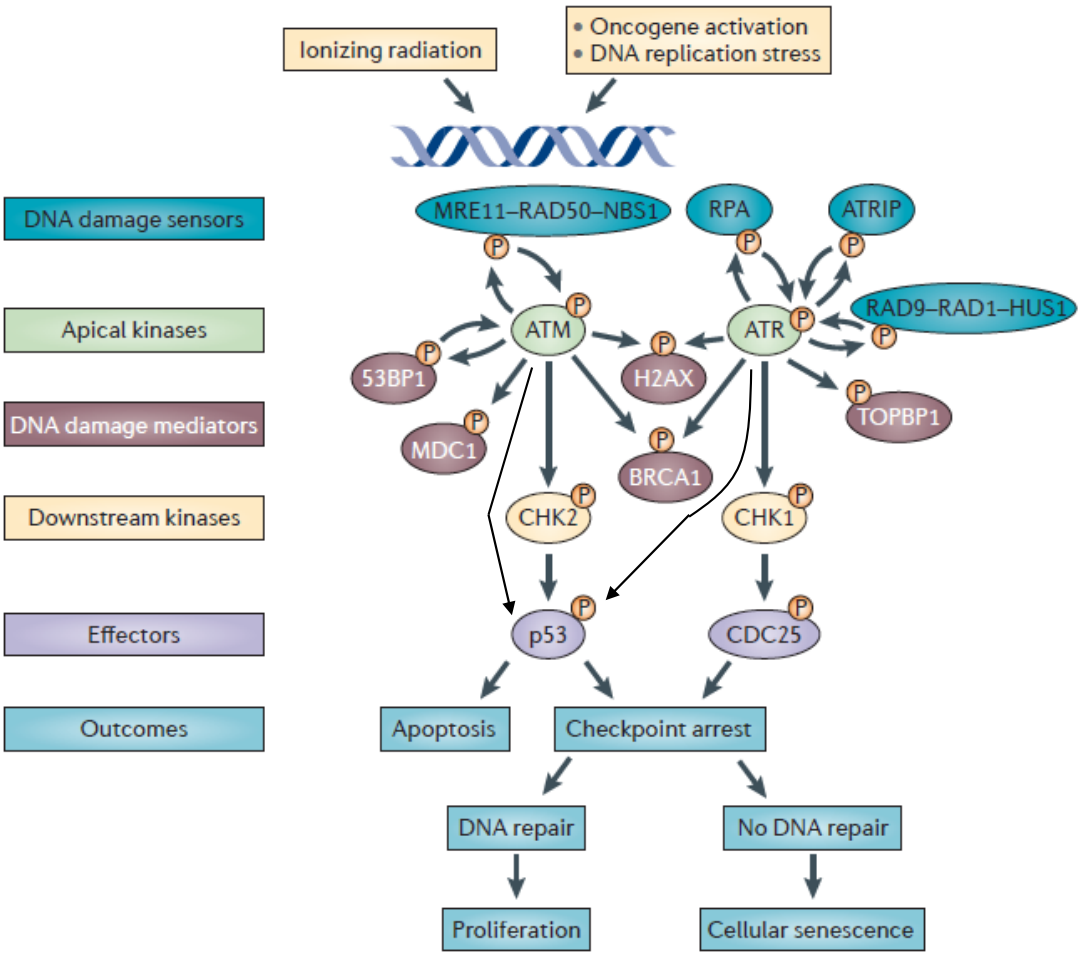


Figure 1.5 DNA damage signalling by ATM and ATR. Activation of ATM or ATR by damage sensors leads to the activation of a signalling network that results in either cell cycle arrest and repair of the damage, or apoptosis and senescence. Adapted from Sulli et al (2012).

1.3 Gemcitabine

Gemcitabine is a chemotherapy drug that has been in clinical use since the mid-1990s and is used to treat a range of solid tumours including breast, bladder, ovarian, non-small-cell lung, pancreatic cancer (Burris 3rd et al., 1997; Carmichael, 1998; Heinemann, 2003; Lorusso et al., 2006; Ramalingam and Belani, 2008) and haematological malignancies such as non-Hodgkin lymphoma and acute leukaemias (Zinzani et al., 2010). It features on the World Health Organisation's list of essential medicines. Despite being the current most effective treatment for several kinds of tumour, many tumours display gemcitabine resistance, they either do not respond to the drug or the duration of the response is short (Toschi et al., 2005). Therefore there is a pressing clinical need to better understand and predict gemcitabine resistance. There are several well characterised resistance mechanisms that involve restricting uptake and activation

of the drug, but the response of the DNA repair pathways to gemcitabine is poorly understood. Cytarabine resistance is also investigated in this project. Cytarabine belongs to the same drug class as gemcitabine and has a similar structure and mechanism of action (see next section) and the two drugs are therefore might elicit similar resistance pathways. Cytarabine has been in clinical use since the 1960s and is an effective treatment for acute myelogenous leukaemia (AML) and lymphocytic leukaemia (Hamada et al., 2002; Lichtman, 2013). Cytarabine treatment is often initially effective in AML patients, but relapse with cytarabine resistant disease is common (Cros et al., 2004).

1.3.1 Mechanisms of action

Gemcitabine and cytarabine belong to a class of drugs known as nucleoside analogues which are used as chemotherapeutics and antiviral agents. Nucleoside analogues resemble endogenous nucleosides and many, including gemcitabine, can be incorporated into DNA during replication and act as replication inhibitors (reviewed in Ewald et al., 2008a). Gemcitabine is a deoxycytidine analogue that differs from deoxycytidine by the exchange of two hydrogen atoms with two fluorine atoms in the 2' position of the sugar moiety (Fig. 1.6). It is a prodrug which requires active conversion to an active form inside the cell. Due to its hydrophilic nature passive diffusion into the cell is slow and gemcitabine needs active uptake across the membrane by human equilibrative nucleoside transporters (hENTs) and human concentrative nucleoside transporters (hCNTs; Mackey et al., 1998). Once inside the cell gemcitabine is mono-phosphorylated by deoxycytidine kinase (dCK), then it is phosphorylated again by nucleotide kinases to di and triphosphate forms, which both disrupt replication via different mechanisms (reviewed in Ewald et al., 2008). Cytarabine is also an analogue of deoxycytidine that has a hydroxyl group in the β-configuration at the 2' position of the sugar moiety (Fig. 1.6). It is transported inside the cell and phosphorylated in the same way as gemcitabine, but it is only the trisphosphate form of cytarabine that inhibits replication (Hamada et al., 2002).

Gemcitabine triphosphate (gemcitabine-TP) and cytarabine trisphosphate are incorporated into replicating DNA in place of cytidine, where they both lead to replication fork stalling (Huang et al., 1991). Fork stalling and the molecular pathways which mitigate it are discussed in detail in the next section. Incorporation into DNA has been shown to be important for gemcitabine cytotoxicity. Gemcitabine-TP incorporation correlates linearly with loss of clonogenicity, and blocking incorporation by inhibiting polymerase activity protects the cell from apoptotic cell

death (Huang and Plunkett, 1995; Huang et al., 1991). The effect of gemcitabine on chain extension has been studied using *in vitro* primer extension assays, in which a radio-labelled sequencing primer was annealed to a complementary sequence, incubated with either human pol α or pol ε and assessed for its ability to extend in the presence of gemcitabine-TP by running the extended primer product on a sequencing gel. These experiments revealed the polymerases could extend the nucleotide chain by one nucleotide after insertion of a gemcitabine-TP molecule, the polymerase then paused but subsequently could extend the chain several nucleotides further. This explained the observation in the same paper that gemcitabine was rarely the terminal nucleotide of DNA strands extracted from gemcitabine treated cells (Huang et al., 1991). Gemcitabine is therefore not a chain terminating molecule, but rather is inhibitory to further chain extension. By contrast, cytarabine was shown to be a more powerful inhibitor of chain extension and was often present at the end of a chain. There are indications that allowing chain extension past a gemcitabine molecule helps shield it from proofreading activity and removal (Gandhi et al., 1996). This demonstrates that although there are structural similarities between gemcitabine and cytarabine there are differences in mechanism which may necessitate the use of different repair mechanisms. Radio-labelled gemcitabine has also been shown to be incorporated into RNA and inhibit RNA synthesis and inhibition of transcription may contribute to its cytotoxic effect (Ruiz van Haperen et al., 1993). Cytarabine has also been shown to inhibit RNA synthesis in B-chronic lymphocytic leukaemia cells, but it was not tested whether this is due to RNA incorporation or inhibition of the polymerase (de Vries et al., 2006). An earlier study showed no cytarabine was incorporated into RNA in acute promyelocytic leukaemia cells HL-60 (Spriggs et al., 1986).

Gemcitabine has a second replication inhibiting mechanism of action. The diphosphate form irreversibly inactivates ribonucleotide reductase (RNR), which is responsible for catalysing the conversion of nucleotides to deoxynucleotides (Baker et al., 1991; Heinemann et al., 1990; Wang et al., 2007). Therefore, the inactivation of RNR lowers intracellular dNTP pools, which in itself causes replication fork stalling. It also increases the amount of gemcitabine triphosphate relative to deoxycytidine trisphosphate thereby having a self-potentiating effect on gemcitabine incorporation into DNA. Cytarabine has been shown to have little effect on dNTP pools (Plunkett et al., 1989).

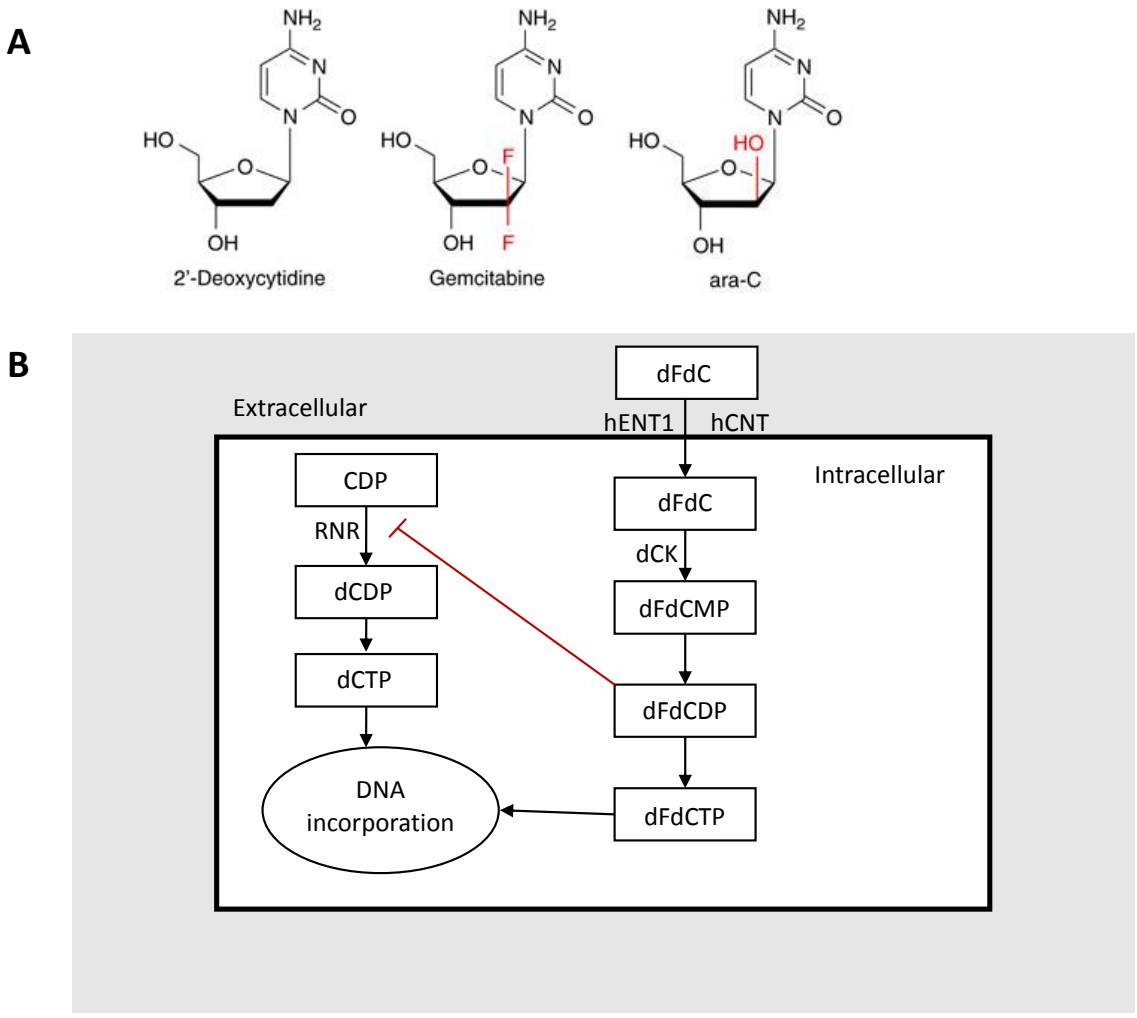


Figure 1.6 The structure and metabolism of gemcitabine. (A) Structure of deoxycytidine and gemcitabine and cytarabine (ara-C, Ewald et al., 2008a). **(B)** Diagram showing activation and actions of gemcitabine (dFdC) inside the cell. dFdC is transported into the cell by human equilibrative nucleoside transporter 1 (hENT1) and human concentrative nucleoside transporter (hCNT). dFdC is mono-phosphorylated by deoxycytidine kinase (dCK) and then further phosphorylated to dFdCDP and dFdCTP. dFdCDP inhibits ribonucleotide reductase (RNR), inhibiting reduction of deoxynucleotides. dFdCTP is incorporated into DNA.

1.3.2 Other replication inhibitors

Comparatively few of the studies investigating replication fork stalling or replication stress have used nucleoside analogues as a fork stalling agent. Many of the sources referenced in this project used the replication inhibitors hydroxyurea (HU) and aphidicolin, which are quite different in structure and mechanism of action to gemcitabine and cytarabine. HU, like

gemcitabine diphosphate inhibits RNR resulting in lowered dNTP pools and replication inhibition, but unlike gemcitabine the inhibition is reversible. HU enters the cell by passive diffusion and does not require uptake by transporters (Gwilt and Tracewell, 1998). A recent study also indicated HU causes oxidative damage, which is likely to contribute to replication inhibition (Huang et al., 2016). Aphidicolin is a metabolite of the mould *Cephalosporium aphidicola* and inhibits replication by binding to the polymerase close to the active site and prevents dNTP binding. It inhibits human polymerases α, ε and δ as well as some viral and bacterial polymerases (Baranovskiy et al., 2014). Although they are often used interchangeably to investigate replication stress, it is plausible that the differences in mechanism between various replication inhibitors will affect the pathways which respond to fork stalling and the DNA repair factors involved.

1.4 Replication fork stalling

As discussed above, gemcitabine induces replication fork stalling via two mechanisms: incorporation into the nascent strand inhibits strand elongation, and inhibition of RNR leads to the depletion of nucleotide pools. Accumulation of stalled forks underpins the cytotoxic effect of gemcitabine, as when forks are not restarted effectively and replication is left unfinished this can lead to cell death by either apoptosis or mitotic catastrophe. Therefore, factors which promote the stabilisation and restart of gemcitabine stalled forks are likely to contribute to gemcitabine resistance. There are many different pathways, which enlist the help of different repair factors, proposed for mammalian replication fork stabilisation and restart and these are discussed below.

1.4.1 Normal replication fork progression

Replication can only initiate at pre-defined sites, termed origins, and mammalian cells specify many more origins than are fired in a normal S phase. Origins are defined by binding of the origin recognition complex (ORC), and then 'licensed' in G1 by the binding of factors which, along with ORC, comprise the pre-replication complex and include the mini chromosome maintenance (MCM) helicase (DePamphilis et al., 2006). Licenced origins are activated during S phase by the action of additional factors including the binding of CDC45 and GINS to the MCM helicase, forming the active CMG helicase complex (Ilves et al., 2010). Origin firing is organised into groups or clusters of origins that fire at different times throughout S phase (Jackson and Pombo, 1998). Mammalian cells are estimated to activate 30-50,000 origins of replication over the course of a typical S phase (Huberman and Riggs, 1966; Méchali, 2010).

Replication forks travel in both directions from the origin and terminate when they collide with another oncoming fork, thereby ensuring that DNA is not replicated more than once. The protein complex responsible for replication, termed the replisome, consists of several components (reviewed in Yao and O'Donnell, 2010). The CMG helicase complex unwinds the double stranded DNA ahead of the fork. DNA polymerases ε and δ synthesise the leading and lagging strands respectively and are tethered to the DNA by PCNA (Kunkel and Burgers, 2008, Fig. 1.7). Both strands are synthesised in a 5' to 3' direction which requires the lagging strand to be synthesised discontinuously as a series of fragments 100-200bp in length known as Okazaki fragments. Polymerases ε and δ also have intrinsic 3' exonucleolytic proofreading activity that is able to excise mismatched bases from the end of the nascent strand and this contributes to the high fidelity of these enzymes (Albertson et al., 2009). The proofreading 3'-5' exonuclease activity of these polymerases was considered a candidate mechanism for gemcitabine removal however *in vitro* experiments showed the proofreading activity of human polymerases ε and γ (a mitochondrial DNA polymerase), or the *E. coli* polymerase Pol I were very inefficient at removing gemcitabine from either the terminal or penultimate position on a strand (Fowler et al., 2008; Gandhi et al., 1996; Huang et al., 1991).

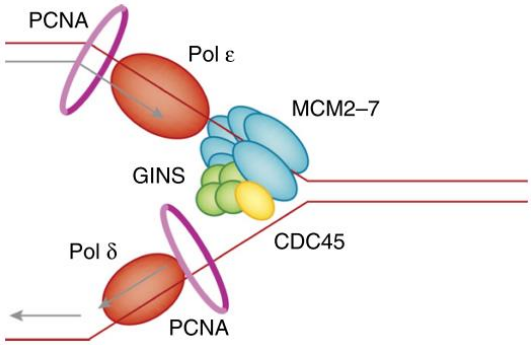


Figure 1.7 Organisation of a normal mammalian replication fork. DNA polymerases ε and δ synthesise the leading and lagging strands respectively and are tethered to the DNA by PCNA. The CMG helicase, made up of MCM2-7, CDC45 and GINS unwinds the duplex DNA ahead of the fork (Berti and Vindigni, 2016).

1.4.2 Stalling and restart

The smooth progression of the replication fork can be interrupted by various obstacles such as a lesion in either of the template strands, secondary structures in the template, collision with transcription machinery or other DNA bound proteins, inhibition of the polymerase or as is the

case with gemcitabine, depletion of nucleotide pools and steric hindrance caused by fraudulent bases in the nascent strand (reviewed in Neelsen and Lopes, 2015). The slowing or stalling of replication in response to such obstacles is termed replication stress (Zeman and Cimprich, 2014). The activation of oncogenes also causes replication stress through the deregulation of origin licensing and firing, leading to under-replicated and re-replicated DNA (Gaillard et al., 2015).

If the movement of the polymerase but not the helicase is inhibited, such as by damaged bases or depletion of nucleotides, this leads to helicase uncoupling. The helicase continues unwinding the DNA ahead of the fork and the polymerases are left behind leaving long stretches of ssDNA which is then bound by RPA (Byun et al., 2005). The increase in ssDNA bound by RPA activates Chk1 checkpoint kinase through recruitment and stimulation of ATR, which orchestrates a global decrease in origin firing, and promotes fork stabilisation and restart (Petermann et al., 2010; Zou and Elledge, 2003). As mentioned previously, mammalian cells have many more licensed origins than they require for a normal S phase. The firing of dormant origins located within active clusters of replication origins, whilst suppressing origin firing in inactive clusters, is a major mechanism for overcoming replication stress (Ge et al., 2007). However, there are not always dormant origins available between two converging stalled forks and so pathways for the restart of stalled forks are also required. There are many different pathways described for replication fork restart and these are discussed below (Fig. 1.8, reviewed in Berti and Vindigni, 2016).

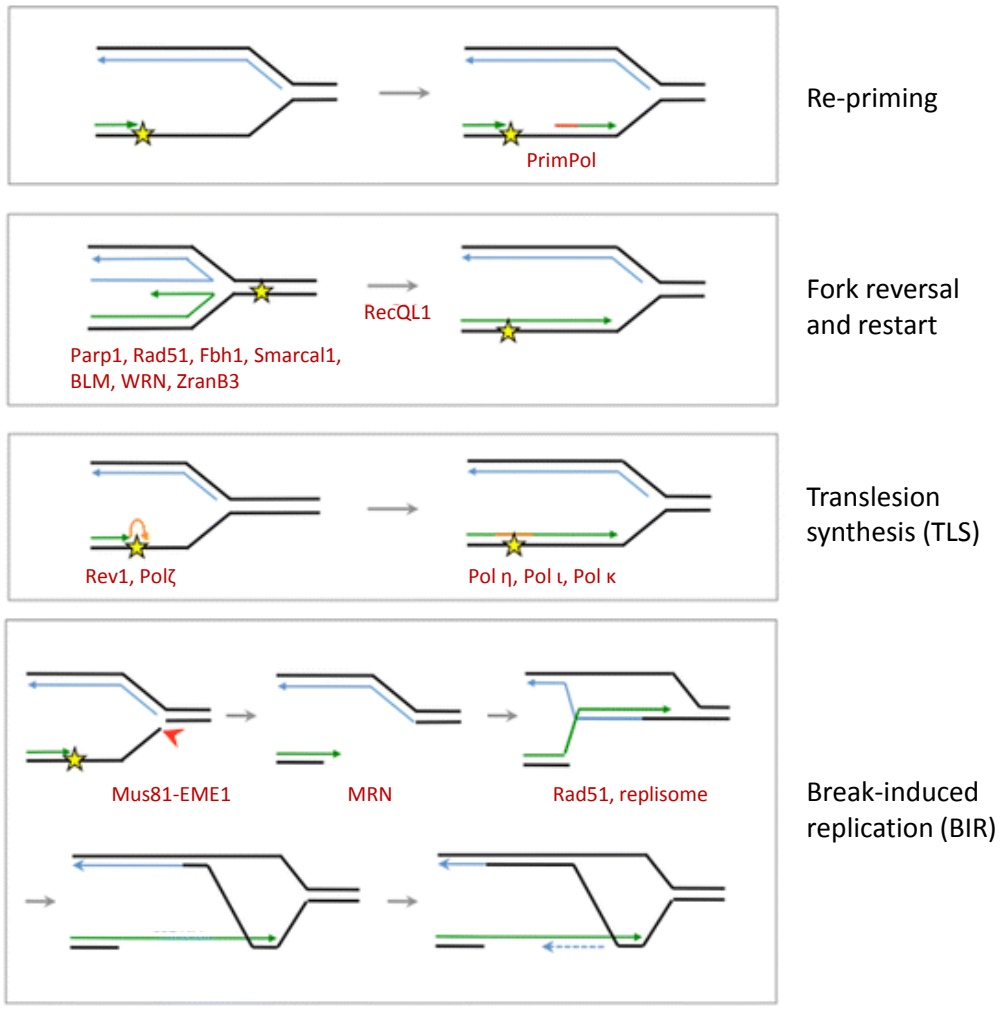


Figure 1.8 Pathways and factors which enable replication forks to overcome stalling. A lesion in the template strand is represented by a star (Munoz and Mendez, 2017).

1.4.2.1 Helicase slowing

The Fanconi anaemia protein FANCD2 has been shown to bind to the helicase MCM upon ATR signalling. Knockdown of FANCD2 had no effect on replication fork speed in untreated cells but after HU treatment forks progressed faster in the absence of FANCD2. This led to the accumulation of DNA damage and reduced proliferation (Lossaint et al., 2013). This suggests FANCD2 binding slows the unwinding activity of the helicase. This may prevent helicase uncoupling and be protective in situations such as gemcitabine induced replication stress, where nucleotides are depleted or polymerisation has slowed, as it will limit the accumulation of ssDNA.

1.4.2.2 Translesion synthesis (TLS)

Damaged bases in the template strand can form a barrier to pol ε and pol δ, but these polymerases can be switched for a translesion polymerase, such as pol η or pol κ. These polymerases have a greater tolerance for damaged DNA but as a trade-off operate with lower fidelity (reviewed in Sale, 2013). The TLS polymerases have been most often studied in the context of bypassing a lesion on the template strand so it is unclear how polymerase switching might play a role in the context of dNTP depletion or fraudulent bases on the nascent strand as is the case with gemcitabine. However, knockdown of pol η in human fibroblasts was shown to lead to gemcitabine sensitivity and biochemical assays showed pol η could efficiently extend a terminal gemcitabine and efficiently bypass a gemcitabine in the template strand (Chen et al., 2006). A second study found that TLS polymerases could allow maintenance of fork speed after treatment with gemcitabine in the absence of MAP kinase- activated protein kinase 2 and this resulted in increased cell viability (Kopper et al., 2013). These findings suggest TLS polymerases contribute to gemcitabine resistance, but as continued nascent strand extension in the presence of gemcitabine may lead to more gemcitabine molecules being incorporated this perhaps conflicts with the findings that show incorporation is important for gemcitabine cytotoxicity (Huang and Plunkett, 1995; Huang et al., 1991).

1.4.2.3 Repriming

Similarly to translesion synthesis, repriming can be used as a way to bypass damaged bases in the template. In this pathway the polymerase reinitiates DNA synthesis downstream of the lesion leaving a small single stranded gap behind it (Elvers et al., 2011). In *Saccharomyces cerevisiae*, mutations in primases lead to an increase in fork reversal under conditions of replication stress, suggesting repriming prevents the need for further fork remodelling (Fumasoni et al., 2015). The enzyme PrimPol has been found to be responsible for repriming in human cells and has also been shown to be important for restart of forks after nucleotide depletion with hydroxyurea (Mourón et al., 2013). Conversely, excessive PrimPol activity is pathological and its activity requires control by RAD51 (Vallerga et al., 2015).

1.4.2.4 Fork reversal

Fork reversal is the process by which the fork backtracks and produces a four branched structure. The nascent strands unwind from the parental strands and the nascent strands then anneal giving rise to a ‘chicken foot’ structure (Fig.1.9). Recent studies have confirmed the formation of these structures in mammalian cells in response to many different kinds of replication stress, where they seem to perform a protective role by preventing double strand

breaks and accumulation of excessive ssDNA (Ray Chaudhuri et al., 2012; Zellweger et al., 2015). These studies also determined that fork reversal is dependent on RAD51 and PARP. Studies suggest there are two mechanisms for fork restart following reversal, the first involves branch migration by REQ1 helicase and preserves the nascent DNA (Berti et al., 2013; Zellweger et al., 2015). The second pathway occurs after prolonged replication arrest and involves nucleolytic processing of the regressed nascent strands by the nuclease DNA2 and requires the helicase activity of WRN (Thangavel et al., 2015). This is hypothesised to promote restart by either recruiting branch migration factors to the partially resected DNA or by an HR like mechanism where a single stranded stretch of the regressed arm invades the duplex ahead of the fork and forms a Holliday junction (Fig. 1.9). Reversed fork structures are also known to be substrates for the endonuclease MUS81 and nucleases which are co-ordinated by SLX4, which leads to double strand break formation. This pathway is negatively regulated by the checkpoint kinases ATR and Chk1 as the accumulation of DSBs is likely to be cytotoxic (Couch et al., 2013; Neelsen et al., 2013).

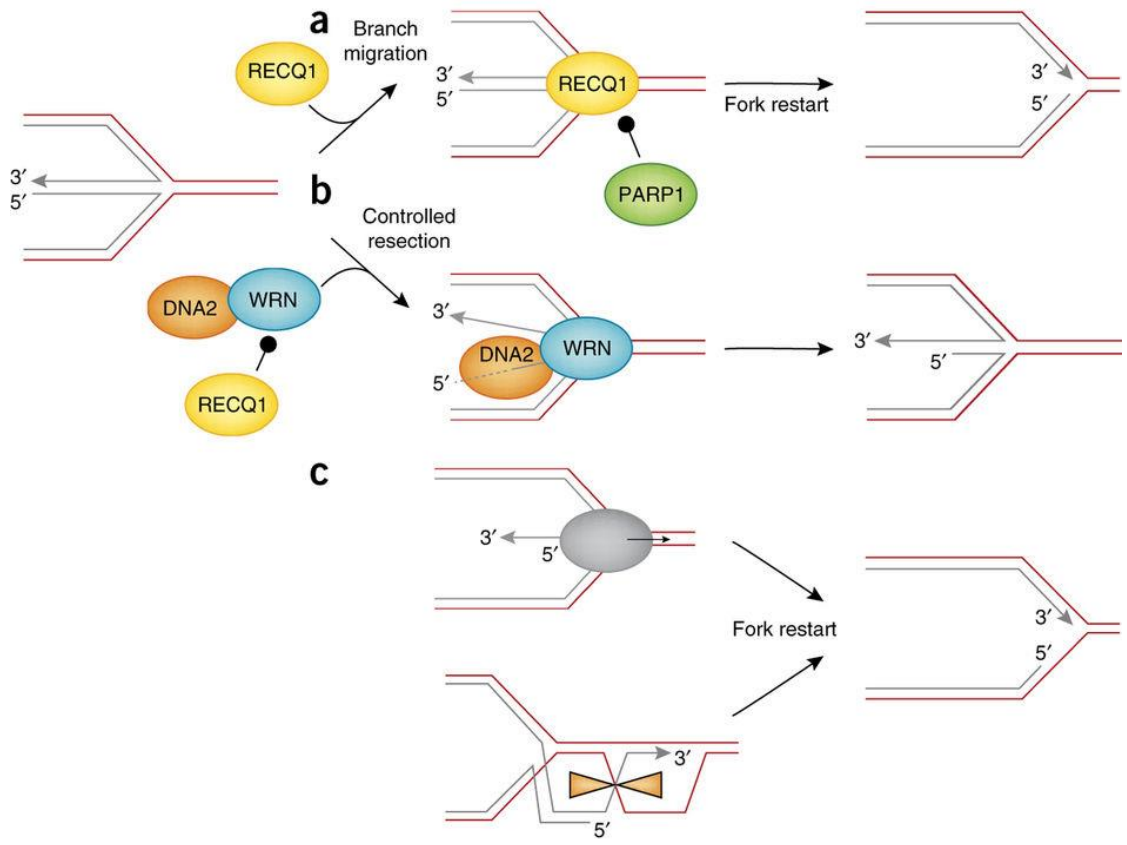


Figure 1.9 Diagram demonstrating restart of reversed forks. (A) Branch migration is carried out by RECQ1 which is inhibited by PARP. **(B)** After prolonged replication arrest resection of the regressed nascent strands is carried out by DNA2 and aided by the helicase activity of WRN. **(C)** Resection leads to restart either by the binding of unknown branch migration factors (grey oval) or by invasion of the duplex DNA by a single stranded stretch of the regressed arm (Berti and Vindigni, 2016).

1.4.2.5 Break induced replication

Processing by nucleases such as that described above can lead to the formation of one-ended double strand breaks at the fork and whilst DSB formation is often considered a pathological response to fork stalling it can also initiate fork restart (Fig.1.8; Hanada et al., 2007; Jones et al., 2014). Break induced replication, a variation of the HR pathway of double strand break repair, can repair the break and continue replication (reviewed in Sakofsky and Malkova, 2017). As in normal HR, one strand is resected and RAD51 is loaded onto the resulting ssDNA, which can then invade the sister chromatid. In one model the D loop is resolved leading to the restoration of a normal fork. Alternatively, replication of the leading strand can then continue in a moving bubble, with lagging strand synthesis following behind the bubble resulting in conservative DNA synthesis. BIR has been shown to be highly mutagenic and the continuation of the BIR D loop is limited in mammalian cells by collision with an oncoming replication fork and cleavage of the D loop by Mus81 (Deem et al., 2011; Mayle et al., 2015).

1.5 Gemcitabine resistance

Several factors are known to mediate gemcitabine resistance. Downregulation of the nucleotide transporter hENT1 or dCK limit the accumulation of gemcitabine di- and triphosphate inside the cell (Bergman et al., 2002; Mackey et al., 1998). These resistance mechanisms are relatively well studied and expression levels of these factors have been shown to have prognostic value (Farrell et al., 2009; Kroep et al., 2002). Several other enzymes such as cytidine deaminase and 5'-nucleotidase also influence the accumulation of intracellular gemcitabine triphosphate and may have a role in resistance (reviewed in Mini et al., 2006). Upregulation of RNR has also been shown to influence gemcitabine resistance by raising the levels of normal nucleotides which compete with gemcitabine for incorporation into DNA (Goan et al., 1999).

However, once phosphorylated and incorporated into DNA, the DNA repair mechanisms which respond to incorporated gemcitabine and the resulting stalled forks are poorly understood. As

previously discussed, incorporation into DNA is important for cytotoxicity and therefore removal of gemcitabine from DNA by nucleases could plausibly lead to resistance. The proofreading 3'-5' exonuclease activity of replicative polymerases was considered a candidate mechanism for gemcitabine removal however *in vitro* experiments showed the proofreading activity of human polymerases ε and γ (a mitochondrial DNA polymerase), or the E. coli polymerase Pol I were very inefficient at removing gemcitabine from either the terminal or penultimate position on a strand (Fowler et al., 2008; Gandhi et al., 1996; Huang et al., 1991). APE1 is a nuclease component of the base excision repair pathway which was shown to be able to excise a terminal gemcitabine from an oligonucleotide in a biochemical assay, but removal was very inefficient so the authors concluded it was unlikely to contribute to gemcitabine resistance (Chou et al., 2000).

Factors associated with double strand break repair (rad51, BRCA2, ATM, Mre11 and Rad50) have been implicated in both gemcitabine resistance and sensitivity in mammalian cells (Choudhury et al., 2009; Ewald et al., 2008b; Jones et al., 2014; Tsai et al., 2010). As discussed above, it has recently become apparent that many HR factors also have important roles in replication fork stabilisation, reversal and HR mediated restart, whether or not a double strand break is formed at the fork. Therefore it is likely these factors can respond to and successfully process gemcitabine stalled fork structures thus mediating resistance. That the same factors are also observed to confer gemcitabine sensitivity is intriguing and hints at the complexity of the replication stress response.

1.6 Nucleotide excision repair and gemcitabine resistance

In order to better understand the DNA repair pathways that influence gemcitabine resistance a screen for gemcitabine sensitivity in *S. pombe* DNA repair mutants was carried out, in a strain which expressed hENT1 and human dCK to enable uptake and activation of the drug (Gasasira, 2013). Several factors belonging to the NER pathway conferred sensitivity when mutated (Fig. 1.10). These were *rhp14*, *swi10*, *rad16*, *rhp41/42* which are the S. pombe homologues of *xpa*, *ercc1*, *xpf* and *xpc* respectively. These factors are all required for the removal of bulky adducts from DNA in *S. pombe*. Interestingly, the *rad13* (*xpg*) mutant, which is also essential for canonical NER, was not sensitive suggesting these factors were acting outside the canonical pathway to mediate gemcitabine resistance. The lack of requirement for *xpg* in gemcitabine resistance suggested a model in which gemcitabine was cleaved off the end of the nascent strand during replication, which would not require an *xpg* mediated excision downstream of

the gemcitabine. The NER pathway is highly conserved between lower and higher eukaryotes, although studies suggest the mechanism of damage recognition and the role of the *xpc* homologues have diverged. In humans XPC is the lesion recognition factor in GG-NER, but dispensable for TC-NER as described earlier in the introduction, but in *S. pombe rhp41* and *42* are essential for both pathways (Marti et al., 2003). Instead, the *S. pombe* genes rhp7 and rhp16, which have no human homologue, are shown to carry out GG-NER lesion recognition (Lombaerts et al., 1999).

In line with the results from the *S. pombe* screen, it was also found that many NER deficient fibroblasts, taken from Xeroderma pigmentosum patients were also sensitive to gemcitabine compared with a normal fibroblast line (Beardmore, 2015). This suggests a role for NER factors in gemcitabine resistance is conserved in humans and could have clinical relevance. However, the mechanism by which NER factors could contribute to gemcitabine resistance was unclear.

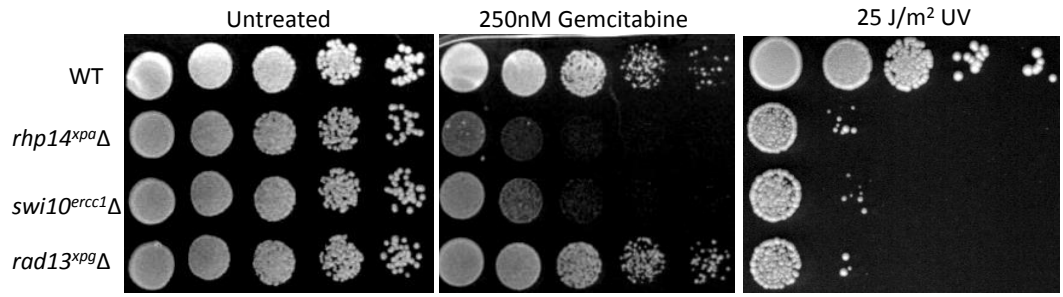


Figure 1.10 Sensitivity of *S. pombe* NER mutants to gemcitabine and UV. Spot test showing sensitivity of several NER mutants after treatment with 250 nM gemcitabine or 25 J/m^2 UV (Gasasira, 2013).

1.6.1 Non-canonical functions of NER factors

That gemcitabine resistance does not require the XPG homologue in the *S. pombe* system suggests the required NER factors are acting in a novel pathway, as XPG is essential for canonical NER. Several NER factors have been shown to have roles outside of the canonical pathway, in transcription, other repair pathways and replication which may be relevant to their putative role in gemcitabine resistance and are therefore discussed below.

1.6.1.1 Transcription

Mutations in subunits of TFIIH (such as XPB and XPD) can give rise to classical XP symptoms but also to the neurological and developmental symptoms of Cockayne syndrome (CS) and Trichothiodystrophy (TTD) such as impaired growth and intellectual disability, which cannot be explained by a failure to repair UV lesions (reviewed in Compe and Egly, 2012). This heterogeneity is explained by TFIIH having an important role in transcription initiation in addition to its role in NER and some mutations being detrimental to one or both functions. The TFIIH complex is essential for transcription initiation by RNA POL II, which is responsible for transcribing most protein coding genes and TFIIH also has a role in the transcription of rRNA by POL I (Feaver et al., 1991; Gerard et al., 1991; Iben et al., 2002). TFIIH forms part of a pre-initiation complex on the promotor together with RNA POL II and other transcription factors. After complex formation the helicase activity of XPB is required to open the DNA at the promotor and allow promotor escape (Douziech et al., 2000; Moreland et al., 1999). TFIIH also phosphorylates the C terminus of RNA POL II which regulates its entry into the preinitiation complex (Serizawa et al., 1993). TFIIH seems to have a different role in the transcription of rRNA by POL I as transcripts can be initiated but not completed in the absence of TFIIH (Iben et al., 2002). XPG, which binds to TFIIH during NER, is also suggested to have a role in transcription. XPG patients display a similar heterogeneity in phenotype to XPB and XPD patients. Mutations which solely affect the nuclease activity of XPG give rise to XP, but mutations which give rise to a truncated protein tend to give rise to a CS phenotype (Schärer, 2008). Concordant with these observations there is evidence supporting a role for the *S. cerevisiae* XPG homologue (Rad2) in transcription elongation that is nuclease independent (Lee et al., 2002).

1.6.1.2 Base Excision Repair

Some observations suggest XPG has a role in aiding the removal of thymine glycol, a radiation induced oxidative lesion removed by the base excision repair pathway. XPG has been reported to stimulate the activity of the DNA glycosylase NTH1, in a nuclease independent manner in *in vitro* experiments (Bessho, 1999; Dianov et al., 2000; Klungland et al., 1999).

1.6.1.3 Interstrand crosslink and double strand break repair

The heterodimer ERCC1-XPF is the 3’ flap endonuclease responsible for the 5’ incision of the NER reaction and it is known to also function as an endonuclease in several other pathways. It has been described previously that NER repairs intrastrand crosslinks but the more cytotoxic interstrand crosslinks are repaired by the Fanconi Anaemia (FA) pathway in which ERCC1-

XPF (also known as FANCQ) has an important role (Fig. 1.4). Indeed, the symptoms of XP-F patients can include FA and chinese hamster ovary (CHO) cells deficient for either factor are highly sensitive to crosslinking agents (Bogliolo et al., 2013; De Silva et al., 2000). The steps of replication coupled ICL repair in the Fanconi anaemia pathway were described previously. Recruitment of ERCC1-XPF is controlled by different partner proteins in NER and ICL repair (Fig. 1.11). During NER ERCC1-XPF is recruited to the lesion by an interaction between ERCC1 and XPA. Abolishing this interaction by mutation of the important residues on either XPA or ERCC1 prevents NER from occurring. However mutation of the same residues does not affect ICL repair and therefore the interaction with XPA is not required in this context (Orelli et al., 2010). ERCC1-XPF is recruited to replication forks stalled at ICLs by the nuclease scaffold protein SLX4 (also known as FANCP) which also binds to nucleases Mus81-EMe1 and SLX1 (Wyatt et al., 2017). Mutations in SLX4 of either MLR or BDB domains which are proposed to mediate binding to XPF cause loss of ICL repair (Guervilly et al., 2015). Similarly mutations of XPF which affect binding to SLX4 also compromise ICL repair without affecting NER incisions (Klein Douwel et al., 2017).

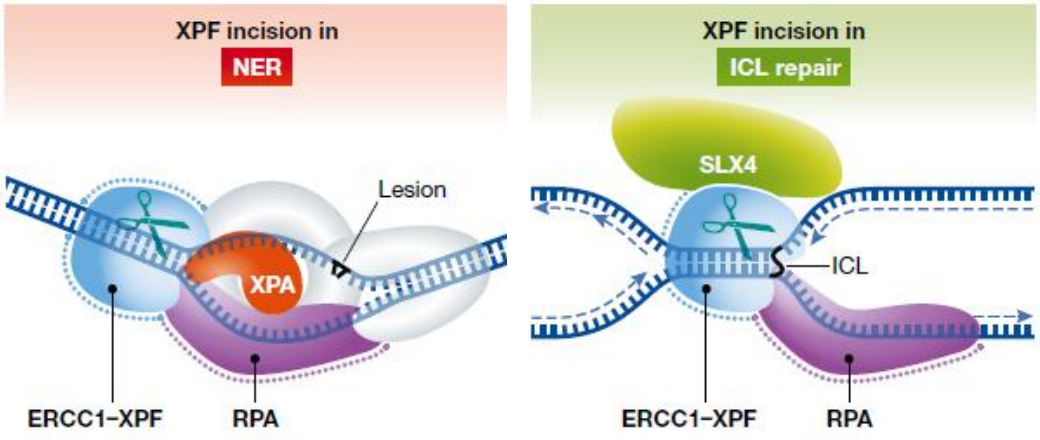


Figure 1.11 The role of ERCC1-XPF in NER and replication-coupled ICL repair. In the NER reaction (left panel) ERCC1-XPF is recruited through an interaction between ERCC1 and XPA, which is not necessary for ICL repair. In ICL repair SLX4 recruits ERCC1-XPF. RPA plays a role in the positioning of ERCC1-XPF that is conserved between both processes (Schärer, 2017).

ERCC1 deficient mouse embryonic fibroblasts (MEFs) and embryonic stem (ES) cells and XP-F patient cells are sensitive to ionising radiation, indicating a role in DSB repair (Ahmad et al., 2008). DSBs can be repaired by several different pathways. Several studies suggest ERCC1-

XPF and their *S. cerevisiae* homologues (Rad1-Rad10) are involved in single strand annealing (SSA) and a Ku-independent end joining pathway (often called alternative end joining) (Ahmad et al., 2008; Al-minawi et al., 2008; Ivanov and Haber, 1995; Ma et al., 2003). Single strand annealing occurs when breaks are positioned in between repeats and adjacent homologous repeated sequences are annealed, leading to the loss of the sequence that was between the repeats. Alternative end joining occurs when annealing of the two broken strands occurs at micro homologies and this pathway often leads to the joining of breaks on different chromosomes (reviewed in Ceccaldi et al., 2016). In both pathways ERCC1-XPF processes the end of the break, removing a 3' flap of non-homologous sequence (Fig. 1.12).

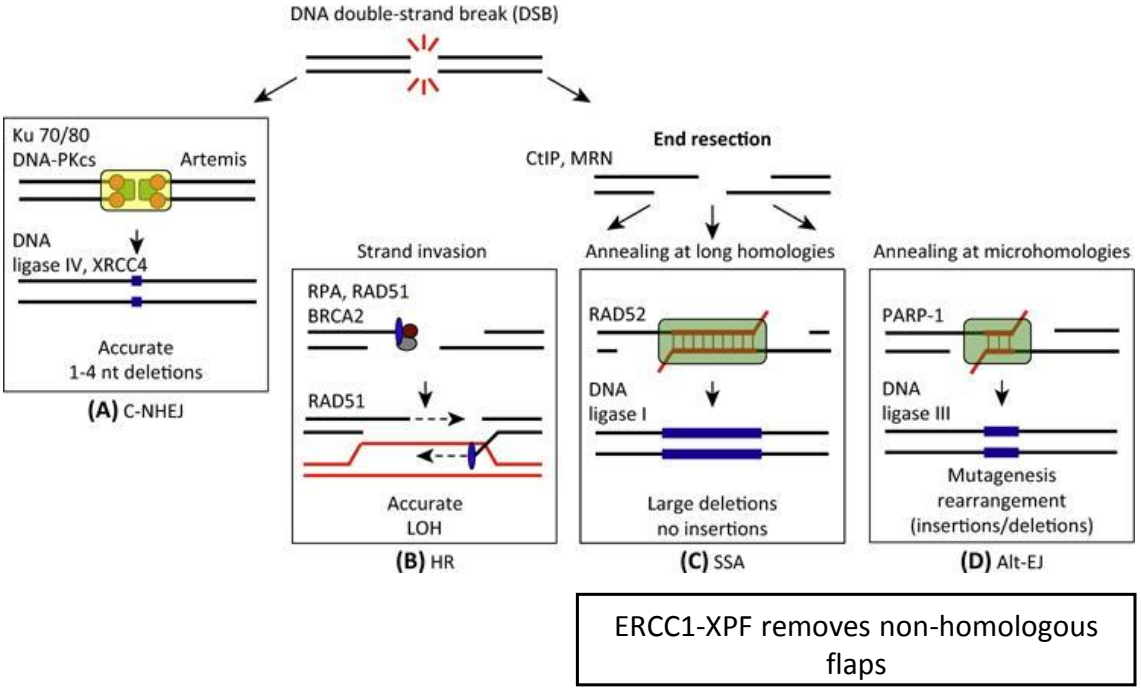


Figure 1.12. The different mechanisms of double strand break repair. Shown here are: classical non homologous end joining (C-NHEJ), homologous recombination (HR), single strand annealing (SSA) and alternative end joining (Alt-EJ) (Ceccaldi et al., 2016b). ERCC1-XPF have been shown to function in SSA and Alt-EJ where they remove the overhanging 3' flaps of non-homologous sequence (shown in red).

1.6.1.4 Replication and mitosis

The participation of ERCC1-XPF in replication coupled ICL repair gives the heterodimer a well-defined role in overcoming replication stress caused by crosslinking agents and its role in double strand break repair may contribute to the repair of broken forks. Other studies provide

additional evidence for the role of ERCC1 in mitigating replication stress. Knockdown of ERCC1 in human fibroblasts has also been shown to result in chromosome segregation defects and misshapen nuclei, an effect that increases after treatment with the replication inhibitor aphidicolin (Naim et al., 2013; Rageul et al., 2011). Depletion of ERCC1, MUS81 or both proteins did not lead to differences in fork speed compared with the control in either untreated or aphidicolin treated cells as assessed by DNA combing. Depletion lead to a decrease in chromosome breaks observed in metaphase spreads, but caused no alteration in the number of breaks observed in G2 cells when they were condensed by treatment with calyculin A. Therefore the authors propose ERCC1 and 3' flap endonuclease MUS81 are responsible for processing under-replicated or interlinked DNA just prior to mitosis to prevent the formation of anaphase bridges and chromosome breaks during mitosis (Fig. 1.13, Naim et al., 2013). A second study also found that depleting MUS81 in U2OS cells and GM637 fibroblasts led to a reduction in metaphase breaks at common fragile sites following low dose aphidicolin treatment (Ying et al., 2013). Both studies concluded that the formation of these breaks is a protective mechanism that is likely to promote genome stability and prevent mitotic catastrophe.

A mechanism for the above described pre-mitotic processing has recently been suggested by work on the SMX tri-nuclease complex (Wyatt et al., 2017). It was described above that ERCC1 associates with the nuclease scaffold protein SLX4 and its partner nuclease SLX1 for its role in inter-strand crosslink repair. This association occurs throughout the cell cycle, but MUS81-EME1 only joins the complex at G2/M in response to cyclin dependent kinase and polo-like kinase 1 phosphorylation (Wyatt et al., 2013). This then allows the complex to efficiently cleave a range of DNA substrates such as Holliday junctions and replication forks via the nuclease action of MUS81. The nuclease action of XPF was not required for efficient cleavage of any of the substrates tested, but the presence of ERCC1-XPF in the complex resulted in a large increase in the efficiency of Holliday junction cleavage, suggesting ERCC1-XPF has a structural role (Wyatt et al., 2017).

In contrast to the mitotic problems experienced by the ERCC1 depleted MRC5 cells described above, and pertinently for this study, ERCC1 deficient Chinese hamster ovary (CHO) cells were shown in one study not to be sensitive to gemcitabine compared with the parent line (Crul et al., 2003a). A separate study showed depletion of XPF in U2OS cells reduced the number of DSBs formed after gemcitabine treatment, an outcome which the authors suggested would increase survival (Jones et al., 2014). Despite there being several studies suggesting a role for

ERCC1-XPF following replication stress, how this affects gemcitabine resistance is unclear and necessitates further investigation. The role of ERCC1 in gemcitabine resistance in human fibroblasts (MRC-5) and human embryonic kidney (HEK293) cells is addressed in chapters 4 and 5 of this project.

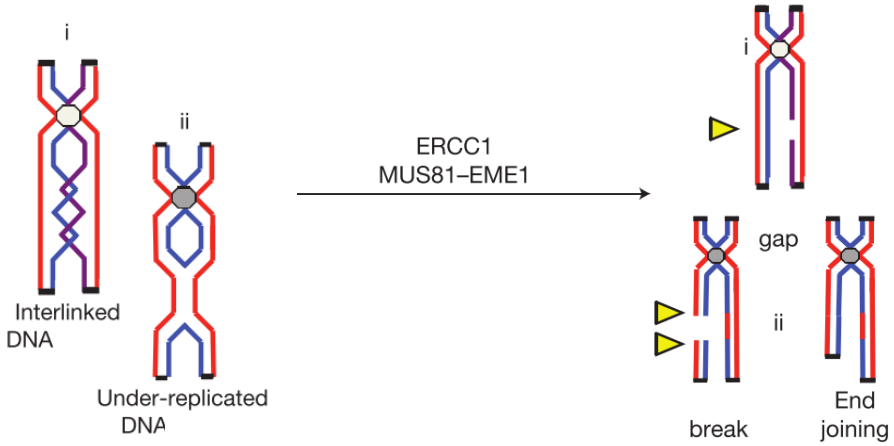


Figure 1.13 Proposed model for the role of ERCC1 and MUS81 in processing chromosomes prior to mitosis. Interlinked DNA (i) is cleaved to leave a single stranded gap on one chromatid. Under-replicated DNA (ii) is cleaved to produce a double stranded break on one chromatid which is repaired by end joining, creating a deletion (Naim et al., 2013).

Aside from ERCC1-XPF several other NER factors are implicated in replication. A recent study showed the colocalisation of PCNA and XPA in untreated proliferating cells and also the presence of XPA, XPD and XPF on nascent DNA using the isolation of proteins on nascent DNA (iPOND) technique, suggesting a role in post-replicative repair (Gilljam et al., 2012).

XPC was shown to be enriched at common fragile sites, sites which are particularly susceptible to replication stress, in a proteomic screen after replication inhibition by aphidicolin. Depleting XPC by siRNA led to a decrease in yH2AX foci on mitotic chromosomes and decrease in phosphorylation of the checkpoint kinase Chk1 as well as an increase in 53BP1 bodies in G1 cells in aphidicolin treated U2OS cells. These effects mirror those which result from ATR inhibition and suggest that harmful replication intermediates at common fragile sites are not being detected, leading to breaks during mitosis and the accumulation of damage in the following G1. This lead the authors to conclude that XPC has a function in damage signalling

after replication stress (Beresova et al., 2016). XPD has been shown to localise to mitotic spindles and spindle formation is impaired by the knockdown of XPD leading to chromosome segregation defects and cells with misshapen nuclei (Ito et al., 2010).

1.7 Aim

A better understanding of how DNA repair interacts with chemotherapy drugs is necessary to improve clinical outcomes. It can also uncover novel functions of DNA repair pathways and give novel insights into the DNA damage response. The sensitivity of *S. pombe* NER mutants and XP patient fibroblasts to gemcitabine is intriguing as it is unclear from our current understanding of NER and gemcitabine how these factors might contribute to resistance and that is the subject of this investigation. This project aimed to find whether or not NER factors localised to repair foci, contributed to replication progression and mitigated gemcitabine incorporation into genomic DNA after gemcitabine treatment by using immunofluorescence microscopy, DNA fibre analysis and mass spectrometry respectively.

2 MATERIALS AND METHODS

2.1 Routine cell culture

Cells were cultured at 37ºC and 5% CO_2 in a humidified incubator. Table 1 contains details of all the cell lines used in this project. All patient fibroblasts, MRC-5 and HEK293 were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma), supplemented with 10% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine and penicillin-streptomycin (both Sigma). They were passaged by washing with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma) before incubation with Trypsin-EDTA solution (Sigma) and collection of detached cells in fresh media. These cells were cultured for a maximum of 3 months. TK6 cells were grown in Roswell Park Memorial Institute 1640 medium (Sigma) supplemented with 5% horse serum (Gibco), 2 mM L-glutamine, penicillin-streptomycin and 2 mM sodium pyruvate (all Sigma). These cells were cultured for a maximum of 6 weeks. All cells were cryopreserved in FBS with 10% dimethyl sulfoxide (DMSO) added and stored in liquid nitrogen after slow cooling.

Table 1. Properties and origins of human cell lines used in the project.

Cell line	Cell type and tissue	Transformation	Source
MRC-5	Fibroblast, lung	SV40	A. Lehmann, University of Sussex

MRC-5 ERCC1-/-	Fibroblast, lung	SV40	R. Beardmore/ this project, Bangor University
XP12RO	Fibroblast, skin	SV40	A. Lehmann, University of Sussex
XP2OS	Fibroblast, skin	SV40	W. Vermeulen, Erasmus MC
XP2OS expressing EGFP-XPA	Fibroblast, skin	SV40	W. Vermeulen, Erasmus MC
XP4PA	Fibroblast, skin	SV40	Alain Sarasin, Paris
XP4PA expressing EGFP-XPC	Fibroblast, skin	SV40	W. Vermeulen, Erasmus MC
XP2YO	Fibroblast, skin	SV40	A. Lehmann, University of Sussex
XPCS1RO	Fibroblast, skin	SV40	A. Lehmann, University of Sussex
GM637	Fibroblast, skin	SV40	Corriell Institute, New Jersey (GM00637)
HEK293	Epithelial, kidney	Adenovirus	T. Caspari, Bangor University

HEK293 ERCC1-/-	Epithelial, kidney	Adenovirus	R. Beardmore/ this project, Bangor University
TK6	Lymphoblast, blood	ICR191 (mutagen)	S. El-Khamisy, Sheffield University
TK6 XPA-/-	Lymphoblast, blood	ICR191	S. Takeda, Kyoto University

2.2 Colony forming survival assay

Colony forming assays were performed as described in Franken et al (2006). Subconfluent cells were trypsinised and passed through a 70 µm cell strainer to achieve a single cell suspension and live cells were counted using a TC20 automated cell counter (Bio-Rad) after staining with 0.4% trypan blue solution (Sigma). 1000-5000 cells were then plated in 5 ml DMEM in 10 cm tissue culture (TC) treated plates and left for several hours to attach. Once attached, the desired concentration of drug was added. After the appropriate incubation time the drug-containing medium was removed and the plate washed with DPBS, then 10 ml of DMEM (20% FBS) was added and plates were incubated for 14 days. In the case of HEK293 cells the DPBS wash step is omitted to avoid excessive loss of cells. After incubation the medium was removed and the colonies stained with 0.4% methylene blue (Sigma) dissolved in 50% ethanol. The stained plates were scanned and the colonies were counted in ImageJ. The plating efficiency (PE, no. of colonies/no. of cells plated) and survival relative to the untreated control (PE treated/PE untreated*100) was calculated. Effective concentration$_{50}$ (EC_{50}) values were calculated using Drfit software (Veroli et al., 2015). Where UVC irradiation (256 nm wavelength) was used in place of a drug, once cells had attached they were washed with DPBS, the DPBS was removed and they were irradiated using a lamp (UVP UVLS-26). 10 ml of DMEM (20%) was added after irradiation and the plates were incubated for 14 days and counted as above. Two technical repeats were included in each independent experiment.

In the case of suspension cells (e.g TK6 cells) a methylcellulose colony forming survival assay was performed as described in Buerstedde and Takeda, 2006. Methylcellulose media was made

by heating RPMI to 55ºC and adding it to 5 g autoclaved methylcellulose powder (Sigma). The methylcellulose was dissolved by stirring overnight at 4ºC and 10% horse serum, 2 mM L-glutamine, penicillin-streptomycin and 2 mM sodium pyruvate was added. A 6 well plate was prepared for each treatment condition by filling each well with 5 ml methylcellulose media and incubating at 37ºC. Cells were diluted to 2 x 10^5 cells per ml and drug treated as appropriate in separate flasks. A sample was taken from each flask and 100 µl of this sample was pipetted into the top two wells of the appropriate 6 well plate and the plate shaken to evenly distribute the cells. Two tenfold dilutions of each sample were made and 100 µl of these dilutions were added to the other wells of the appropriate plate. Plates were then incubated for 12 days and any colonies were counted and calculations performed as above.

2.3 ATP based cell viability assay

The Cell Titer Glo 2.0 (Promega) assay was used as per the manufacturer's protocol. This assay uses luciferin which is luminescent after reacting with the adenosine triphosphate (ATP) contained with viable cells and luciferase. An approximately linear relationship exists between luminescence and cell number over a large range of cell numbers for TK6 and MRC-5 cells, showing the assay is appropriate for use with these cells lines across a range of seeding densities (Fig. 2.1). Cells were plated in wells of a white opaque 96 well plate in 80 µl medium. 20 µl of drug-containing or drug-free control medium was added to each well and the plate was incubated for 3 days. 100 µl of Cell Titer Glo 2.0 reagent was added to each well, and a control well with no cells but 100 µl media to check for background luminescence. The plate was placed on an orbital shaker for 2 mins before incubation at RT for 15 mins. The luminescence was recorded using a Wallac 1420 Victor^2 microplate reader (Perkin Elmer). Percentage viability was calculated (treated luminescence/ untreated luminescence*100). Three technical repeats were included in each independent experiment.

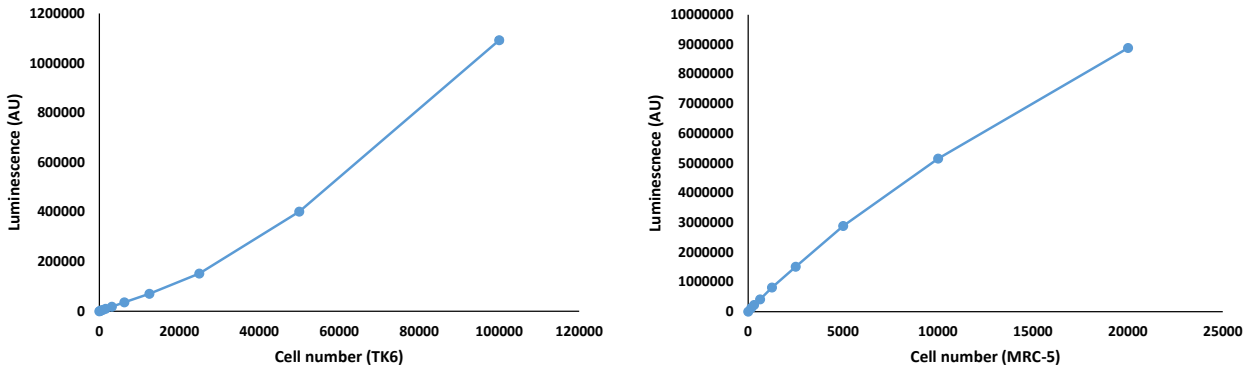


Figure 13. ATP viability assay optimisation. Luminescent signal obtained over a range of seeding densities for TK6 lymphoblasts (left) and adherent MRC-5 fibroblasts (right). Data are from one experiment with 3 technical repeats.

2.4 Drug preparation

All drugs used in this project were diluted from stocks as shown in table 2.

Table 2. Sources and preparation of drugs used in this project

Drug	Supplier	Stock solution (solvent)
Cytarabine (AraC)	Tocris Bioscience	100 mM (water)
Gemcitabine (dFdC)	Tocris Bioscience	10 mM (water)
Hydroxyurea (HU)	Sigma	1 M (water)
Mirin	Abcam	10 mM (DMSO)
Mitomycin C (MMC)	Abcam	10 mM (DMSO)
Phleomycin	Abcam	5 mM (water)
G418	Sigma	100 mg/ml (water)
Puromycin	Sigma	1 mg/ml (water)
Ampicillin	Sigma	50 mg/ml (water)

2.5 Liquid chromatography tandem mass spectrometry (LC/MS/MS)

This assay was performed to measure the amount of gemcitabine incorporated into DNA and the protocol was adapted from Pettersen et al (2011). $4x10^6$ cells were treated with gemcitabine and ^{13}C ^{15}N labelled deoxycytidine (dCyd$^{13}C^{15}N$, Silantes) for 24hrs. The cells were harvested and 6-20 µg DNA was extracted using the Qiagen DNeasy kit. The DNA was then precipitated using ethanol precipitation and the resulting pellet solved in 20 µl ddH_2O.

The next steps enzymatically hydrolysed the DNA into single nucleotides so that the relative abundance of the individual nucleotides can be measured by LC/MS/MS. The DNA was denatured at 95ºC for 3 minutes then cooled on ice. Ammonium acetate was added to the

dissolved DNA to a final concentration of 10 mM and $MgCl_2$ to a final concentration of 5 mM. 0.8 U Nuclease P1 (2 U/µl, Sigma N8630) was added and the sample was incubated at 45ºC for 60 minutes. Following this, ammonium bicarbonate was added to a final concentration of 100 mM and then 0.0008 U of snake venom phosphodiesterase (0.002 U/µl, Sigma P3243) and 2 U of alkaline phosphatase (1 U/µl, Sigma P5931) were added. This reaction was incubated at 37ºC for 90 minutes. After the enzymatic digestions the nucleosides were purified by adding 90 µl ice cold methanol, mixing and centrifuging at 14000g at 4ºC for 20 minutes. The resulting supernatant was transferred to a new tube and dried in a vacuum centrifuge. The residues were dissolved in 50 µl 5% methanol in water (v/v). Samples were then processed by Dr Rolf Kraehenbuehl at Bangor University on a Sciex Api 4000 Triple Quadrupole LC/MS/MS System. Contamination of extracted DNA by free gemcitabine was tested by treating with dCyd$^{13}C^{15}N$ only and then spiking with gemcitabine after cell lysis, but before DNA extraction. In these cases gemcitabine was not detectable above background demonstrating the column extraction effectively removed free gemcitabine (Fig.2.2).

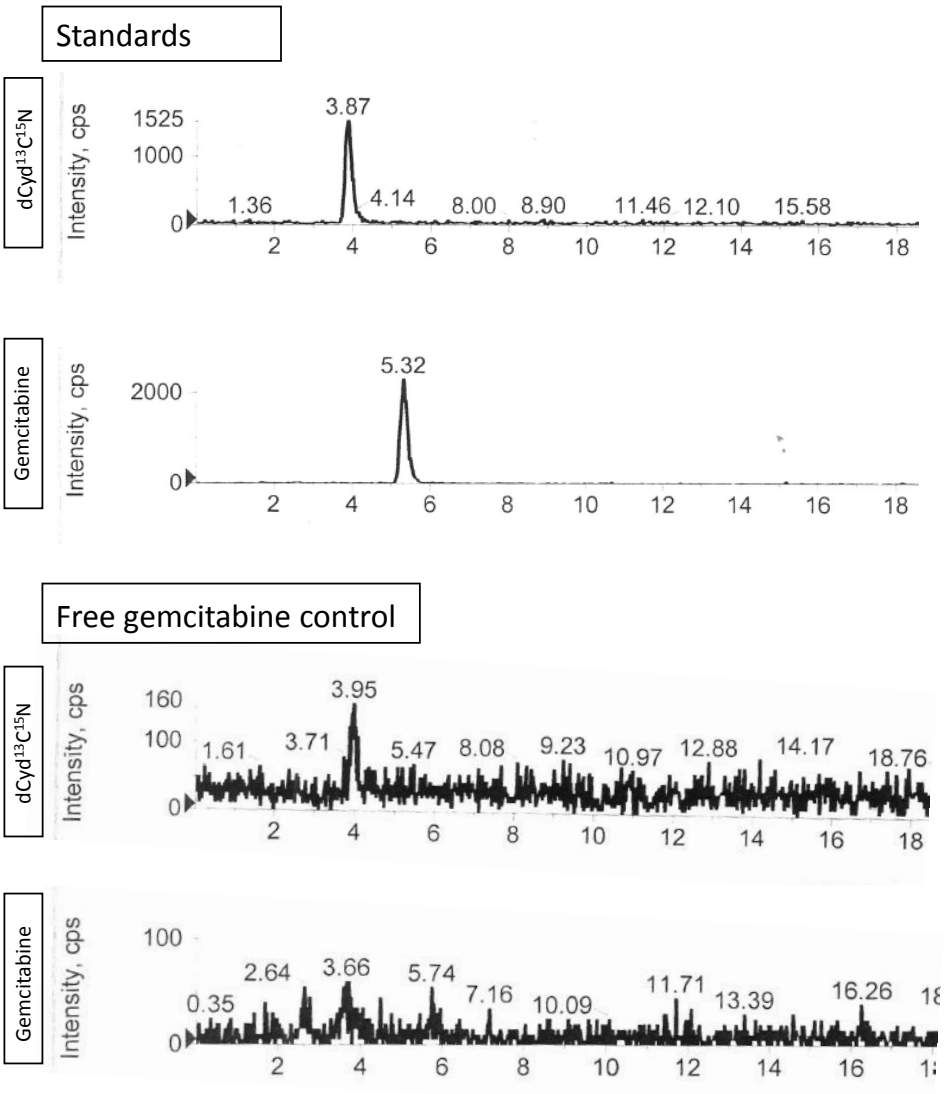


Figure 2.2 LC MS/ MS Controls. Standards show position of dCyd^{13}C^{15}N and gemcitabine peaks (top). Analysis of DNA from cells treated with dCyd^{13}C^{15}N only, where the cell lysate was spiked with gemcitabine before column extraction to show that this process removed unincorporated gemcitabine. Chromatograms from Dr. Rolf Kraehenbuehl.

2.6 DNA fibre analysis

This technique allows measurement of replication fork parameters such as fork speed and stalling. It was carried out as described in (Jones et al., 2014) and the technique was learned in the lab of Dr Eva Petermann with instruction from Dr Rebecca Jones and Dr Panos Kotsantis. Cells were plated at a non-confluent density into 2 wells of a TC treated 6 well plate and left for several hours to attach. To measure fork speed or stalling under normal conditions 5-chloro-2'-deoxyuridine (CldU) (Sigma, C6891) was added to a final concentration of 25 µM and cells

returned to the incubator for 20 mins. 5-iodo-2'-deoxyuridine (IdU) (Sigma, I7125) was then added to a final concentration of 250 µM and the cells were returned to the incubator for a further 20 mins. In order to measure the effect of gemcitabine on fork speed it was added alongside IdU and in the fork stalling experiment it was added together with CldU and incubation times adjusted as detailed in chapter 3. Replication was stopped by washing twice with ice cold PBS, then cells were trypsinised and collected in 250 µl cold PBS. The collected cells were counted and diluted or concentrated to a density of $5x10^5$ cells/ml before spreading.

5 slides of DNA fibre spreads were prepared for each experimental condition. In order to spread the fibres 2 µl of the cell suspension was spotted onto one end of a microscope slide (Menzel-Glaser Superfrost) and left for 5-7 minutes until starting to dry around the edge. 7 µl spreading buffer (200mM Tris-HCl, 50mM EDTA, 0.5% SDS, pH 7.4) was added to the spot, mixed with it using a pipette tip and left for 2 minutes. The slides were tilted to approximately 30º to allow the drop containing the lysed cells to run slowly down the slide over 3-5 minutes and then left to dry completely. The slides were fixed in 3:1 solution of methanol: acetic acid for 10 mins and then allowed to dry. The slides were stored at 4ºC before immunostaining.

For the immunostaining steps the slides were washed twice with H_2O (all washes were 5 minutes) followed by 1h 20 min incubation with 2.5 M HCl. Slides were washed twice with PBS before incubating with blocking solution (PBS, 1% BSA, 0.1% Tween20) for 1h. After removing the blocking solution 115 µl of a mixture of rat αBrdU (1:1000), which has greater affinity for CldU, and mouse αBrdU (1:250), which has greater affinity for IdU, in blocking solution was pipetted down one margin of the slide and spread over the whole slide by laying a large rectangular coverslip on top (antibody details in Table 3). The fibres were incubated with the primary antibodies for 2 hrs at room temperature (RT) after which they were washed three times with PBS. They were then fixed for 10 mins with 4% formaldehyde in PBS, washed three times with PBS and once with blocking solution. After removal of blocking solution 115 µl of a mixture of αRat AlexaFluor 555 (1:500) and αMouse AlexaFluor 488 (1:500) in blocking solution was pipetted down one margin of the slide, covered with a large coverslip and incubated at RT for 2.5 hrs. The slide was washed with PBS four times and a large coverslip mounted with Fluoroshield mounting medium (Abcam) and sealed with nail polish. Immunostained slides were stored at -20ºC before imaging. Fibres were imaged using a Zeiss LSM 710 confocal with a x63 objective lens and fibres were measured and counted in ImageJ.

Table 3. Antibodies used in DNA fibre analysis (DFA), immunofluorescence microscopy (IF) and western blotting (WB).

Antibody	Supplier	Dilution
αBrdU Clone BU1/75(ICR1), rat	Bio-rad	1:1000 (DFA)
αBrdU Clone B44, mouse	BD Biosciences	1:250 (DFA)
αXPA (12F5), mouse	Abcam (ab2352)	1:100 (IF)
αXPA (FL-273), rabbit	Santa Cruz (sc-853)	1:200 (WB)
αgamma H2A.X (phospho S139), mouse	Abcam	1:1000 (IF)
αERCC1 (EPR7277), rabbit monoclonal	Abcam (ab129267)	1:5000 (WB), 1:100 (IF)
αXPF, mouse	Abcam (ab85140)	1:1000 (WB)
αGapDH (G-9), mouse	Santa Cruz (sc-365062)	1:50 000 (WB)
αRat AlexaFluor 555, donkey	Abcam	1:500 (DFA, IF)
αMouse AlexaFluor 488, donkey	Abcam	1:500 (DFA, IF)
αRabbit HRP-linked	Cell Signalling (70745)	1:3000 (WB)
αMouse HRP-linked	Dako (P0260)	1:3000 (WB)
α53BP1, rabbit polyclonal	Abcam (ab36823)	1:100 (IF)

2.7 Immunofluorescent staining

Training in immunofluorescent staining and microscopy was obtained in the lab of Dr Catherine Green, Oxford University. 5-ethynyl-2′-deoxyuridine (EdU) staining was carried out using the Click-iT EdU Alexa Fluor 555 Imaging Kit (Invitrogen). Cells were seeded onto 11 mm^2 circular coverslips in 4 well plates at a density that allowed them to be subconfluent at the time of fixation and left to attach overnight. In the case of EdU labelling, EdU was added to the culture media to a final concentration of 10 μM 5 minutes prior to any drug treatment.

Before fixation cells were washed once in cytoskeletal (CSK) buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 7.0, and 3 mM $MgCl_2$), if pre-extraction was required they were incubated for 20 s in 0.1% Triton in CSK buffer and then washed once again in CSK buffer. Cells were fixed by incubation with 2% formaldehyde in CSK for 25 mins at RT. This was followed by permeabilisation in PBS 0.5% Triton for 2 minutes. The cells were then blocked in 5% bovine serum albumin (BSA) in PBS 0.1% Tween20 (PBS-Tw) for 1 hr at RT. EdU fluorescent labelling, if performed, was carried out as per the manufacturer's instructions and then cells were washed with blocking solution before antibody labelling. Cells were incubated with primary antibodies in blocking solution diluted as indicated in table 3 at 4ºC overnight. Coverslips were washed with PBS-Tw 3 times 5 mins, before 2 hr incubation at RT, protected from light, with secondary antibodies diluted in blocking solution. If ToPro3 (ThermoFisher) was used as a nuclear stain this was added alongside the secondary antibodies at 1:1000 dilution. Cells were then washed 3 times 5 mins with PBS-Tw and then mounted on a microscope slide using fluoroshield mounting medium, containing DAPI (abcam) if this was to be used as a nuclear stain. Coverslips were sealed with nailpolish and slides kept at 4ºC until imaging. Imaging was performed using a Zeiss LSM 710 inverted confocal microscope and images were analysed using the software ImageJ.

2.8 Protein extraction

300µl lysis buffer (50 mM Tris 7.4, 200 mM NaCl, 2 mM $MgCl_2$, 0.5% Triton) was used per 5 x 10^6 cells. 2µl benzonase (≥250 U/µl, Sigma) and complete protease inhibitor cocktail (Roche) was added to the lysis buffer before each experiment. Cells were collected, pelleted by centrifugation (250 x g, 5 mins) and then lysed by the addition of ice cold lysis buffer and vortexing. The samples were incubated on ice for 1 hr vortexing every 15 mins and then centrifuged at 14,000 x g for 10 mins. The supernatant was then transferred to a new tube and stored at -80ºC until needed.

2.9 Western blotting

The Pierce BCA Protein Assay kit (ThermoFisher Scientific), which is a colorimetric assay read using the NanoDrop 2000 (ThermoFisher Scientific), was used to determine protein concentration prior to western blotting. 50µg protein dissolved in 1x Laemmli buffer was denatured at 95ºC for 5 mins. Denatured samples were then run on 4-15% precast polyacrylamide gels (Bio-Rad) at 120 V for 1 hr 10 mins before transfer to nitrocellulose

membrane at 60 V for 2 hrs using the Mini-PROTEAN electrophoresis system (Bio-Rad). The membrane was blocked in milk buffer (PBS, 0.05% Tween20, 5% milk powder) for 30 mins at RT then incubated with primary antibody in milk buffer at 4ºC overnight. The membrane was then washed 3x for 10 mins in wash buffer (PBS, 0.05% Tween20) followed by incubation with horse radish peroxidase (HRP) conjugated secondary antibody in milk buffer for 1 hr at RT. Antibodies were used at dilutions indicated in table 3. This was followed by 3 more washes before activation of chemiluminescence with Amersham ECL Western Blotting Detection Reagent and developing onto photographic film or imaging using the Bio-Rad ChemiDoc system. The housekeeping protein GAPDH or staining with Pierce Reversible Protein Stain was used as a loading control.

Where the membrane needed to be stripped and reprobed the membrane was washed once with PBS to remove the detection reagent and then incubated 2 x 10 mins at RT in stripping buffer (200 mM glycine, 3.5 mM SDS, 1% Tween20, pH 2.2). The membrane was washed 3 x 10 mins in wash buffer and was then ready for blocking and probing as described above.

2.10 PCR and sequencing

PCR amplification and sequencing of resulting fragments was used to characterise ERCC1-/- CRISPR lines. PCR was carried out using MyTaq polymerase and Red Mix mastermix (Bioline), following the manufacturers instructions. The following PCR conditions were used: initial denaturation 95ºC 1 min, then 30 cycles of denaturation (95ºC, 15 s), annealing (55ºC, 15 s) and extension (72ºC, 20 s). The following primers were used as described 5'>3': (1) Forward AGAGATCGCCCTGCTCTATG, (2) Reverse GGTGATGCGGCACTCGATC (for detection of inserted selection marker) and (3) ACCCGTCGATTGTTTTCCCT. As described in chapter 4, fragments that did not contain the selection marker were sequenced to understand what mutation was present in the cells which did not express ERCC1. PCR products were cleaned prior to sequencing using spin column clean up (Nucleospin, Macherey-Nagel), then sequenced using Eurofins Mix2Seq service. Samples were prepared as per the Mix2Seq protocol and sequenced in both forward the reverse directions using the following primers: Forward GTTACAGAGCCTCTAGCG, Reverse GATCCTATCCTCTTCGTCC.

2.11 Complementation of MRC-5 ERCC1-/- with *ercc1* cDNA

Transfection of MRC-5 ERCC1 -/- cells with ERCC1 cDNA was carried out to test whether the MRC-5 ERCC1 -/- phenotype was indeed due to loss of ERCC1.

2.11.1 Plasmid transformation and purification

The expression vectors used were pcDNA3.1+ plasmids purchased from Genscript, with an ERCC1 open reading frame (table 4) inserted in the multiple cloning site. The plasmid also contained an ampicillin resistance sequence under the control of a bacterial promotor and a G418 resistance sequence under the control of a mammalian promotor for selection purposes. Bacterial transformation was used to amplify the plasmids. Chemically competent *E. coli* (NEB 10-beta) were thawed on ice for 10 mins. 10 ng plasmid DNA in 1-5 µl was added to the thawed bacteria and the tube was flicked to mix. The mixture was left on ice for 30 mins, heat shocked at 42ºC for 30 seconds and placed back on ice for 5 mins. 950 µl SOC growth medium (LB, 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.19g KCl, 10mM $MgCl_2$, 10mM $MgSO_4$/ L) was added and 60 mins of outgrowth in a shaking incubator at 37ºC was allowed before selection plating. 2 1:10 dilutions in SOC of the bacteria containing mixture were made. 100 µl of each dilution was spread on a LB agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 20 g agar/ L) plate containing ampicillin (100 µg/ ml) and incubated O/N at 37ºC. An ampicillin resistant colony was picked the following day and used to inoculate 40 ml of LB which was placed in a 37ºC shaking incubator O/N. 0.5 ml of this culture was mixed with 50% glycerol in a cryotube to make a bacterial stock. Another 3 ml was harvested for extraction of the plasmid DNA using a Nucleospin plasmid kit (Macherey-Nagel). The DNA concentration of the resulting extract was then measured using the Qubit (ThermoFisher Scientific).

2.11.2 Transfection of MRC5 ERCC1-/- cells

Cells were plated in a 6 well plate at a density of 400 000 cells per well in DMEM without added antibiotics. Lipofectamine 2000 (ThermoFisher) was used as the transfection reagent. For every well transfected 1 µg DNA was added to 100 µl DMEM (without any additives) and in a separate tube 4 µl Lipofectamine 2000 was added to 100 µl DMEM (without additives). Both tubes were mixed by inversion five times and left to stand for 5 mins. The contents of the tube holding the transfection reagent were then added to the tube containing the DNA solution. This tube was then mixed by inversion five times and left to stand for 25 mins. 200 µl of DNA/liposome complex was then added to the cells which were then returned to the incubator for 24 hrs.

After 24, 48 and 72 hrs cells were collected and checked for ERCC1 expression by western blot and compared with cells transfected with an empty vector and untransfected MRC-5. This

determined the expression levels and persistence of expression after transfection. At 24 hrs post transfection, cells were harvested and seeded for use in survival assays.

In the case of stable transfection the cells were passaged five times before selection to dilute out cytoplasmic expression of the plasmid. A concentration of 600 µg/ml G418 was found to be effective in killing unmodified MRC-5 cells and was therefore used to select for those cells which had incorporated the expression vector into their genomic DNA. The resistant population of cells was then seeded into 10 cm plates at low density and grown for 14 days when several clones could be picked and amplified into a clonal population. Clonal populations were then tested for ERCC1 expression by western blotting.

Table 4. ERCC1 cDNA sequences used for attempted rescue of the MRC-5 ERCC1 -/- phenotype.

Isoform (Uniprot)	**ORF Sequence**
Isoform 1	AAGCTTATGGACCCTGGGAAGGACAAAGAGGGGGTGCCCCAGCCCT CAGGGCCGCCAGCAAGGAAGAAATTTGTGATACCCCTCGACGAGGA TGAGGTCCCTCCTGGAGTGGCCAAGCCCTTATTCCGATCTACACAG AGCCTTCCCACTGTGGACACCTCGGCCCAGGCGGCCCCTCAGACCTA CGCCGAATATGCCATCTCACAGCCTCTGGAAGGGGCTGGGGCCACG TGCCCCACAGGGTCAGAGCCCCTGGCAGGAGAGACGCCCAACCAGG CCCTGAAACCCGGGGCAAAATCCAACAGCATCATTGTGAGCCCTCG GCAGAGGGGCAATCCCGTACTGAAGTTCGTGCGCAATGTGCCCTGG GAATTTGGCGACGTAATTCCCGACTATGTGCTGGGCCAGAGCACCT GTGCCCTGTTCCTCAGCCTCCGCTACCACAACCTGCACCCAGACTAC ATCCATGGGCGGCTGCAGAGCCTGGGGAAGAACTTCGCCTTGCGGG TCCTGCTTGTCCAGGTGGATGTGAAAGATCCCCAGCAGGCCCTCAA GGAGCTGGCTAAGATGTGTATCCTGGCCGACTGCACATTGATCCTC GCCTGGAGCCCCGAGGAAGCTGGGCGGTACCTGGAGACCTACAAGG CCTATGAGCAGAAACCAGCGGACCTCCTGATGGAGAAGCTAGAGCA GGACTTCGTCTCCCGGGTGACTGAATGTCTGACCACCGTGAAGTCA GTCAACAAAACGGACAGTCAGACCCTCCTGACCACATTTGGATCTC TGGAACAGCTCATCGCCGCATCAAGAGAAGATCTGGCCTTATGCCC

	AGGCCTGGGCCCTCAGAAAGCCCGGAGGCTGTTTGATGTCCTGCAC GAGCCCTTCTTGAAAGTACCCTGAGGATCC
Isoform 3	AAGCTTATGGACCCTGGGAAGGACAAAGAGGGGGTGCCCCAGCCCT CAGGGCCGCCAGCAAGGAAGAAATTTGTGATACCCCTCGACGAGGA TGAGGTCCCTCCTGGAGTGGCCAAGCCCTTATTCCGATCTACACAG AGCCTTCCCACTGTGGACACCTCGGCCCAGGCGGCCCCTCAGACCTA CGCCGAATATGCCATCTCACAGCCTCTGGAAGGGGCTGGGGCCACG TGCCCCACAGGGTCAGAGCCCCTGGCAGGAGAGACGCCCAACCAGG CCCTGAAACCCGGGGCAAAATCCAACAGCATCATTGTGAGCCCTCG GCAGAGGGGCAATCCCGTACTGAAGTTCGTGCGCAATGTGCCCTGG GAATTTGGCGACGTAATTCCCGACTATGTGCTGGGCCAGAGCACCT GTGCCCTGTTCCTCAGCCTCCGCTACCACAACCTGCACCCAGACTAC ATCCATGGGCGGCTGCAGAGCCTGGGGAAGAACTTCGCCTTGCGGG TCCTGCTTGTCCAGGTGGATGTGAAAGATCCCCAGCAGGCCCTCAA GGAGCTGGCTAAGATGTGTATCCTGGCCGACTGCACATTGATCCTC GCCTGGAGCCCCGAGGAAGCTGGGCGGTACCTGGAGACCTACAAGG CCTATGAGCAGAAACCAGCGGACCTCCTGATGGAGAAGCTAGAGCA GGACTTCGTCTCCCGGGTGACTGAATGTCTGACCACCGTGAAGTCA GTCAACAAAACGGACAGTCAGACCCTCCTGACCACATTTGGATCTC TGGAACAGCTCATCGCCGCATCAAGAGAAGATCTGGCCTTATGCCC AGGCCTGGGCCCTCAGAAAGTAAGAGCTCTGGGAAAGAACCCAAGG AGTTGGGGGAAGGAGAGAGCCCCAAATAAACACAACCTGAGACCCC AAAGTTTTAAGGTGAAAAAAGAACCAAAGACCAGACACAGTGGCTT CCGCCTGTAAGGATCC

2.12 Chromosome segregation assay

This assay assessed the frequency of visible segregation defects (such as lagging chromosomes and anaphase bridges) occurring in ana/ telophase cells before and after gemcitabine treatment. Cells were seeded onto 11 mm^2 circular coverslips in 4 well plates at a density that allowed them to be subconfluent at the time of fixation and left to attach overnight. The growth medium was removed and the coverslips were washed with PBS and then fixed with 4% formaldehyde

in PBS for 20 mins at RT. The cells were permeabilised with 0.5% Triton in PBS for 2 mins then washed with PBS and mounted in Fluoroshield with DAPI (Abcam). The edges of the coverslip were sealed with nail polish and slides kept at 4ºC until imaging. Images of approximately 50 cells in anaphase or telophase were collected per condition for each experiment, using a Zeiss LSM 710 inverted confocal microscope. The images were then scored in ImageJ.

3 GEMCITABINE SENSITIVITY IN XP PATIENT CELL LINES

This chapter begins by demonstrating that XP patient cell lines, which are deficient in core NER factors, are sensitive to gemcitabine compared to a normal cell line (MRC-5) as was described in previous work (Beardmore, 2015). This was also consistent with previous work in *S. pombe* where several NER mutants were found to be sensitive to gemcitabine (Gasasira, 2013). As discussed in the introduction NER factors do not have a well characterised role in replication or mitigating replication stress so in this chapter different techniques are employed to investigate the basis of this sensitivity. Mass spectrometry enables measurement of the amount of gemcitabine incorporated into newly replicated DNA and can therefore indicate a difference in either incorporation or removal. DNA fibre analysis examines replication forks directly and provides an insight into whether differences in fork stalling underpin sensitivity. Finally immunofluorescence microscopy experiments investigate whether NER factors form repair foci or are present at stalled forks.

3.1 XP patient fibroblasts are sensitive to UV and gemcitabine compared to normal human fibroblasts MRC-5

3.1.1 Patient cell lines

The NER deficient fibroblast cultures used in this study were established from skin biopsies from XP patients. They carry mutations in core NER factors and have been used extensively to study NER. Defective NER is diagnosed using a repair assay known as Unscheduled DNA Synthesis (UDS) which measures the amount of newly synthesised DNA that replaces damaged patches of DNA after UV exposure relative to a normal cell line. Cells have been assigned to a complementation group by fusion with cells representative of each complementation group and observing whether or not their repair defect is corrected (reviewed Lehmann et al., 2011). The origin of these cell lines and the details of the genetic defects which they carry are described below and the complementation groups to which they are assigned are given in

brackets. All of the patient fibroblasts used in this project were immortalised by SV40 transformation.

3.1.1.1 XP12RO (XP-A)

These cells are skin fibroblasts isolated from a 12 year old Palestinian male who exhibited severe XP symptoms and also neurological symptoms of de Santis-Cacchione syndrome (Kaloustian et al., 1974; Satokata et al., 1992a). The cells are homozygous for a C to T transition at nucleotide 619 of exon 5 of the *xpa* gene. This changes Arg207 to a stop codon resulting in a truncated protein containing 206 out of 273 amino acids (Satokata et al., 1992b). The mutated gene produces a reduced amount of mRNA and no XPA protein is detectable by immunoprecipitation or immunofluorescent staining (Miyamoto et al., 1992; Satokata et al., 1992b) It has been demonstrated that exons 2-6 are essential for UV lesion repair and in accordance with this the fibroblasts are highly sensitive to UV light and exhibit reduced unscheduled DNA synthesis (UDS) that has been reported to be between <5% or 12.5% of normal (Kaloustian et al., 1974; Muotri et al., 2002; Satokata et al., 1992b).

3.1.1.2 XP2OS (XP-A)

Skin fibroblasts were isolated from a 7 year old Japanese female who, like the previous patient, suffered severe XP symptoms and neurological symptoms of Santis-Cacchione syndrome (Takebe et al., 1974, 1977). The cells carry a homozygous AG to AC transition at the splice acceptor site of intron 3 of the *xpa* gene. This causes abnormal splicing, mRNA instability and loss of activity of the resulting protein. No normal mRNA can be detected (Satokata et al., 1990, 1992a). The fibroblasts are reported to be highly sensitive to UV light and undergo <2% of normal UDS after UV exposure (Takebe et al., 1977).

3.1.1.3 XP4PA (XP-C)

This cell line is comprised of skin fibroblasts which were cultured from a newborn Algerian male who was diagnosed with XP prenatally. The boy received UV protective measures but no neurological or developmental problems were mentioned (Halley et al., 1979). The full length XPC protein is 823 amino acids in length but XP4PA is homozygous for a dinucleotide deletion which causes a frameshift and premature stop codon at amino acid 430. This mutation also leads to a 6.5 fold reduction in mRNA transcript level as measured by northern blot and a truncated protein (Li et al., 1993). The cells are sensitive to UV light and show reduced UDS which has been reported as 12.5% (Halley et al., 1979) and 30% (Daya-Grosjean et al., 1987).

The truncated protein appears to retain some function as cells expressing more severely truncated XPC protein are more sensitive to UV light (Li et al., 1993).

3.1.1.4 XP2YO (XP-F)

These fibroblasts were established from a 64 year old Japanese female who exhibited mild XP symptoms and no neurological abnormalities. The cells are heterozygous, with one *xpf* allele containing a single nucleotide deletion leading to a frameshift and premature stop codon at amino acid 646 (from a normal length protein of 905 amino acids). The second *xpf* allele has a base substitution which results in a Thr556 to Ala change. mRNA levels are described as normal but no full length protein can be detected by western blot (Matsumura et al., 1998), however the same authors demonstrate that low levels of full length protein can be detected by coIP with ERCC1 (Yagi et al., 1997). They hypothesise that the mutated protein retains some repair function and this is responsible for the mild clinical phenotype. The cells are sensitive to UV and have a reported UDS of 17% (Matsumura et al., 1998).

3.1.1.5 XPCS1RO (XP-G)

Skin fibroblasts were obtained from a Moroccan boy exhibiting severe developmental and neurological abnormalities of Cockayne Syndrome who died aged 7 months. The cells were hypersensitive to UV and also exhibited UDS 4% of normal (Hamel et al., 1996). XPCS1RO are homozygous for a single nucleotide deletion in *xpg*. This causes a frameshift at amino acid 925 and a premature stop at amino acid 980 compared to a full length XPG protein of 1186 amino acids. No full length or truncated protein was detected from these cells by western blotting with an antibody raised against the N terminus of the protein leading to the conclusion that the truncated protein is unstable (Nouspikel et al., 1997).

3.1.2 Survival after UV exposure

The XP patient fibroblasts described above were tested for sensitivity to UV light relative to the normal fibroblast line MRC-5. This assay acts as a control to confirm these cell lines have an NER phenotype. It also provides an indication of differing levels of residual repair the cells may have as, as was described above, the patient cells may not be truly null for their defective genes. The colony forming survival assay measures survival by counting the fraction of cells that form colonies (Fig. 3.1), and are therefore able to survive, continue to divide and produce large numbers of progeny after treatment with a cytotoxic agent (Franken et al., 2006).

All the XP fibroblasts were found to be sensitive to UV as expected, validating their deficiency in NER (Fig. 3.2). XP-F (XP2YO) was found to be slightly less sensitive than the others, which indicates a greater level of residual repair capacity than the other cell lines. This is consistent with the cell line having come from a very mildly affected individual. XP4PA, the XPC deficient cell line, is very similar in sensitivity to the XPA deficient lines. This is somewhat at odds with the finding in (Beardmore, 2015) that XP4PA is not at all sensitive to the UV mimetic drug 4NQO, which was ascribed to XPC deficient cells still being proficient in TC-NER. However this finding is consistent with UV dose of 0.86 J/m causing a 37% drop in survival, as described by Li et al., 1993. This suggests 4NQO and UV are not interchangeable as DNA damaging agents and that loss of GG-NER is just as problematic for the cell as loss of both NER pathways after UV irradiation.

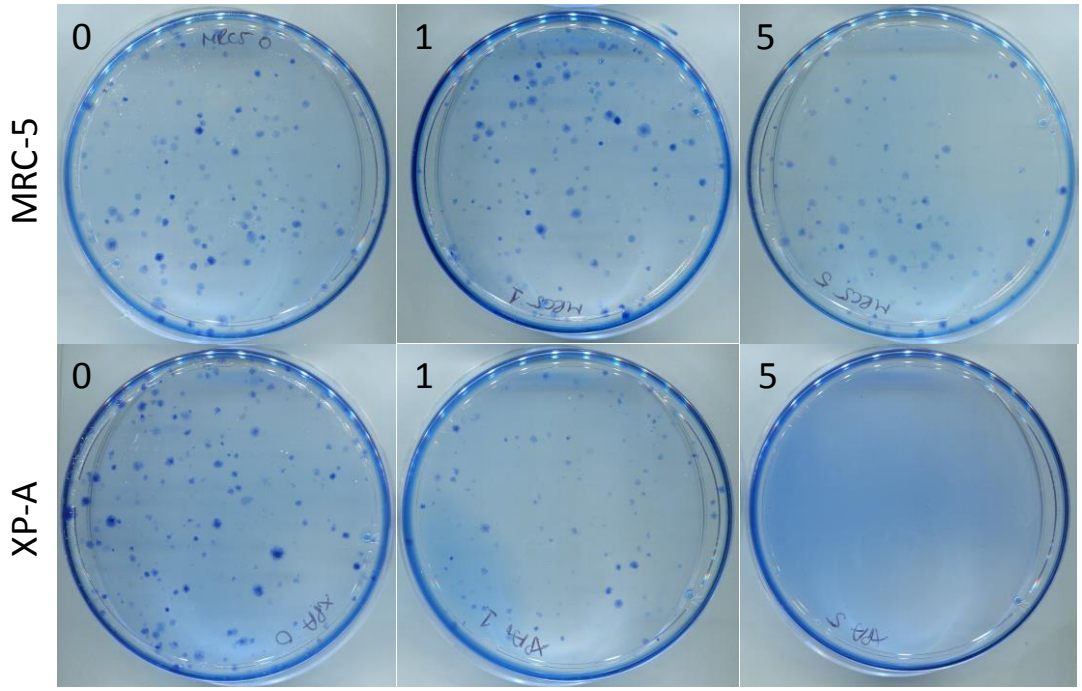


Figure 3.1 Colony forming survival assay. Example images showing colonies formed by normal cells (MRC-5) and XP-A (XP12RO) cell lines after treatment with different doses of UV light (J/m^2).

3.1.3 Survival after gemcitabine

The fibroblasts were tested in the same manner for sensitivity to a 24hr treatment with gemcitabine (Fig. 3.2). A 24 hr treatment was used so that all the cells would enter S phase

while exposed to the drug. The results resemble what is seen after UV treatment. All but one of the XP patient cell lines tested show high sensitivity, with an EC_{50} value between 9 and 22 fold higher than that of MRC-5 (Table 5). The exception is XP-F (XP2YO) which shows much milder sensitivity than the rest, only 2 fold that of MRC-5. These data replicate those presented in (Beardmore, 2015). This indicates that the NER deficiency in these cell lines may sensitise them to gemcitabine and that the NER pathway has an uncharacterised role in gemcitabine resistance.

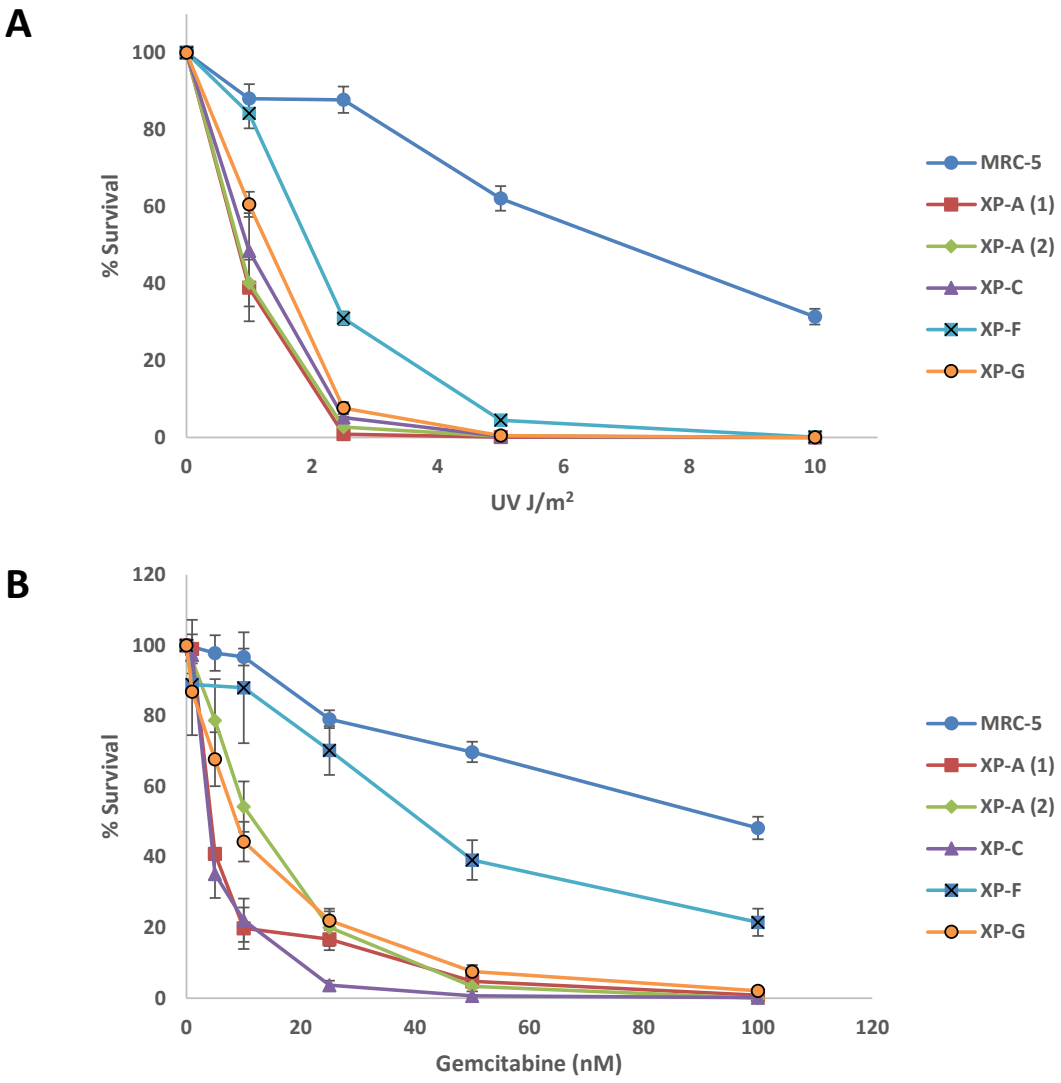


Figure 3.2 Sensitivity of XP patient fibroblasts to UV and gemcitabine. Colony forming survival assays showing (A) UV sensitivity and (B) gemcitabine sensitivity of 5 XP patient cell lines, XP-A (1, XP12RO), XP-A (2, XP2OS), XP-C (XP4PA), XP-F (XP2YO), XP-G (XPCS1RO)

compared with MRC-5. Cells were exposed to gemcitabine for 24hrs. Data from 2-6 independent experiments. Error bars show standard error.

Table 5. Gemcitabine EC_{50} values for XP patient fibroblasts and fold decrease when compared with MRC-5.

Cell line	EC50 (nM)	Fold decrease
MRC-5	96.5	0
XP-A (1) (XP12RO)	4.31	22
XP-A (2) (XP2OS)	10.9	9
XP-C (XP4PA)	3.98	24
XP-F (XP2YO)	40.3	2
XP-G (XPCS1RO)	8.35	12

3.2 Liquid chromatography tandem mass spectrometry (LC/MS/MS) indicates defective gemcitabine removal from DNA in XP-A and XP-G cell lines.

There is evidence to suggest gemcitabine cytotoxicity is mediated by incorporation of the analogue into DNA (Huang and Plunkett, 1995; Huang et al., 1991). Given the canonical role of NER in which damaged DNA is excised, it is plausible NER factors may excise gemcitabine from DNA, thus leading to resistance. A defect in gemcitabine excision would lead to an increase in the amount of gemcitabine incorporated into DNA which can be measured by LC MS/MS.

3.2.1 Optimisation of LC/MS/MS protocol

This technique has been used previously to study the amount of nucleoside analogues, 5-fluorouracil and 5-fluoro-2-deoxyuridine, incorporated into DNA after treatment (Pettersen et

al., 2011). For this study cells were treated simultaneously with gemcitabine (dFdC) and ^{13}C ^{15}N labelled deoxycytidine (dCyd$^{13}C^{15}N$ or hdC) for 24hrs. This enabled all cells time to enter S phase in the presence of gemcitabine and dCyd$^{13}C^{15}N$ gave a measure of newly synthesised DNA so differences in rates of replication could be corrected for by determining the gemcitabine: dCyd$^{13}C^{15}N$ ratio. Following treatment DNA was extracted from cells using a procedure which excluded free nucleotides and drug molecules from the sample. The DNA was then enzymatically digested into single nucleosides. The different nucleosides and analogues were distinguished and quantified by LC/MS/MS. An increased amount of gemcitabine relative to dCyd$^{13}C^{15}N$ could represent either increased incorporation or decreased removal from DNA. As NER is known to excise damaged sections of DNA it was hypothesised that an increased relative amount of gemcitabine in the XP patient cell lines represents decreased removal.

Before a comparison of the different cell lines could be made, gemcitabine and heavy deoxycytidine doses needed to be optimised. The addition of exogenous cytidine, in this case dCyd$^{13}C^{15}N$, has the potential to affect replication (Gemble et al., 2015). For this reason the lowest dose that reliably yielded a clear signal on the mass spectrometer was sought and determined to be 10nM. Gemcitabine incorporation relative to dCyd$^{13}C^{15}N$ incorporation was calculated for a range of gemcitabine doses (Fig. 3.3). At a dose of 1 µM gemcitabine and 10 nM dCyd$^{13}C^{15}N$, gemcitabine and dCyd$^{13}C^{15}N$ were incorporated into DNA in approximately equal amounts in MRC-5 cells. When the relative gemcitabine incorporation of MRC-5 and XP-A were compared XP-A cells showed greater gemcitabine incorporation at 10 nM, 100 nM and 1 µM gemcitabine. These are concentrations where there is a difference in survival between the cell lines (Fig. 3.2). This supports the idea that higher levels of gemcitabine incorporated into DNA leads to poorer survival. At 10 µM gemcitabine XP-A cells show slightly less gemcitabine incorporation relative to MRC-5 (0.82), however at this concentration it is unlikely either cell line will survive the treatment. 1 µM gemcitabine was chosen as the dose used in the subsequent experiments as at this dose there were high relative levels of gemcitabine incorporated into DNA and a survival difference between MRC-5 and XP-A.

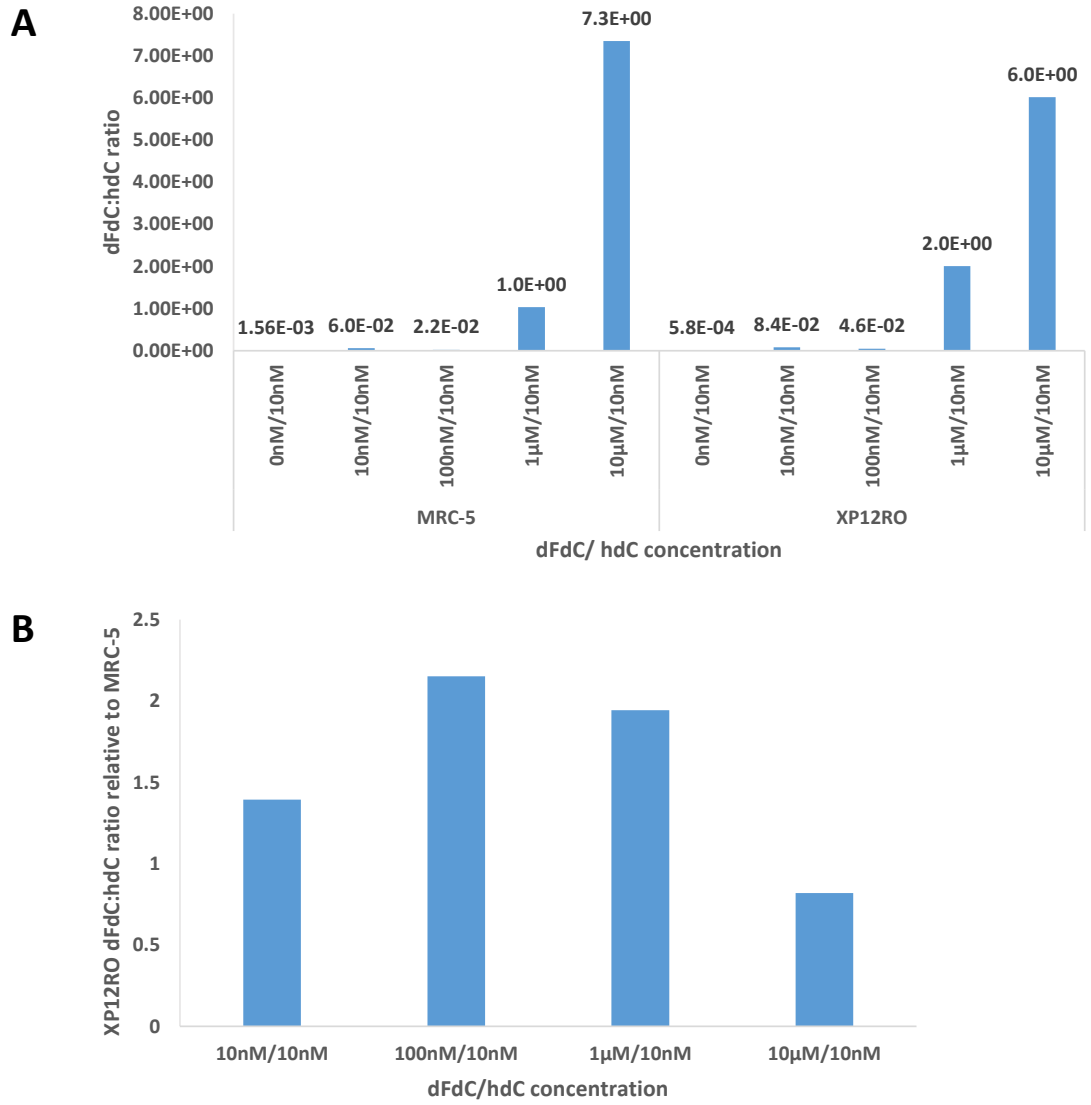


Figure 3.3 Optimisation of gemcitabine dose for the LC/MS/MS assay. (A) dFdC: dCyd$^{13}C^{15}N$ (hdC) ratio after 24hr treatment with 10nM dCyd$^{13}C^{15}N$ and different concentrations of gemcitabine for MRC-5 and XP-A (XP12RO) cells. (B) XP-A (XP12RO) dFdC: dCyd$^{13}C^{15}N$ (hdC) ratio relative to MRC-5. Data are from a single experiment.

3.2.2 XP-A and XP-G cells show increased gemcitabine incorporation compared to MRC-5

Figure 3.4 shows the gemcitabine to dCyd$^{13}C^{15}N$ ratio of several XP patient cell lines after a 24 hr treatment with 1µM gemcitabine and 10 nM dCyd$^{13}C^{15}N$. XP-A (XP12RO) and XP-G (XPCS1RO) show respectively 1.79 and 1.46 fold increases in gemcitabine incorporation compared to MRC-5. Whereas XP-F (XP2YO) shows a slight decrease compared to MRC-5. This suggests XPA and XPG may have a role in gemcitabine removal and lack of these proteins

leads to higher levels of gemcitabine in DNA and consequent poorer survival. However, the statistical significance of the differences in dFdC: dCyd$^{13}C^{15}N$ (hdC) ratio between the patient cell lines and MRC-5 is weak when analysed with a t test (XP-A, p=0.07, XP-G, p= 0.07). Therefore this conclusion is only weakly supported by the current data and requires further work to clarify the situation, such as increasing the number of repeats performed and testing different cell lines which are deficient for the same gene to see if the same changes in gemcitabine incorporation are observed. From these data it appears that XPF does not have a role in gemcitabine removal, although it cannot be ruled out that residual XPF activity in XP2YO is sufficient to carry out this function.

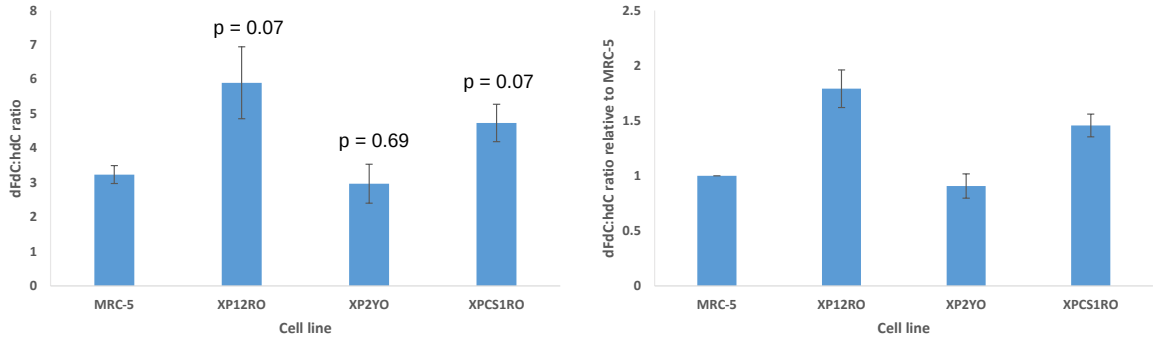


Figure 3.4 Gemcitabine incorporation in XP patient cell lines compared with MRC-5. Lefthand chart shows dFdC: dCyd$^{13}C^{15}N$ (hdC) ratio in MRC-5 (control), XP-A (XP12RO), XP-F (XP2YO) and XP-G (XPCS1RO). Right hand chart shows the same data with values for XP patient cell lines expressed relative to MRC-5 (control). Data is from 3 independent experiments. Error bars show standard error. P values given are compared with MRC-5 using a t test.

3.3 DNA fibre analysis suggests XPA and XPG have a role in mitigating fork slowing and stalling

Gemcitabine is a replication inhibitor (Ewald et al., 2008a), so in order to understand the role of the XP proteins in mitigating gemcitabine sensitivity it is informative to look at differences in replication dynamics in the XP mutant fibroblasts. DNA fibre analysis allows observation of individual replication forks and quantification of replication fork parameters such as speed and the occurrence of different fork structures (reviewed in Nieminuszczy et al., 2016). Labelling with two different analogues for short periods of time allows the distinction of initiations and ongoing or stalled forks (Fig. 3.5). Replication initiates at an origin and extends

in both directions from the origin. A replication fork terminates when two forks travelling towards each other meet or the fork encounters a problem. The assays described here were carried out to determine whether XP proteins had a role in fork progression, specifically in preventing the slowing and stalling of forks. Alternatively they could have no role in fork progression, but a function in processing persistently stalled forks later on, as was found for BRCA2 and RAD51 (Jones et al., 2014). The evidence presented here suggests the former.

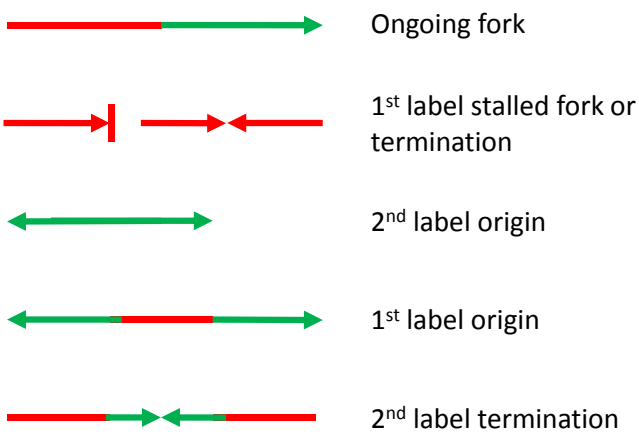


Figure 3.5 Key to the appearance of different fork structures that can be seen by DNA fibre analysis. Red represents the 1st label (CldU), green represents the 2nd label (IdU).

3.3.1 Fork speed decreases within an hour of treatment with 1µM gemcitabine

Fork speed is calculated by measuring the length of the green IdU labelled segment of ongoing forks and dividing by IdU incubation time (Fig. 3.6). This gives a measure of normal fork progression and can show if fork progression is impeded by a drug if the drug is added alongside the IdU. Fork speed under normal conditions was measured for NER proficient fibroblasts (MRC-5) and XP-A cells (XP12RO). XP-A cell forks were shown to be slower (0.77 kb/min) compared with MRC-5 forks (1.2 kb/min). This could indicate a role for XPA in normal replication fork progression (as has been suggested by Gilljam et al., 2012). When 1 µM gemcitabine (dFdC) is added alongside the IdU it causes a slowing of forks in both cell lines, although XP-A cells (65% of no gemcitabine control) see a more dramatic decrease than MRC-5 (84% of no gemcitabine control). This is what would be expected if XPA had a role in maintaining fork progression under replication stress.

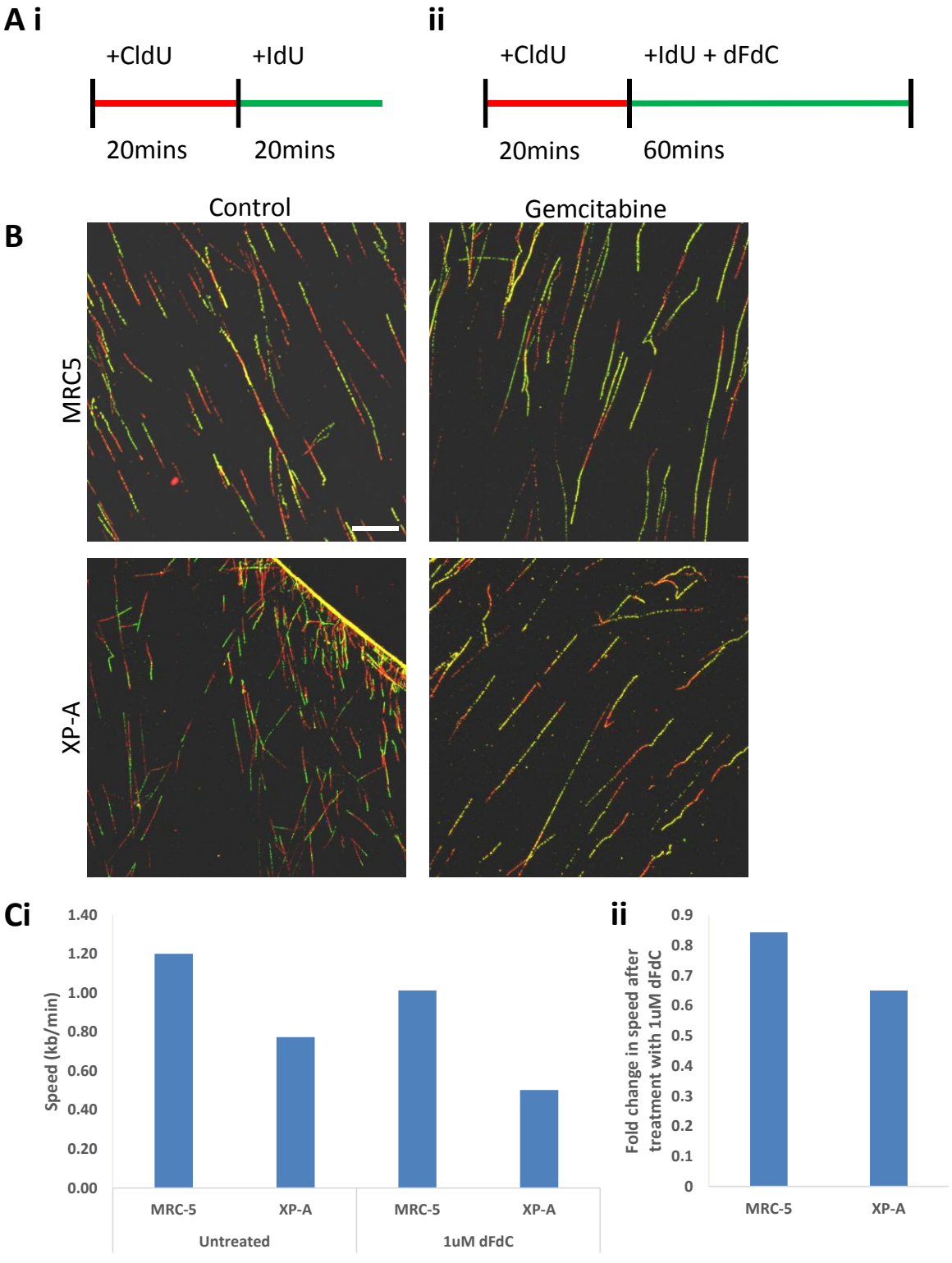

A i
+CldU
+IdU
20mins
20mins
ii
+CldU
+IdU + dFdC
20mins
60mins
B
Control
Gemcitabine
MRC5
XP-A
Ci
Speed (kb/min)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
MRC-5
XP-A
MRC-5
XP-A
Untreated
1uM dFdC
ii
Fold change in speed after treatment with 1uM dFdC
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MRC-5
XP-A

Figure 3.6 Replication fork speed in MRC-5 and XP-A cells. (A) Scheme showing experimental setup for (i) control and (ii) gemcitabine (dFdC) treated cells. Length of the green IdU labelled portion is used to calculate fork speed. **(B)** Representative images for MRC-5 (WT) and XP-A (XP12RO) with and without 1 µM gemcitabine (dFdC) treatment. Scale bar shows 20 µm. **(C)** Bar charts showing (i) fork speed and (ii) fold change in fork speed with gemcitabine (dFdC) treatment. Approximately 150 forks and 10 cells were assayed per condition. Data is from a single experiment.

3.3.2 Increased fork stalling after gemcitabine treatment in XP patient cell lines

Measuring fork speed does not show whether forks are continuing slowly or if they have stopped or reversed before the end of the incubation. If XP proteins act to mitigate stalling, more stalling would be expected in the cell lines where these factors are mutated. An assay was carried out to measure fork stalling in MRC-5 (WT) and XPA, XPF and XPG deficient patient fibroblasts, under normal conditions, after a 1 hour 100 nM gemcitabine treatment and after a 1 µM treatment. Figure 3.7 shows a diagram of how the assay was executed. Gemcitabine was added 20 minutes after the first label (CldU, red) and then the second label was added after an hour of gemcitabine treatment (IdU, green). Forks that had stopped before the end of the hour in gemcitabine would not incorporate IdU and show as red only labelled structures (Fig. 3.5). The proportion of stalled forks and terminations was calculated by counting all fork structures and dividing the number of stalled forks by all red (CldU) labelled structures. Stalled forks cannot be distinguished from 1st label terminations, where two converging forks collide, which is a weakness of this analysis. However as a replication inhibitor, gemcitabine would be expected to lower the incidence of terminations and therefore any increase in the number of red (CldU) only structures after gemcitabine can reasonably be attributed to an increase in stalling.

Figure 3.7 shows without gemcitabine treatment the incidence of stalled forks/ terminations is small in all the cell lines tested and there is no statistically significant difference between MRC-5 and XP12RO (XP-A, p= 0.72), XP2YO (XP-F, p= 0.14) or XPCS1RO (XP-G, p=0.58) as assessed by a t test. Gemcitabine treatment reveals differences between the cell lines. After 1 hour of 100nM gemcitabine treatment all the XP patient cell lines show a mild increase in fork stalling compared to MRC-5, but the increase is only statistically significant in XP12RO (XPA, p=0.0099) and XP2YO (XPF, p=0.026). After 1 hour of 1 µM gemcitabine treatment XP12RO

(XP-A) and XPCS1RO (XP-G) have 67% and 77% of forks stalled respectively compared to 47% of forks stalling in MRC-5, whereas XP2YO (XP-F) shows a similar level to MRC-5 ($p=0.37$). Increased fork stalling in XPA and XPG deficient cell lines at both 100 nM and 1µM suggests these factors have a role in fork progression and that increased numbers of stalled forks may be responsible for their gemcitabine sensitivity. The role of XPF in fork progression is less clear. After 100 nM gemcitabine treatment it appears to have a role in fork progression, but when treated with the higher dose it does not.

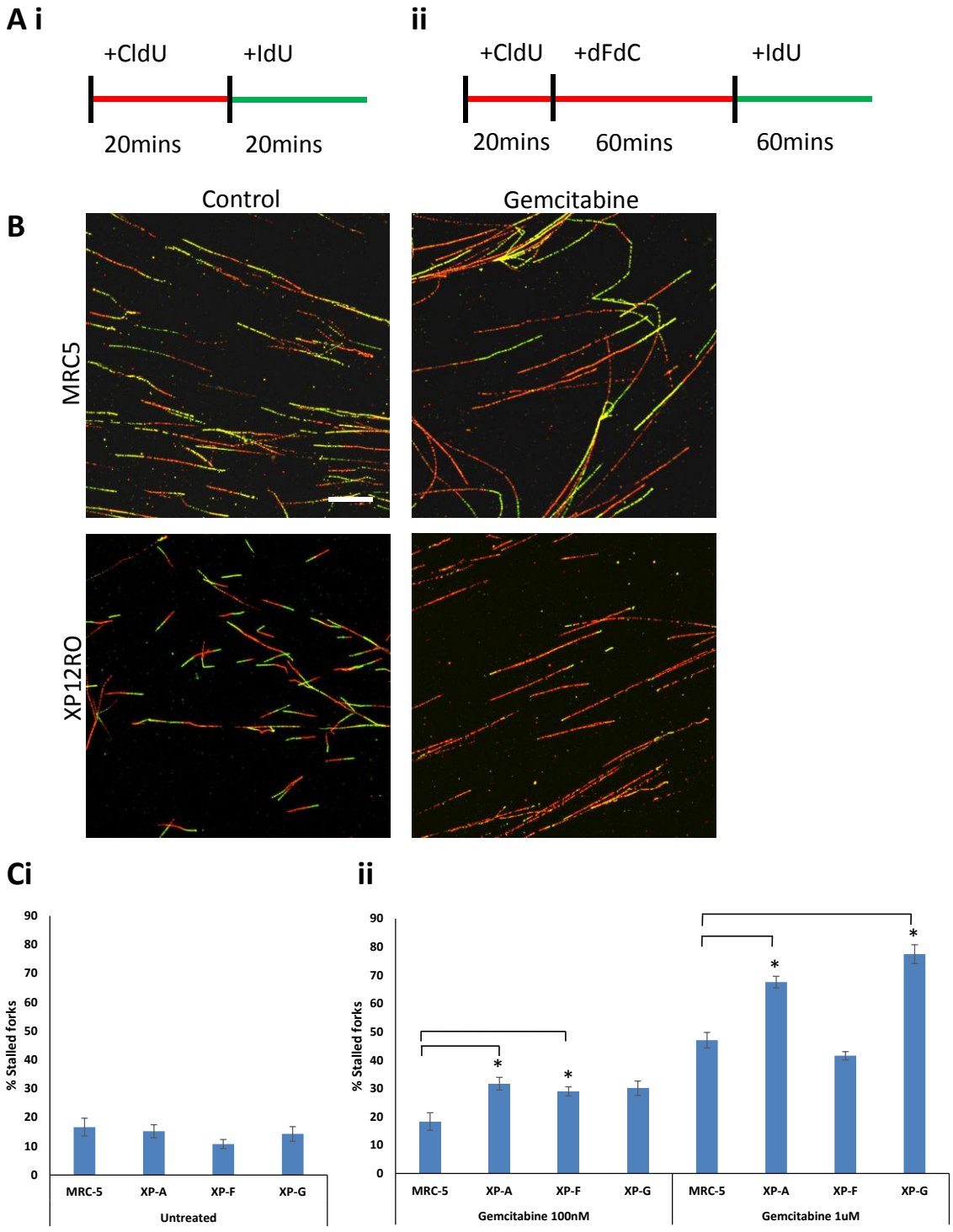


Figure 3.7 Replication fork stalling after gemcitabine treatment. (A) Diagram showing method for fork stalling assay. **(B)** Representative images of fork stalling in MRC-5 (WT) and XP12RO (XPA) under normal conditions and with 1 µM gemcitabine. Scale bar represents 20 µm **(Ci)** Bar chart showing % of stalled forks under normal conditions in MRC-5 (WT), XP-A

(XP12RO), XP-F (XP2YO) and XP-G (XPCS1RO). **(Cii)** Percentage of stalled forks after gemcitabine treatment in the same cell lines. Data is from 3-5 independent experiments. Approximately 200 fork structures were counted from 10 cells per condition. Error bars show standard error. Asterisk indicates p value determined by a t test <0.05.

3.4 Localisation of XPA after nucleoside analogue treatment

Confocal microscopy has been used extensively to investigate the dynamics of NER proteins after UV irradiation (reviewed in Vermeulen, 2011). Several studies have shown that NER factors localise to sites of damage after local UV irradiation (Staresincic et al., 2009; Volker et al., 2001). However, the localisation of NER factors after replication stress or nucleoside analogue treatment has been poorly characterised. Localisation to stalled forks after gemcitabine treatment would implicate NER factors in having a role in fork progression and further explain the sensitivity of the NER deficient patient cell lines to gemcitabine. The following results describe the localisation of XPA after gemcitabine or cytarabine treatment. XP-A fibroblasts (XP12RO, XP2OS) show extreme sensitivity to gemcitabine in the colony forming assays compared with NER proficient fibroblasts (MRC-5) and XPA has been shown to bind to sites of UV damage (Volker et al., 2001), so might be expected to localise to stalled forks upon nucleoside analogue treatment.

3.4.1 Pre-extraction uncovers XPA foci

Antibody staining of fixed MRC-5 (wt) cells demonstrates XPA is abundant within the nucleus of both untreated cells and cells treated with 2 μM gemcitabine for 2 hrs and shows a pan nuclear distribution (Fig. 3.8). This distribution is similar to that observed by Rademakers et al. (2003) and Gilljam et al. (2012) and there was no evidence of nuclear exclusion or import upon damage. Absence of any staining in XP-A (XP12RO) cells demonstrates the specificity of the antibody (Fig. 3.8). There appears to be some variation in intensity of fluorescence between nuclei but there is no statistically significant increase in mean gray value of nuclei ($p=0.11$) upon a 2 hr treatment with 2 μM gemcitabine. This is evidence against any nuclear import or upregulation occurring in response to gemcitabine treatment.

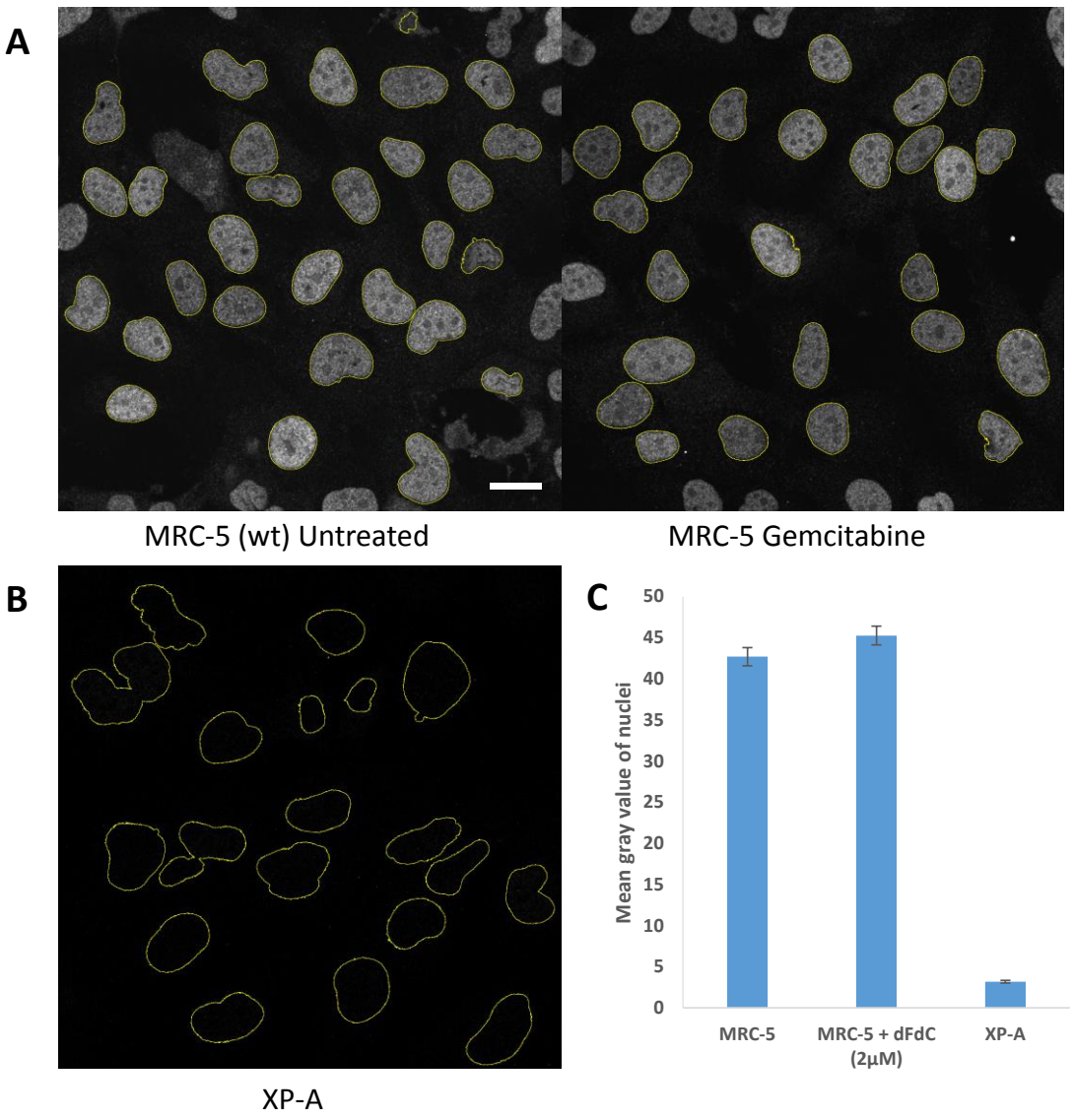


Figure 3.8 Localisation of XPA protein in MRC-5 cells. (A) αXPA staining in MRC-5 (wt) cells with and without gemcitabine (dFdC) treatment (2µM, 2hrs). **(B)** αXPA negative control staining in XP-A (XP12RO) cells. Yellow lines outline DAPI stained areas (nuclei). Images captured with 40x objective, scale bar shows 20µm. **(C)** Bar chart showing mean gray value (intensity) of nuclear αXPA staining in MRC-5 (wt, n=77 nuclei), MRC-5 (wt) gemcitabine treated cells (n=89) and XP-A (XP12RO, n= 50). Mean gray value is measured in ImageJ. Data is from a single experiment. Error bars show standard error.

No discrete foci are observed in figure 3.8 but the XPA staining is not completely uniform across the nucleus and has a speckled appearance that may be indicative of damage foci obscured by an excess of unbound XPA protein. For this reason in subsequent assays pre-extraction was used to remove unbound protein before fixation. The pre-extraction protocol

was tested by replicating work which shows that XPA binds to areas of local UV damage, inflicted by irradiation through a micropore filter (Volker et al., 2001). Figure 3.9 (left panel) shows that this was successfully replicated and that pre-extraction did not remove the bound protein. Using pre-extraction, XPA foci were uncovered in small numbers of untreated and cytarabine treated cells (Fig. 3.9). However, it was not clear whether these foci represented stalled forks and therefore γH2AX was investigated as a possible marker of stalled forks.

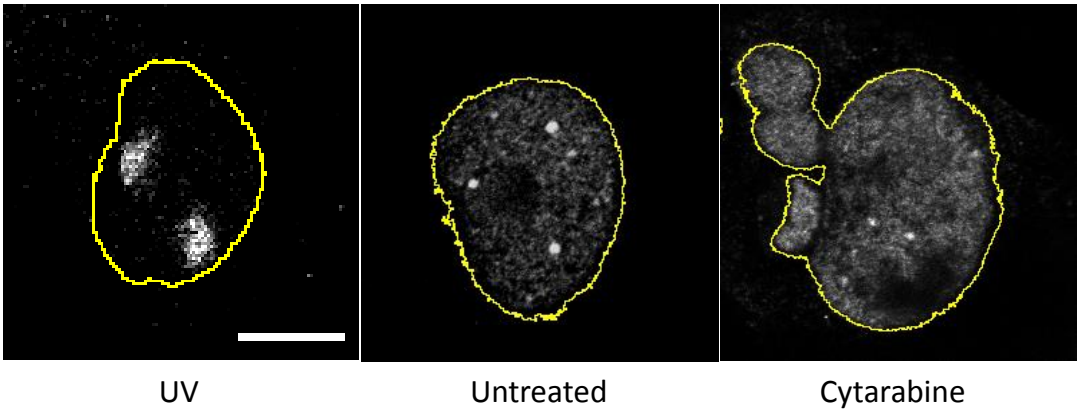


Figure 3.9 Localisation of XPA to sites of damage and repair foci. MRC-5 (wt) cell pre-extracted and stained with αXPA after local UV irradiation (30 J/m^2 UVC, left). Examples of XPA foci occurring in both untreated (centre) and cytarabine (5 µM, 30 mins) treated cells. Yellow outlines indicate extent of DAPI staining. Scale bar shows 10 µm.

3.4.2 γH2AX does not form discrete foci in response to gemcitabine

γH2AX foci have long been accepted as a marker of double strand breaks and been used in numerous studies for quantification of breaks and investigating the factors involved in break resolution (Paull et al., 2000; Sedelnikova et al., 2002). More recently it has been identified that H2AX is phosphorylated in response to many different kinds of damage including stalled forks after gemcitabine treatment (Ewald et al., 2007). Therefore γH2AX was investigated as a possible marker of stalled forks with which NER factors could colocalise.

Ewald et al., 2007 described distinct γH2AX foci at stalled forks after gemcitabine treatment, similar to those formed by DSBs after ionising radiation (IR). Discrete foci, such as those seen after IR, would be ideal for colocalisation studies so this claim was investigated using MRC-5 (wt) and XP-A (XP12RO) cells. Figure 3.10 shows that the distribution of γH2AX staining is very different after gemcitabine than after a known DSB causing agent, phleomycin (Giunta et al., 2010). Phleomycin causes the formation of large, discrete γH2AX foci in the majority of

cells whereas after gemcitabine, a large proportion of cells show a pan nuclear distribution (Fig. 3.10). This is the case for both MRC-5 cells and XP-A cells, although XP-A cells show a pan nuclear distribution of γH2AX after gemcitabine more frequently (84%) than do MRC-5 cells (71%). It is unclear what this pan nuclear response represents but it may be more similar to the γH2AX response after UV irradiation than after IR. De Feraudy et al., (2010) noted three types of γH2AX staining after UV irradiation. There were some large γH2AX foci which co-localised with 53BP1, which are likely to represent DSBs, small γH2AX foci which did not co-localise with 53BP1 and intense pan nuclear staining which occurred in a proportion of S phase (PCNA positive) cells. Their results indicated that the intense pan nuclear staining was a pre-apoptotic response and that the small γH2AX foci may mark stalled replication forks. Pan nuclear γH2AX has also been shown to occur after the induction of clustered IR damage, which similar to the previous study is dependent on ATM phosphorylation, but in contrast is not associated with subsequent apoptosis (Meyer et al., 2013). In conclusion, there is still much ambiguity about what γH2AX staining represents and it is not a good specific marker of stalled replication forks.

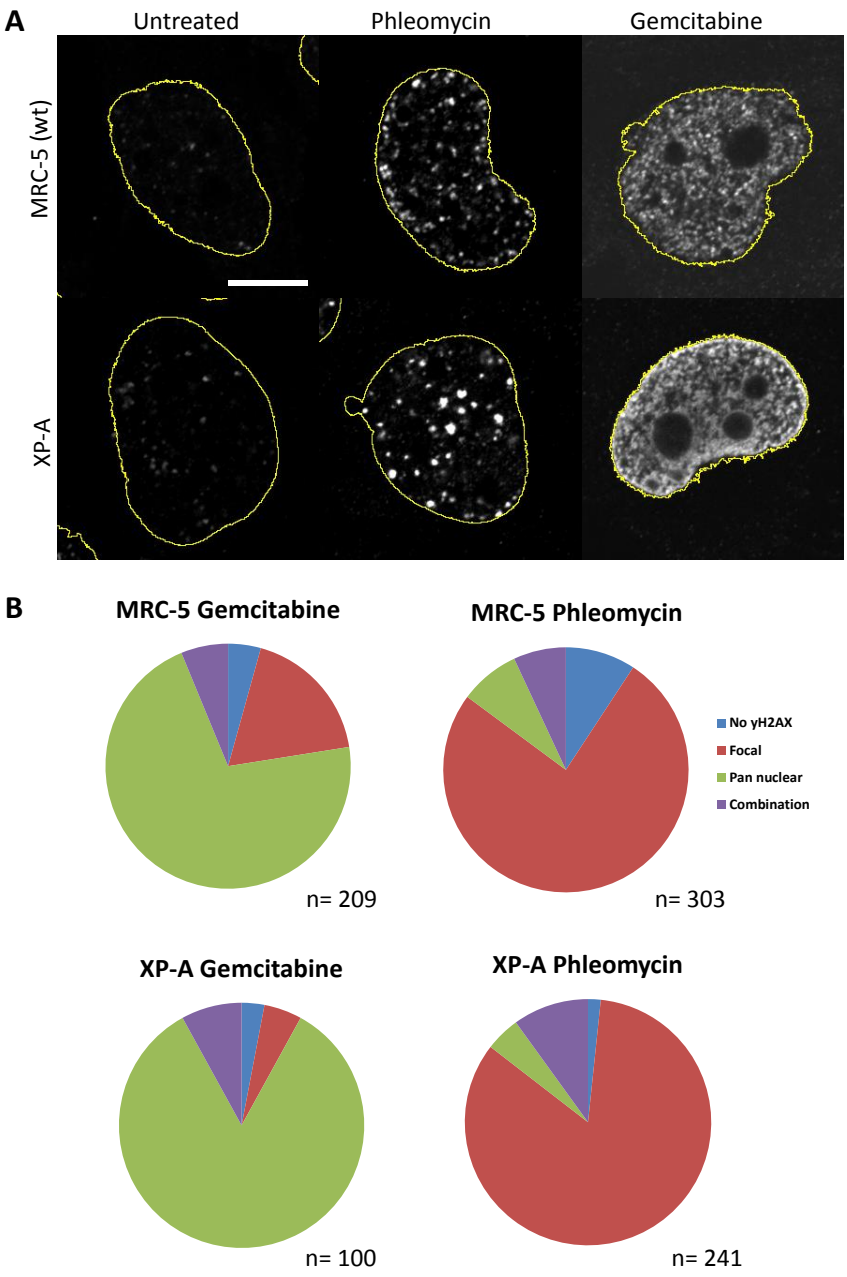


Figure 3.10 Phosphorylation of histone H2AX after gemcitabine treatment. (A) MRC-5 (wt) and XP-A (XP12RO) nuclei antibody stained for γH2AX after no treatment (left), or drug treatment with either phleomycin (30 µg/ml) or gemcitabine (2 µM). In the case of drug treatment cells were treated for 2hrs then imaged 24 hrs later. Yellow outlines indicate the DAPI stained area. Images were captured with a x60 objective. Scale bar shows 10µm. **(B)** Pie charts showing the qualitative differences in γH2AX pattern after treatment with gemcitabine and phleomycin in both MRC-5 and XP-A (XP12RO) cells. Data are from a single experiment.

3.4.3 Colocalisation of XPA foci with EdU labelled forks after gemcitabine treatment

Whatever the significance of the γH2AX distribution, the widespread distribution of γH2AX makes it difficult to locate NER factors at stalled forks, therefore a different marker of forks was chosen. EdU (5-ethynyl-2'-deoxyuridine) is a thymidine analogue that is incorporated into replicating DNA, but does not block replication and can be fluorescently labelled. This is therefore a good tool to label replication forks (although it does not indicate which are stalled) and in doing so allows S phase cells to be distinguished. Distinguishing cells in S phase, where replication is occurring, is important as these are the only cells which will experience gemcitabine mediated fork stalling. If XPA forms foci at stalled forks after a short treatment with gemcitabine, their numbers would increase in S phase cells only and including cells outside of S phase in the analysis may obscure any potential effect.

Figure 3.11 shows that the XPA foci which are visible after pre-extraction sometimes colocalise with EdU foci, suggesting XPA is sometimes present at the replication fork. This has previously been suggested by iPOND and colocalisation studies (Gilljam et al., 2012). However, this is not always the case and many cells have EdU foci without colocalising XPA and vice versa (not shown). It was thought possible that the colocalising EdU and XPA foci represented stalled forks, where XPA was binding to fulfil a role in stabilising or repairing the fork. To test this the number of S phase cells containing XPA foci was scored before and after a 2 hr 2 μM gemcitabine treatment. This is a dose higher than that shown to decrease fork speed and increase fork stalling in DNA fibre analyses. If XPA foci represent stalled forks their incidence should increase upon this treatment. As figure 3.11 shows there is a very small and statistically insignificant increase in the proportion of S phase cells containing XPA foci. This is at odds with the large percentage of forks that are stalled in these cells (47%), as judged by the DNA fibre analysis after only 1 μM treatment for 1 hour. Therefore, it appears XPA foci do not occur at the majority of stalled forks after gemcitabine treatment. However, this conclusion merits further investigation as there are alternative explanations. XPA could be present only transiently at the fork in which case foci would not accumulate, although when responding to UV damage XPA remains bound to DNA for 2-4 hrs after exposure (Rademakers et al., 2003). In addition, this experiment looked at a short period of gemcitabine exposure, however XPA may only respond to a particular subset of stalled fork structures, such as collapsed forks which occur after prolonged fork stalling. Live imaging of individual cells with

GFP tagged XPA after gemcitabine and collecting images at many more time points after treatment are ways in which these alternatives could be addressed in future.

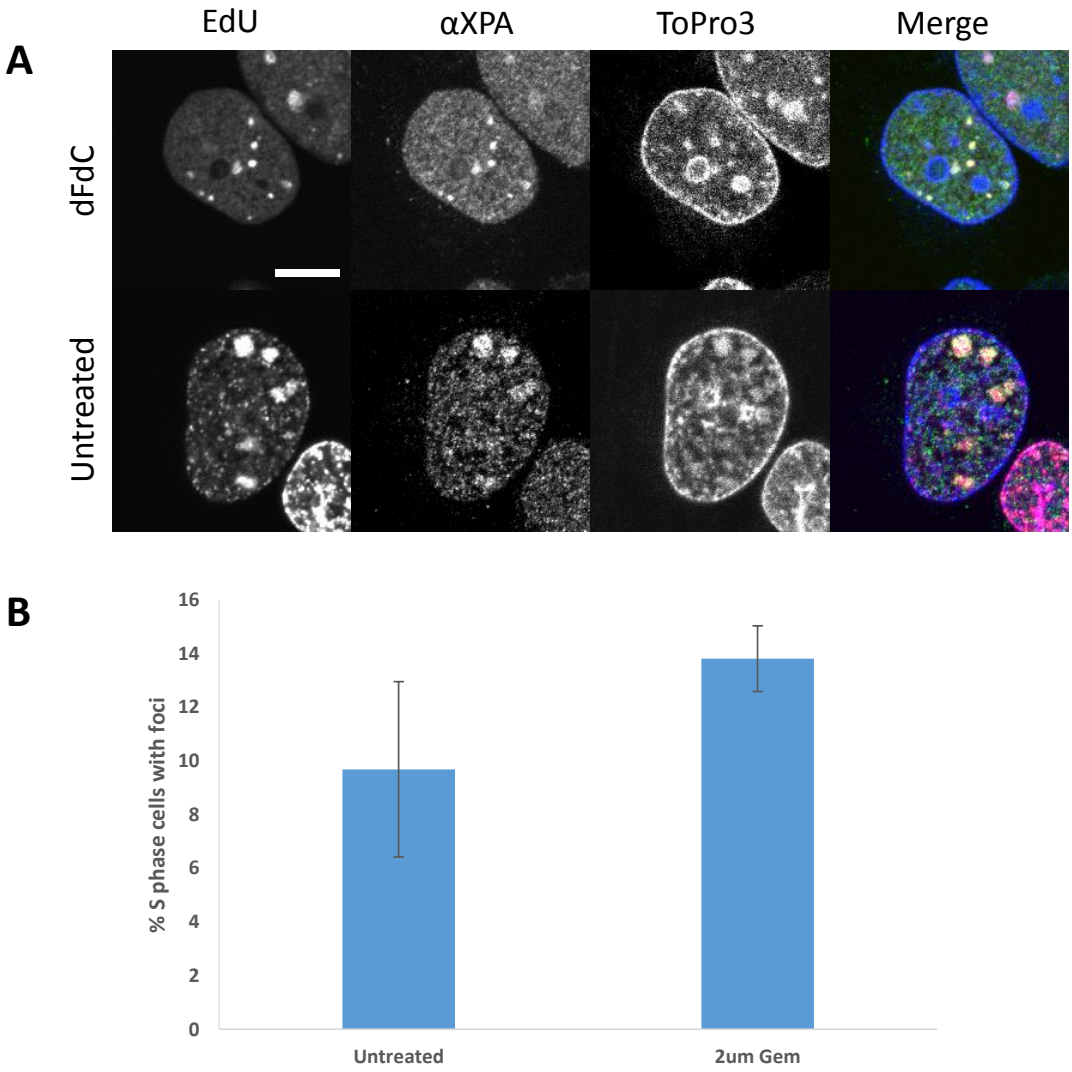


Figure 3.11 Co-localisation of XPA and EdU. (A) Example images of nuclear XPA foci colocalising with EdU observed in both gemcitabine treated and untreated cells. ToPro3 is a nuclear stain. Scale bar shows 10µm. **(B)** Quantification of S phase cells exhibiting XPA foci. Data is from 2 independent experiments, >100 cells were scored per condition. Error bars show standard error.

3.5 'Normal' fibroblast cell line GM637 is very sensitive to gemcitabine when compared to MRC-5

The NER deficient patient cell lines had been compared with an NER proficient fibroblast line MRC-5, a setup which is common to much published work investigating NER (for example

Marti et al., 2006; Oh et al., 2011; Shen et al., 2009; Volker et al., 2001). MRC-5 has no known genetic defects and is therefore considered ‘normal’ and differences between MRC-5 and XP patient cell lines were attributed to the presence or absence of an NER factor. However, the normal and patient lines are from different individuals and in this case different tissues making their genetic and epigenetic backgrounds potentially quite different. For this reason a second normal fibroblast line, proficient in NER, (GM637) was purchased and tested for UV and gemcitabine alongside MRC-5. It showed similar sensitivity to UV but alarmingly, was drastically more sensitive to gemcitabine (Fig. 3.12). This strongly implies there is another factor in the genetic background of MRC-5, aside from NER proficiency that causes it to be unusually gemcitabine resistant. This makes comparisons between MRC-5 and XP patient fibroblasts uninformative when investigating gemcitabine resistance and casts doubt on the premise that NER factors have a role in gemcitabine resistance in human cells. A new experimental approach was required and this is developed in Chapter 2.

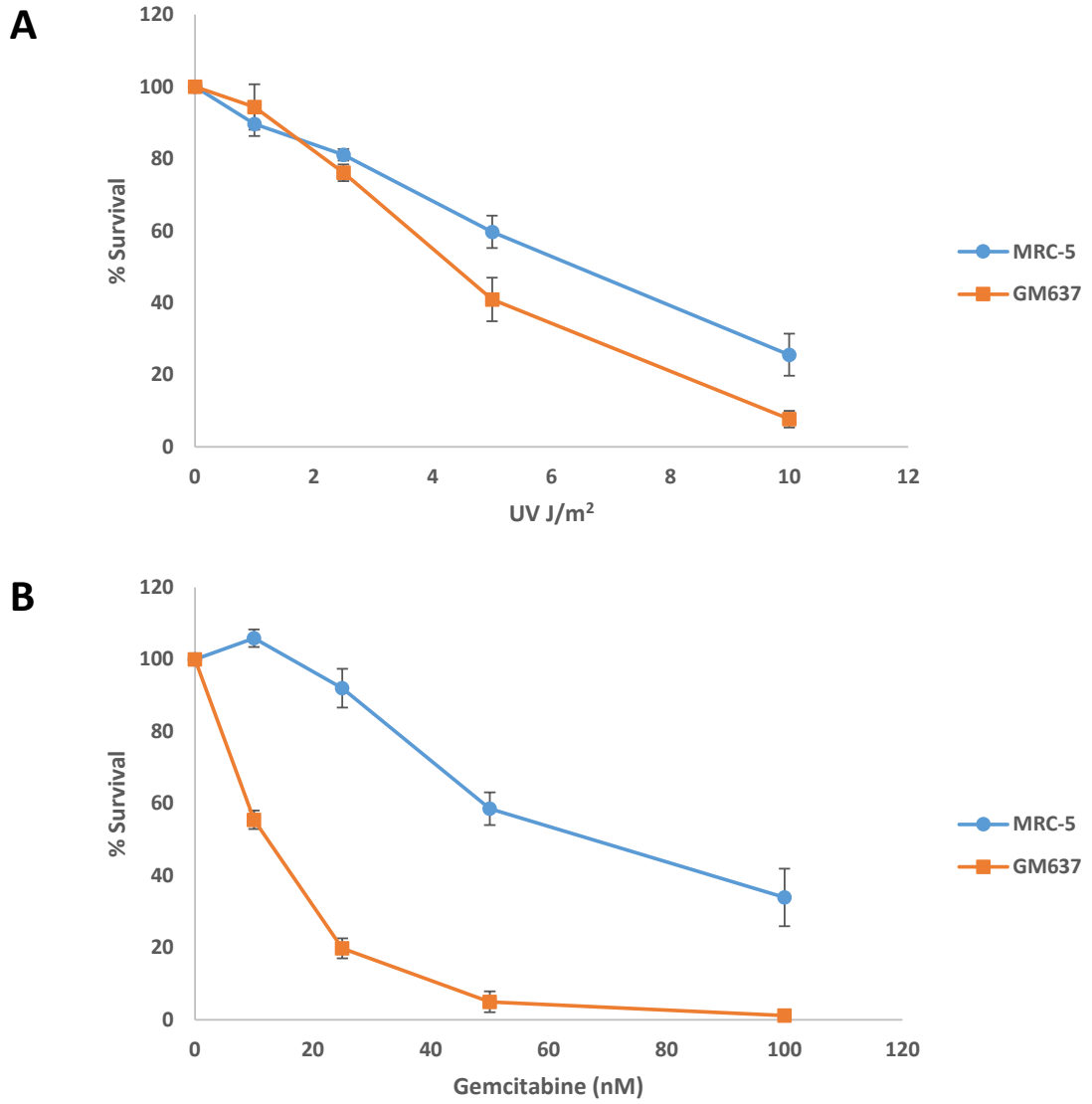


Figure 3.12 Survival of two SV40 transformed normal human fibroblasts after UV and gemcitabine. Colony forming assays showing survival of SV40 transformed normal fibroblasts MRC-5 and GM637 after **(A)** UV-C irradiation and **(B)** 24hr gemcitabine treatment. Data is from 3 independent experiments. Error bars show standard error.

3.6 Chapter Discussion

The results in this chapter aimed to build upon previous work, which showed *S. pombe* NER mutants and NER deficient human cells were sensitive to gemcitabine (Beardmore, 2015; Gasasira, 2013) and elucidate the mechanisms behind the unexplained gemcitabine sensitivity of XP patient fibroblasts. LC MS/MS data suggested that XP-A and XP-G fibroblasts incorporate more gemcitabine into genomic DNA, perhaps indicating a defect in gemcitabine removal. These results correlated with increased fork stalling in the same cell lines after a short

gemcitabine treatment compared with normal fibroblasts MRC-5. Taken together with work in *S. pombe* (described in the introduction) showing sensitivity of several NER mutants, this suggested a role for *xpa* and *xpg* in gemcitabine removal and replication fork progression. However, the LC MS/MS and DNA fibre assays both used MRC-5 as a normal control and at the end of the chapter it was shown that there are large differences in gemcitabine sensitivity between different 'normal' human fibroblast lines and MRC-5 may well be abnormally resistant to gemcitabine. Therefore the differences observed are not necessarily due to XPA or XPG function, potentially invalidating the original interpretation of these results and demonstrating a need for isogenic controls to circumvent this issue. The studies of XPA by immunofluorescent microscopy are unaffected by this issue, however no upregulation, and only a small and statistically insignificant increase in foci formation was observed after treatment with gemcitabine.

The observation of large differences in gemcitabine resistance between 'normal' cell lines is a likely result of there being several different mechanisms of gemcitabine resistance, aside from a possible role of NER, and these resistance mechanisms are likely to vary between cell lines. This will make generalising results obtained from cell culture experiments difficult and necessitate conclusions be tested on a wide panel of cell lines.

4 INVESTIGATING THE ROLE OF NER FACTORS IN GEMCITABINE RESISTANCE USING ISOGENIC CONTROLS

4.1 Introduction

The previous chapter concluded with the observation that the two normal fibroblast cell lines (GM637 and MRC-5) showed very different sensitivities to gemcitabine and are therefore unsuitable as controls for comparison with XP patient cell lines. Therefore different approaches needed to be taken to establish whether or not NER factors had a role in gemcitabine resistance in human cells, as is seen for *xpa*, *xpc*, *xpf* and *ercc1* homologues in *S. pombe*. Experiments described in this chapter make use of XP patient cell lines which stably express a tagged functional copy of the protein they are deficient in. This was in order to investigate if expression of a functional copy can rescue gemcitabine sensitivity. However, it was thought that these results could still be confounded by residual NER activity or problems with expression from the exogenously introduced gene and therefore another approach was taken. The CRISPR-Cas9 technique allows site specific genetic alterations, allowing the creation of knockout cell lines which can be compared with the parent cell line (Jinek et al., 2012). In this chapter XPA-/- and ERCC1 knockout cell lines, generated via CRISPR-Cas9, were tested for gemcitabine sensitivity.

4.2 Complementation of XP patient cell lines by the stable expression of a functional protein

The UV sensitivity of XP patient cell lines can be rescued by stable transfection with a functional copy of the appropriate gene. If this complementation also rescued gemcitabine sensitivity, it would be strong evidence for a role of the factor in gemcitabine resistance and does not suffer from the same methodological problems as comparing cell lines from different individuals. XP-A (XP2OS) and XP-C (XP4PA) cells stably transfected with GFP tagged XPA

(Fig. 4.1) and XPC respectively were obtained from W. Vermeulen (Erasmus M.C, Rotterdam). Previous publications had shown the XPA fusion protein still localised to the nucleus and restored UDS and UV sensitivity despite the large GFP tag (Rademakers et al., 2003). The XPC-GFP construct has also been shown to rescue the UV sensitivity of the XP-C (XP4PA) cell line (Hoogstraten et al., 2008). In addition, XP-G (XPCS1RO) cells stably transfected with XPG cDNA, which has been shown to rescue UDS after UV, were obtained from O. Scharer (S. Korea, Staresincic et al., 2009). It was confirmed in this study that all the transfected cell lines showed restored UV resistance as reported, showing the transfected genes were functional in classical NER (Fig. 4.1). However, restored XPA expression did not alter gemcitabine sensitivity suggesting either XPA is not required for gemcitabine resistance, or there could be a problem with expressing the gene from the introduced construct. The GFP tag could potentially interfere with the function of the protein and western blotting shows it is overexpressed which could also interfere with its function. The cells may also produce a small amount of endogenous protein that retains some function. That UV resistance is restored suggests that these issues do not occur, but it may be that different interactions are required of the protein after gemcitabine than those in classical NER.

The expression of XPC-GFP in the XPC deficient line (XP4PA) appears to elicit a small rescue of gemcitabine sensitivity at lower doses of gemcitabine that is significant ($P=\leq 0.05$) at 25 nM. Given that the effect size is small a larger number of repeats would be needed to confidently assert that a rescue has been achieved and it may not be biologically relevant, but this is an indication that XPC may have a role in gemcitabine resistance. It is also possible that other factors such as those discussed above, limit the size of the rescue observed.

Conversely, the expression of XPG in the XPG deficient cell line (XPCS1RO) causes a small sensitisation of this cell line to gemcitabine that is statistically significant at 10 and 25 nM doses of gemcitabine. This suggests XPG may be creating a cytotoxic intermediate and operating in a different pathway to XPC. The expressed XPG protein in this experiment was not tagged, but it still suffers from the limitation that the endogenous protein in the patient cell line may retain some function and the level of protein expression was not tested.

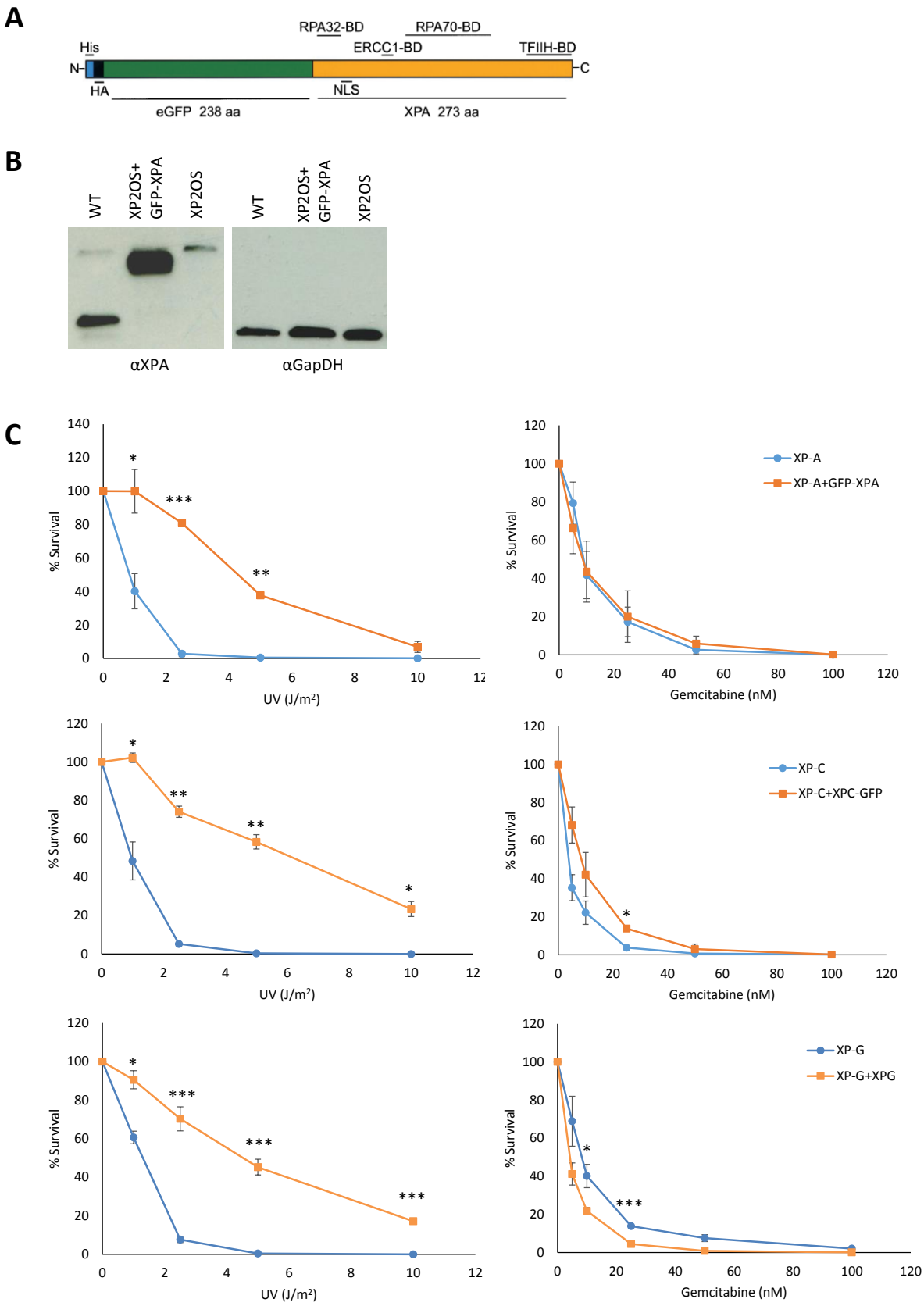

A
RPA32-BD
RPA70-BD
His
ERCC1-BD
TFIIH-BD
N
C
HA
NLS
eGFP 238 aa
XPA 273 aa
B
WT
XP2OS+ GFP-XPA
XP2OS
WT
XP2OS+ GFP-XPA
XP2OS
αXPA
αGapDH
C
% Survival
UV (J/m²)
Gemcitabine (nM)
XP-A
XP-A+GFP-XPA
XP-C
XP-C+XPC-GFP
XP-G
XP-G+XPG

Figure 4.1 Complementation of XP patient cell lines by stable expression of the functional gene. (A) Diagram showing GFP-XPA fusion protein (Rademakers et al., 2003). **(B)** Western blot analysis of protein extracts from fibroblasts wild type for XPA (XP4PA), XP-A patient cells + GFP-XPA and XP-A patient cells. Membrane was stripped and reprobed with αGapDH as a loading control. **(C)** Colony forming survival assays after UV-C irradiation or 24hr gemcitabine treatment. XP-A (XP2OS) cells with and without GFP-XPA construct (n=2-4). XP-C (XP4PA) cells with and without XPC-GFP construct (n=2-3). XP-G (XPCS1RO) cells with and without XPG construct (n=2-4). Error bars show standard error. The differences between cell lines were significant at P values *P > 0.05, **P > 0.005, ***P > 0.001.

4.3 CRISPR-Cas9 knockout cell lines

The role of XPA and ERCC1 in gemcitabine sensitivity was investigated by using knockout cell lines generated by CRISPR-Cas9. This approach allows comparison of the knockout line with an isogenic control without the limitations of the rescue experiments described previously. CRISPR-Cas9 is a recently developed method for making sequence specific mutations (Jinek et al., 2012; Ran et al., 2013a). In contrast to the use of patient cell lines, the knockout line can be designed so that the target gene is mutated early in the open reading frame in order to minimise the chance of the gene producing any product. The technique utilises the microbial nuclease Cas9 which is targeted to a particular sequence, such as the start of a key exon using a guide RNA, and cleaves DNA to form a double strand break (Fig. 4.2). The double strand break is most usually repaired by NHEJ which is error-prone and frequently leads to insertion or deletion events at the site of the cut, creating a frameshift and premature stop just downstream of the target. The break can also be repaired by HR and this provides an opportunity to replace the target sequence with a homology directed repair (HDR) construct, which may include a selectable marker. This is achieved by designing the construct so it has homology arms at either end and is introduced by transfection alongside the Cas9 nuclease. A noteworthy limitation of the CRISPR-Cas9 technique is the occurrence of off-target effects, where the Cas9 nuclease cuts at other sequences across the genome which are similar to the target sequence and can therefore give rise to mutations in other genes (Fu et al., 2013). These effects can be reduced by careful design of the guide sequences, to minimise the number of similar sequences and by use of a Cas9 mutant known as Cas9 nickase (Cas9n). Cas9n makes only a single strand break at the target sequence, so Cas9n must be guided to adjacent positions

on each strand by two different guide RNAs in order to make a DSB. This significantly decreases off-target effects (Ran et al., 2013b, Fig. 4.2).

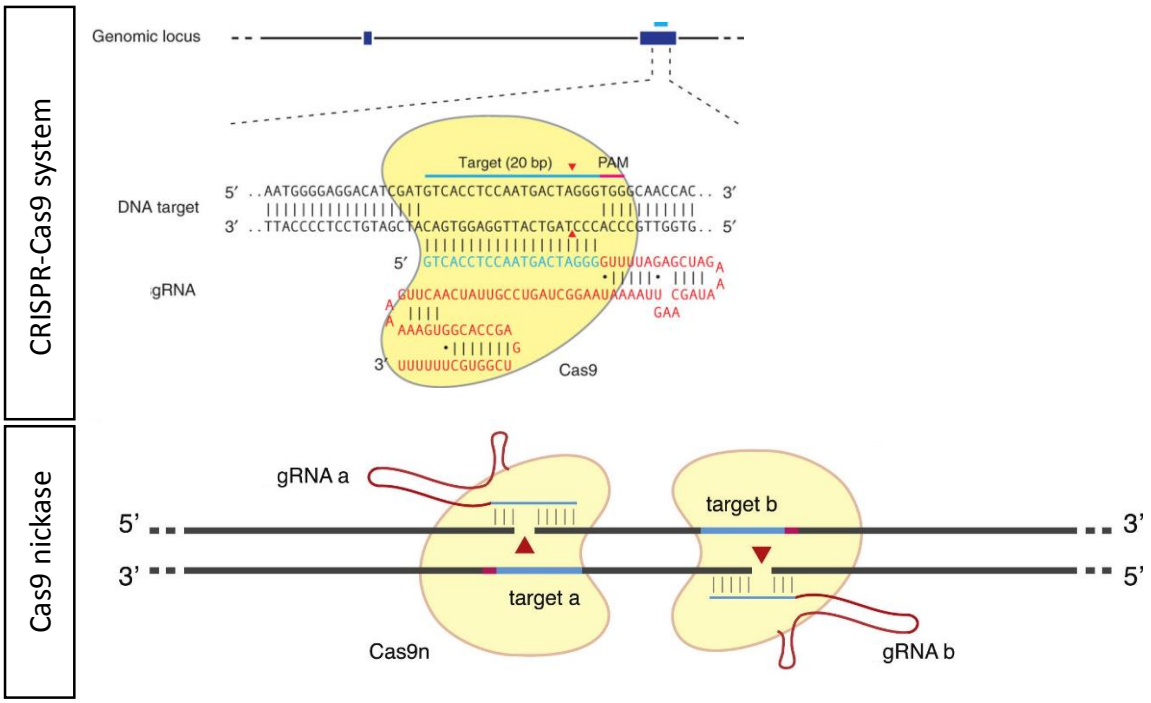


Figure 4.2 Introduction of sequence specific breaks by CRISPR-Cas9 gene editing. Top image shows introduction of a DSB (cut site denoted by red arrows) by the Cas9 nuclease (represented in yellow, Ran et al., 2013a). The cleavage is targeted by a guide RNA (gRNA) which comprises of a guide sequence (blue) and scaffold sequence (red). Bottom image shows introduction of two offset SSBs by Cas9 nickase (Cas9n), which together produce a DSB (Ran et al., 2013b).

4.3.1 CRISPR-Cas9 knockout strategy for *ercc1*

ERCC1 knockout cell lines in MRC-5 and HEK293 backgrounds were generated by R. Beardmore (2015) and clonal homozygous ERCC1 null populations were selected and characterised as part of this project. The cell lines were generated using a Cas9 nickase strategy to limit off-target effects and an HDR construct. The HDR construct included a puromycin resistance marker to aid selection and was designed so that it replaced exon 1, including the start codon, minimising the chance of any gene product being formed (Fig. 4.3). The guide RNAs were designed to target exon 1 which is present in all transcript variants of *ercc1* described in the NCBI gene database. The homology arms, which facilitated the inclusion of the HDR construct in the correct place by homologous recombination were each 600bp in

length. The left hand homology arm targeted base pairs 45423375 to 45423974 of chromosome 19 (NCBI, GRCh38.p7 Primary Assembly). The right hand homology arm targets base pairs 45422680 to 45423279 of chromosome 19 (NCBI, GRCh38.p7 Primary Assembly). After transfection with all the CRISPR-Cas9 components and selection of puromycin resistant colonies, the resulting populations were tested by western blot for absence of ERCC1. Initially the selected populations had undetectable levels of ERCC1 expression, but after repeated passages ERCC1 expression returned, indicating these were mixed populations and required a further round of selection (Fig.4.4, Beardmore, 2015).

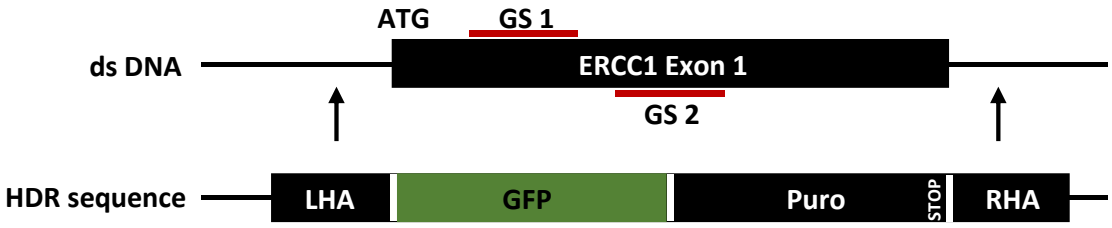


Figure 4.3 Targeting *ercc1* by CRISPR-Cas9n. Diagram showing the position of the guide sequences (GS) for double nicking by Cas9n and the design of the homology directed repair (HDR) sequence. The HDR sequence contains left and right homology arms (LHA, RHA) that enable it to replace *ercc1* exon 1, a GFP marker and puromycin resistance marker (Puro).

4.3.2 Isolating an MRC-5 ERCC1-/- CRISPR-Cas9 knockout

A second round of clonal selection was carried out by plating the cells out at low density in 10 cm plates and allowing the cells to grow into colonies. Individual colonies were then transferred into individual wells of a 24 well plate and expanded to form separate populations, which were then tested for ERCC1 expression by western blot. Figure 4.4 shows a clonal population with no detectable ERCC1 protein expression was isolated (Clone 3), alongside populations that showed reduced ERCC1 expression, confirming the original population had consisted of a mix of genotypes. Expression of ERCC1 protein in clone 3 did not return over 3 weeks in culture, strongly suggesting this population consisted only of cells in which ERCC1 had been entirely knocked out. Clone 3, henceforth referred to as MRC-5 ERCC1-/-, was then selected for further characterisation to understand the nature of the deletion.

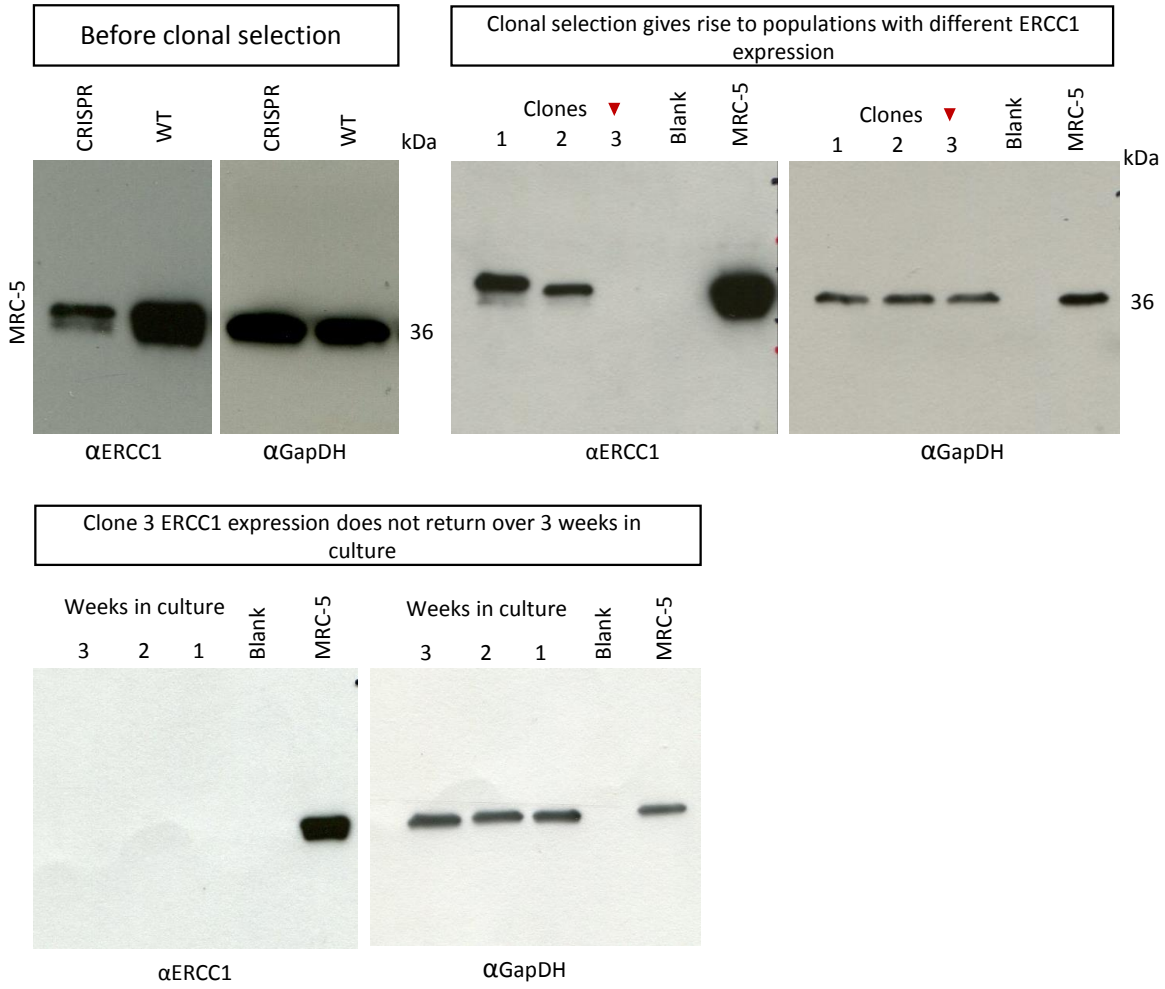


Figure 4.4 Isolation of MRC-5 cells with no detectable ERCC1 protein expression. Upper left hand panel shows western blot analysis of ERCC1 protein levels of a population of cells resulting from CRISPR-Cas9 targeting ERCC1 generated by R. Beardmore (2015) compared with unmodified MRC-5 (WT). Upper right hand panel shows ERCC1 protein expression of populations resulting from clonal selection. Selected clone showing no detectable ERCC1 expression is marked with red arrowhead. Lower panel shows ERCC1 expression remains absent in this clone over 3 weeks in culture. All membranes were stripped and probed with αGapDH as a loading control.

As shown in figure 4.3 an HDR insert was used to replace exon 1 and aid selection. PCR was used to detect whether it was present at the correct position on one or both alleles. Figure 4.5 shows how the primers were designed to enable detection of the insert. A forward primer was positioned upstream of where the HDR sequence should be inserted. This was used in conjunction with two reverse primers. Reverse primer 2 primed inside the HDR insert which would produce a 657 bp fragment if the HDR sequence was present and no fragment would be produced if it was not. Reverse primer 3 primed downstream of where the HDR sequence should finish and would produce a 3606 bp fragment if it were present or a 1110 bp fragment if it were absent, as the HDR sequence is longer than the endogenous sequence it replaces. Gel electrophoresis of the PCR products showed that none of the fragments associated with the presence of the insert were amplified from MRC-5 wt DNA as expected. However, a product of approximately 657 bp was amplified from MRC-5 ERCC1-/- DNA using reverse primer 2 and a product approximately 1110bp in size using reverse primer 3. The 3606 bp fragment was not detected, but the comparatively large size of this fragment may have prevented its efficient amplification. These results indicate that the HDR insert is present in one allele only. As MRC-5 ERCC1-/- lacked detectable ERCC1 protein expression it was supposed that the second allele must contain a mutation that abrogates ERCC1 protein expression and this was confirmed by sequencing the PCR product.

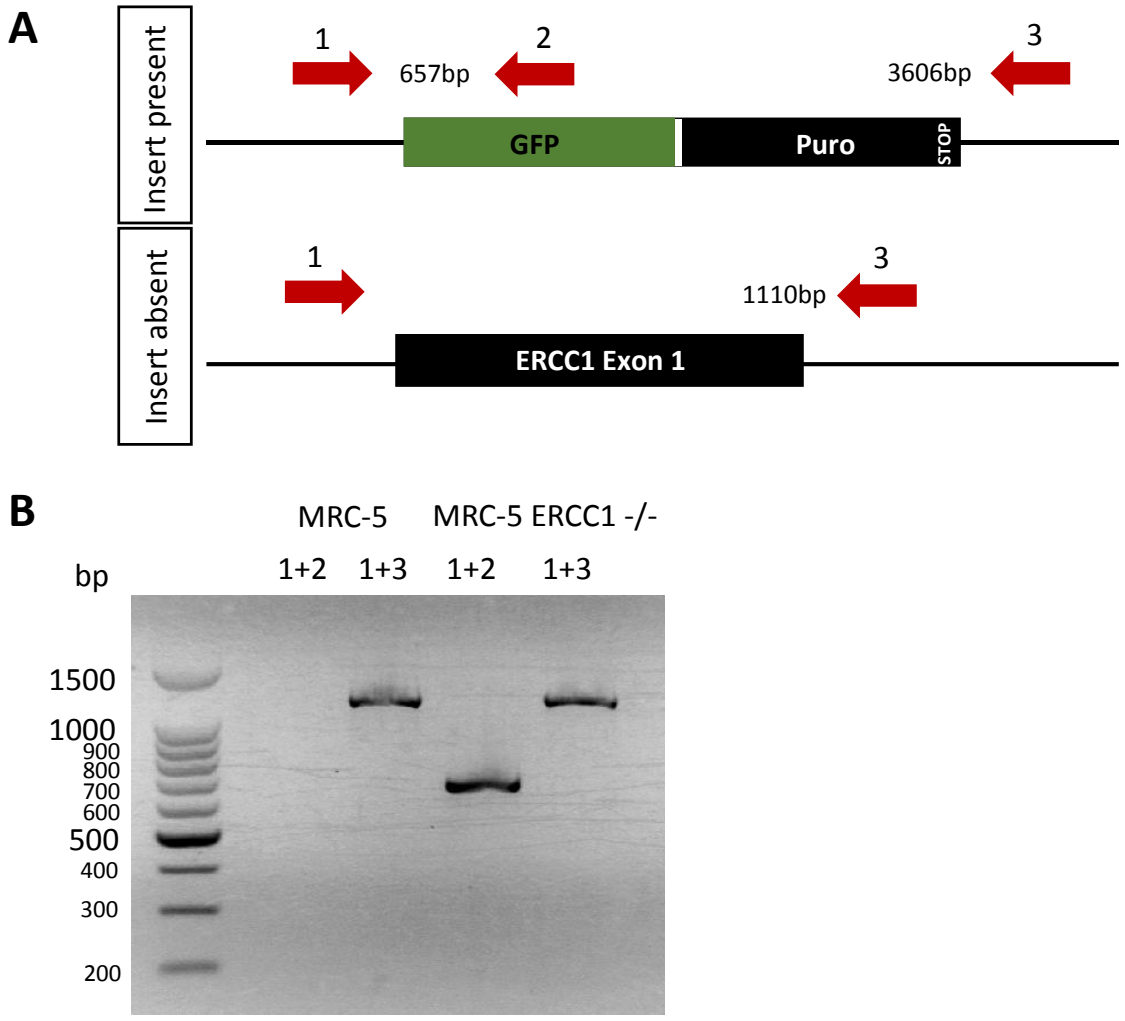


Figure 4.5 Detection of the HDR insert by PCR. (A) A forward primer positioned upstream of the potential site of the insert was used in conjunction with two reverse primers, one priming inside the insert and the other beyond the insert. Primers are represented with red arrows alongside the predicted size of the PCR products they would produce in the presence or absence of the insert. **(B)** Gel electrophoresis of PCR products obtained using the above described primers in MRC-5 wt and MRC-5 ERCC1-/-.

The PCR product from the allele lacking the insert was then sequenced alongside the corresponding product amplified from MRC-5 wt DNA. The fragments were sequenced in both forward and reverse directions and the consensus sequences of wild type and mutant alleles were compared. This showed a 19 nucleotide deletion in MRC-5 ERCC1 in exon 1 (Fig. 4.6). This deletion leads to a frameshift and a change in amino acid sequence from amino acid 26 onwards and a premature stop codon after 85 amino acids. In summary, the MRC-5 ERCC1-/- cells contain the HDR insert in place of exon 1 on one allele, and a frameshift causing a

premature stop codon in exon 1 on the other allele. The results of the western blotting and sequencing together make it very unlikely that any functional gene product is produced and the line can be considered a true ERCC1 knockout.

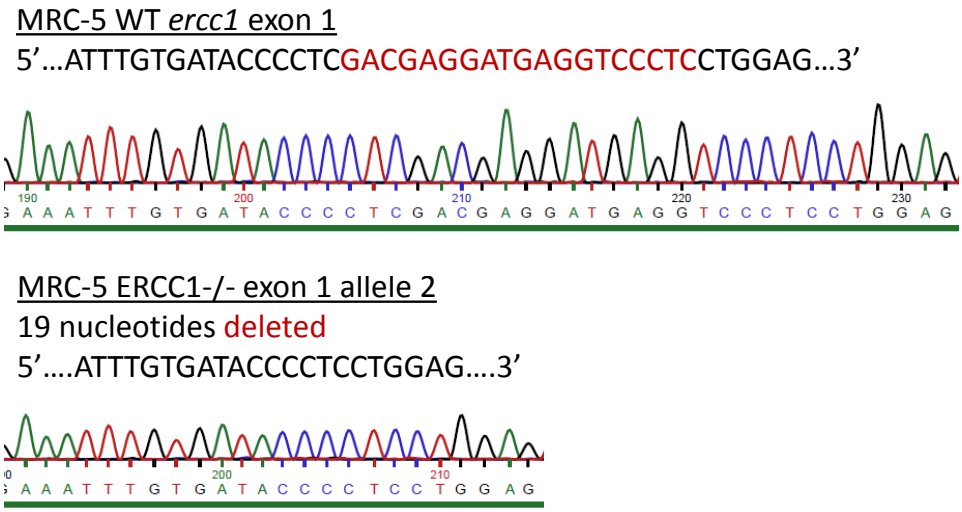


Figure 4.6 Small deletion present in *ercc1* exon 1 in MRC-5 ERCC1-/- cell line. Sequencing results for the PCR product amplified using primers 1+3 from MRC-5 wt and MRC-5 ERCC1-/- DNA. Bases shown in red are deleted in MRC-5 ERCC1-/-

4.3.3 Isolating a HEK293 ERCC1-/- CRISPR-Cas9 knockout

Similarly to the MRC-5 CRISPR-Cas9 population, the HEK293 CRISPR-Cas9 population that was received from R. Beardmore showed considerable ERCC1 expression (Fig 4.7). It was also believed to be a mix of genotypes that included some homozygous knockouts for ERCC1, as it originally had very low ERCC1 expression that increased with repeated passaging. Isolation of clonal populations was carried out by plating in 96 well plates at 1 cell per well. This yielded a clone (Clone 1) which showed no ERCC1 expression which will henceforth be referred to as HEK293 ERCC1-/-. The nature of the knockout was investigated by PCR detection of the insert, using the primers described in the previous section and template DNA from HEK293 wt and HEK293 ERCC1-/-. Figure 4.7 shows HEK293 wt DNA did not produce any of the fragments associated with the insert as expected. HEK293 ERCC1-/- DNA produced a fragment of approximately 657bp when reverse primer 2 was used, consistent with the presence of the insert on at least one allele. The use of reverse primer 3 produced a fragment

of approximately 1000bp, slightly smaller than the corresponding wt fragment, suggesting one allele did not contain the insert but instead contained a deletion of approximately 100bp.

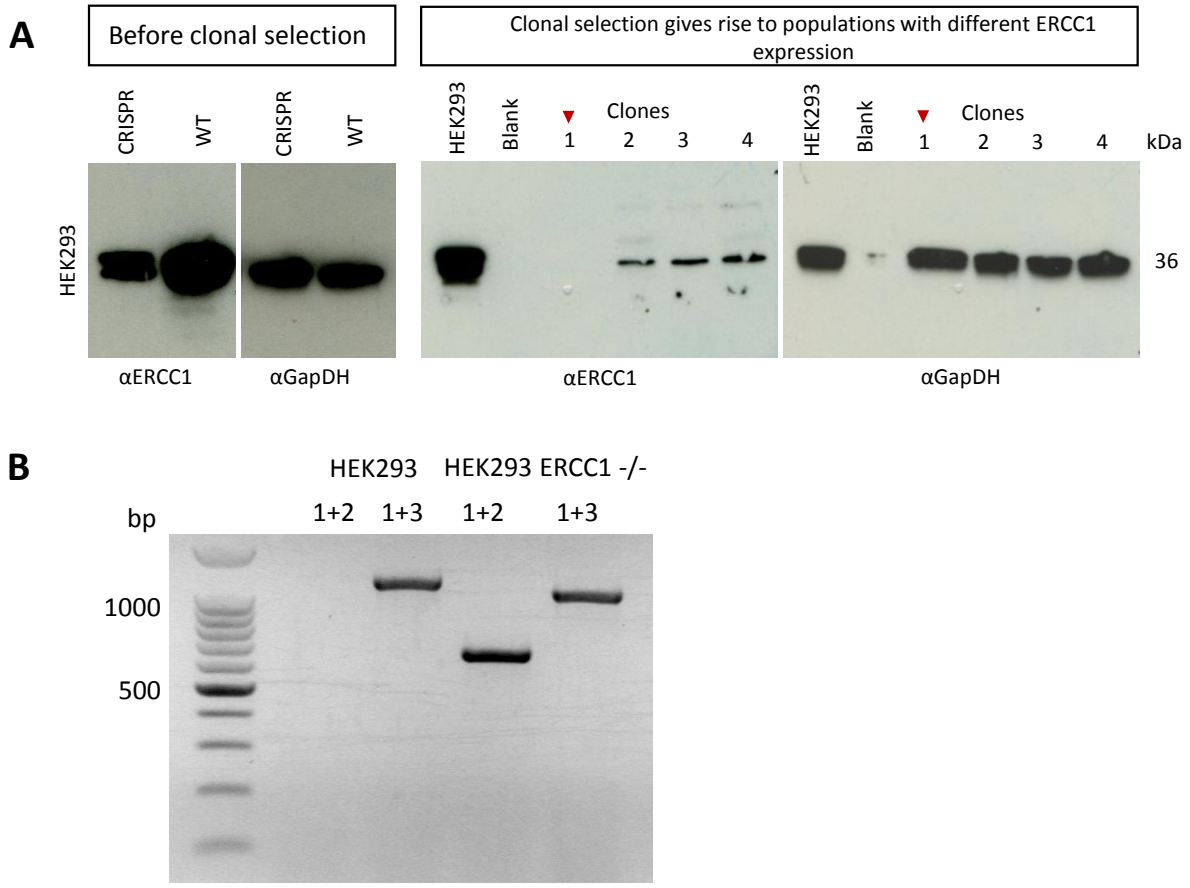


Figure 4.7 Isolation of HEK293 cells with no ERCC1 protein expression and detection of the HDR insert by PCR. (A) Left hand panel shows western blot analysis of ERCC1 protein levels of the population of HEK293 ERCC1 CRISPR-Cas9 cells received from R. Beardmore (CRISPR) compared with unmodified HEK293 (WT). Right hand panel shows ERCC1 protein expression of populations resulting from clonal selection. Selected clone showing no detectable ERCC1 expression is marked with red arrowhead. Both membranes were stripped and probed with αGapDH as a loading control. **(B)** Gel electrophoresis of PCR products amplified by the primers described in figure 4.5 from HEK293 wt and HEK293 ERCC1-/- DNA.

In accordance with this, sequencing showed a large deletion of 228 nucleotides and the addition of 134 nucleotides in their place (Fig. 4.8). The deleted segment contained the start codon for exon 1 so it is likely that no protein is translated. The inserted nucleotides blast to the *E. coli* genome, so are likely to have originated on the plasmid. HEK293 has been characterised as being hypotriploid by the European collection of authenticated cell cultures (ECACC

85120602). The ercc1 gene is situated on the q arm of chromosome 19 (NCBI). A karyotype analysis of HEK293 cells in a recent study showed 3 copies of chromosome 19 in 50% of cells and chromosome number and aberrations can vary considerably between HEK293 cells from different sources (Stepanenko and Dmitrenko, 2015; Stepanenko et al., 2015). Therefore there may be 3 *ercc1* alleles in HEK293 ERCC1-/-. Karyotyping was not performed as part of this study but there was no evidence of overlapping peaks in the sequencing chromatogram or different size PCR products, which would have indicated the presence of another allele with a different sequence. In summary, HEK293 ERCC1-/- has incorporated the HDR insert into one *ercc1* allele, eliminating the start codon and has a deletion which also eliminates the start codon on the other allele. Again the western blotting and sequencing results in this cell line make it very unlikely any ERCC1 protein is produced and HEK293 ERCC1-/- can be considered a knockout.

HEK293 WT *ercc1* exon 1

5'…GGGCGAGCCGAAGGTGGAGGTCAAAGGGGCGTGGCGTTACAGAGCCTCTAGCGCTG
GGTGTTGGGGACCTGACGCTATGGAGCTCTCGGAGTTTTGTGGGGGACGGCTGTGAGTGG
GGGGTTCCTGCTGCGGGATGAGAACGTAGACGCCAGTGGCTCACTCGCTCCTGGCACCTTC
CCTTTCAGGCTCCAG*ATG*GACCCTGGGAAGGACAAAGAGGGGGTGCCCCAGCCCTCAGG
GCCGCCAGCAAGGAAGAAATTTGTGATACCCCTCGACGAGGATGAGGTCCCTCCTGGAGT
GGTAGGACAAGGAGATGCGGGGCCCCTGGGAGGCTGGGGGCTGTTAGGACGAAGAGGA
TAGGATGGGGCCTGTGGGACCAGGGTGTGGGTTAGTGGATTTGGGGGCCACGGACGACT
TGGGGAAACAGTCCTTGGTCCTCCCCAGGTCCCAGTTTCCCCATCTGTGAAATGGATGGGT
GGTTCTAAGAGAGGGCTAAGGCAGAGGCCAGACACTGGCATTCAGCAGGTAGCCCCTGAT
GTGTTTTACGAGGCCAGCACCCTGATTTTTCAAGACATGAATTTATTGCTCGTGTTTAAGAAT
CGCCAGGTTTTGCAATCTTAAAAAAA…3'

HEK293 ERCC1-/- *ercc1* exon 1 allele 2

5'…GGGCGAGCCGAAGGTGGAGGTCAAAGGGGCGTGGCGTTACAGAATAGTAGTTACATA
ACCGCTGGACGCCGCAAACCTGAGCAACCGTCAGGGCCGTCGACAAGCGTTCATAAGCGG
TGAAGGTCTGTGAACGGGTCACGGTAAGCTGGTCAGGGAACAAATCCACTGCAAGAACAC
ACGAGGATGAGGTCCCTCCTGGAGTGGTAGGACAAGGAGATGCGGGGCCCCTGGGAGGC
TGGGGGCTGTTAGGACGAAGAGGATAGGATGGGGCCTGTGGGACCAGGGTGTGGGTTAG
TGGATTTGGGGGCCACGGACGACTTGGGGAAACAGTCCTTGGTCCTCCCCAGGTCCCAGT
TTCCCCATCTGTGAAATGGATGGGTGGTTCTAAGAGAGGGCTAAGGCAGAGGCCAGACAC
TGGCATTCAGCAGGTAGCCCCTGATGTGTTTTACGAGGCCAGCACCCTGATTTTTCAAGACA
TGAATTTATTGCTCGTGTTTAAGAATCGCCAGGTTTTGCAATCTTAAAAAAA…3'

Figure 4.8 Sequence alterations in HEK293 ERCC1-/- cell line. Sequencing results obtained from the PCR products amplified using primers 1+3 from HEK293 wt and HEK293 ERCC1-/- DNA. Nucleotides in black are identical in wt and mutant, (228) nucleotides shown in red are deleted in the mutant and (134) nucleotides shown in blue are added in the mutant. The start codon (ATG) of exon 1 is underlined and in italics. The CRISPR-Cas9 guide sequences are underlined.

4.3.4 MRC-5 ERCC1 -/- is sensitive to UV and gemcitabine

Following characterisation of the genetic changes in the MRC-5 ERCC1-/- line, the ERCC1-/- phenotype was tested by measuring survival after UV exposure. As ERCC1 is a key protein in the NER pathway, ERCC1-/- cells were expected to be very sensitive to UV compared with the parent line and the results shown in figure 4.9 are consistent with this. MRC-5 ERCC1-/- was also sensitive to gemcitabine compared with the parent line which is consistent with the gemcitabine sensitivity of the *ercc1* homologue (*swi10*) mutant in *S. pombe*, but a link between ERCC1 deficiency and gemcitabine sensitivity has not been shown before in human cells (Gasasira, 2013). A possible second explanation for the increased gemcitabine sensitivity of the MRC-5 ERCC1-/- line could be the occurrence of an off-target mutation in another gene which affects gemcitabine sensitivity. This possibility is addressed in the next chapter.

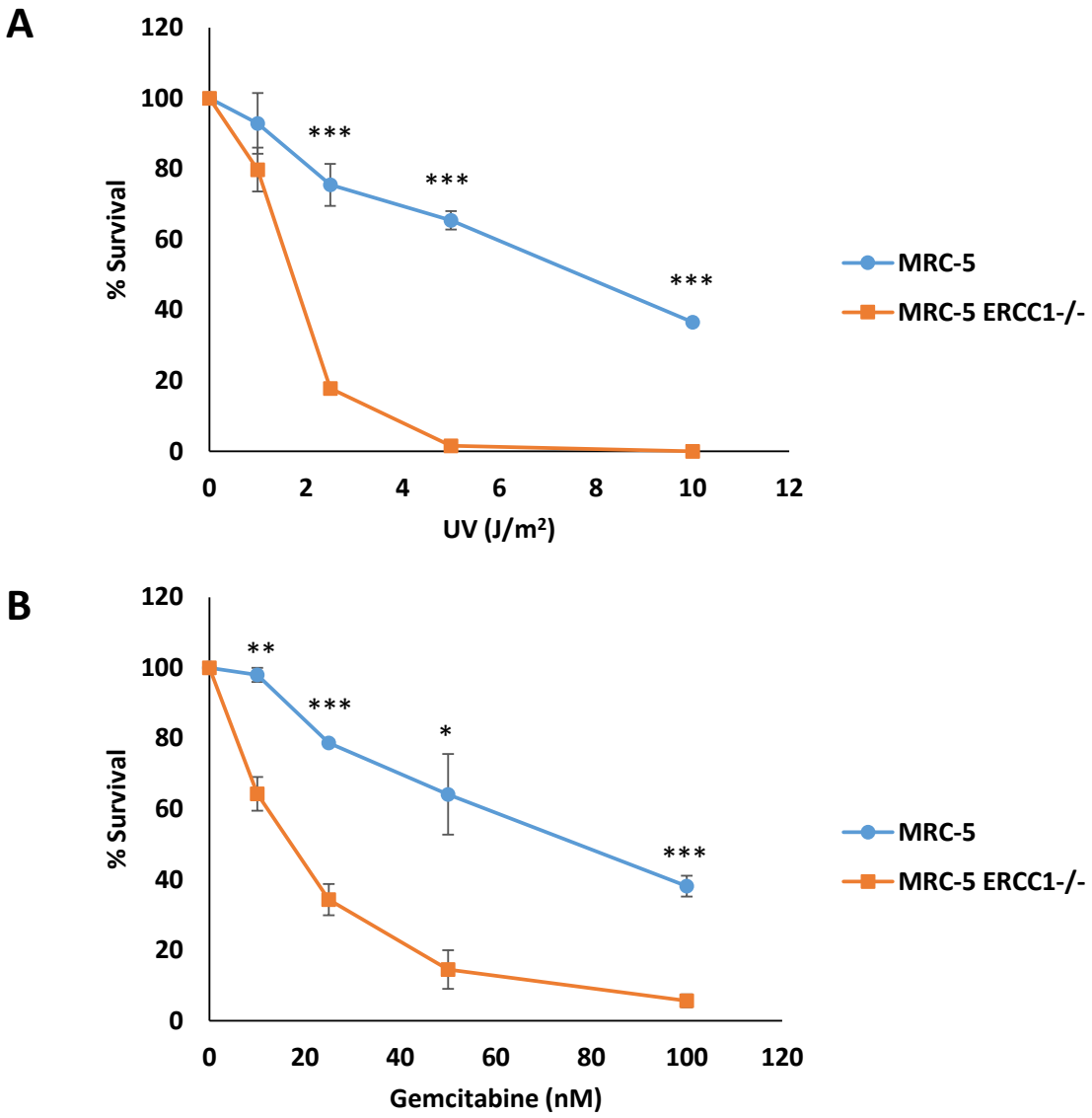


Figure 4.9 MRC-5 ERCC1-/- is sensitive to UV and gemcitabine. Colony forming survival assays showing survival after **(A)** UV-C irradiation and **(B)** 24hr gemcitabine treatment. Data is from 3 independent experiments. Error bars show standard error. Difference between cell lines is significant at * P = 0.05, ** P = 0.005, ***P = 0.001 when t-test is applied.

4.3.5 HEK293 ERCC1-/- is sensitive to MMC but not to gemcitabine

ERCC1 plays an important role in crosslink repair and several studies have confirmed that ERCC1 deficient cells are sensitive to crosslinking agents such as mitomycin C (MMC, Crul et al., 2003b; Friboulet et al., 2013; Klein Douwel et al., 2014). HEK293 ERCC1-/- was sensitive to MMC as expected, which is phenotypic evidence to support the loss of ERCC1 (Fig. 4.10). MMC was used instead of UV as HEK293 are only semi-adherent and easily

dislodged during wash steps of the colony forming assay and the UV protocol involves extra wash steps. In contrast to their sensitivity to MMC, HEK293 ERCC1-/- showed no sensitivity to gemcitabine in a colony forming assay and appeared mildly resistant to gemcitabine when tested with a luminescence based viability assay (Fig. 4.10). These results contrast with the gemcitabine sensitivity of MRC-5 ERCC1-/- and suggest that the role of ERCC1 after gemcitabine treatment differs between cell lines.

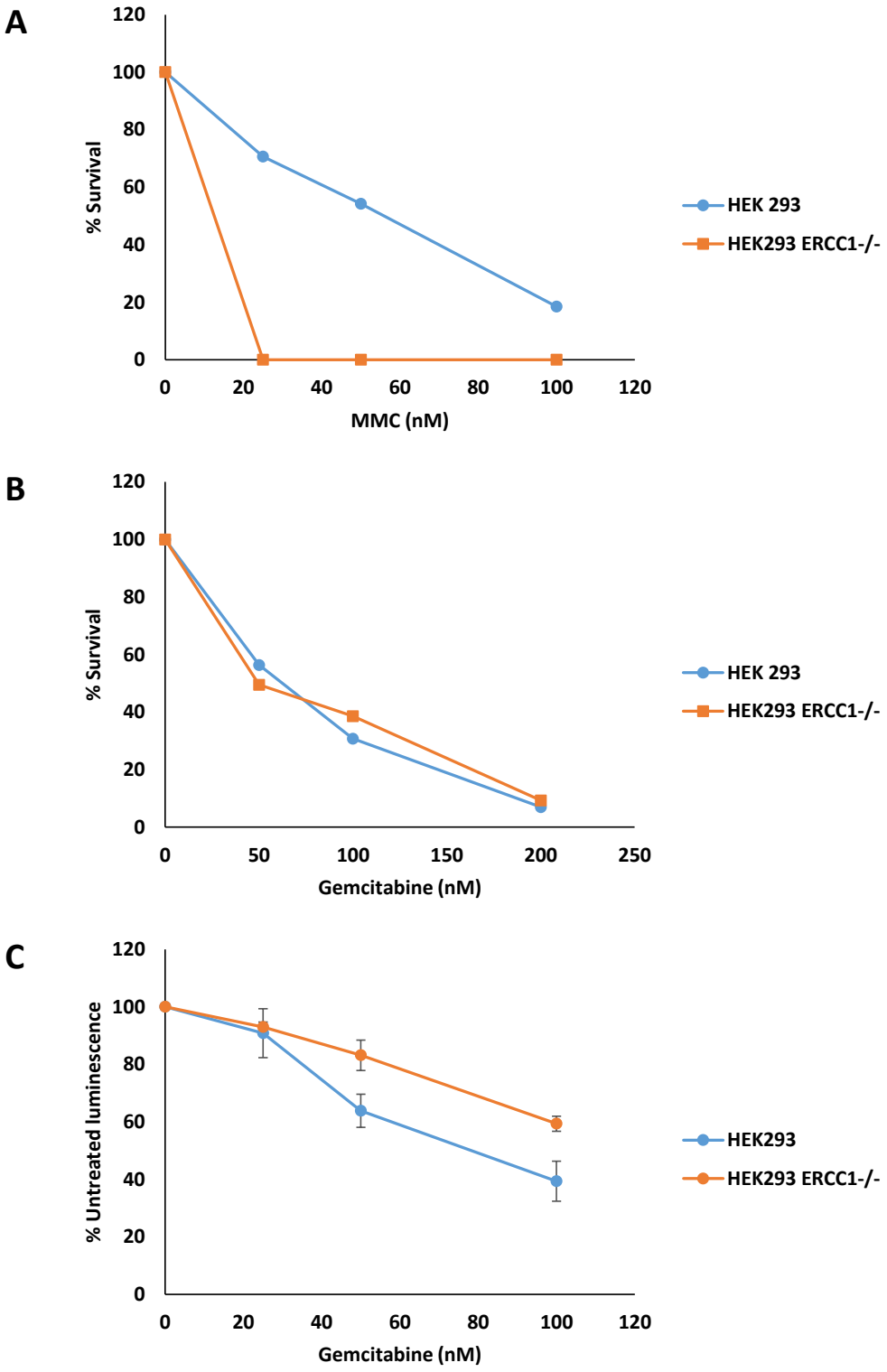


Figure 4.10 HEK293 ERCC1-/- is sensitive to MMC but not to gemcitabine. Colony forming assays show survival after 24 hr treatment with (A) mitomycin C (MMC) or (B) gemcitabine. Data is from a single experiment. (C) Cell titer glo 2.0 assay showing viability after 72 hrs in gemcitabine. Data is from 2 independent experiments. Error bars show standard error.

4.4 XPA-/- CRISPR-Cas9 knockout is not sensitive to gemcitabine or other replication inhibitors

The *S. pombe xpa* homologue has been shown to be involved in gemcitabine resistance, making *xpa* an interesting candidate for involvement in gemcitabine resistance in human cells (Gasasira, 2013). However, it was described at the beginning of this chapter how reintroduction of wild type *xpa* failed to rescue the sensitivity of an XPA deficient patient cell line, suggesting it does not have a role. Given the disagreement with the *S. pombe* data and the previously discussed problems with the rescue experiment, testing a knockout cell line for *xpa* was important. An XPA-/- CRISPR-Cas9 knockout cell line was obtained from S. Takeda (Kobe University) which had been made from the human lymphoblast cell line TK6. The line was first tested by western blot to confirm the lack of XPA expression (Fig. 4.11) and then tested by two different assays for viability after UV and gemcitabine. The luminescence based viability assay and the colony forming assay gave concordant results. The XPA-/- cells were very sensitive to UV, as expected for cells with an NER defect, but not sensitive to gemcitabine. These results generally support the conclusion of the rescue experiment that *xpa* does not have an important role in gemcitabine resistance. In *S. pombe xpa* homologue (*rhp14*) mutant cells are also sensitive to the replication inhibitors hydroxyurea (HU) and cytarabine (AraC), which operate with different mechanisms of action to gemcitabine (Gasasira, 2013). The TK6 XPA-/- cells were not sensitive to these drugs either, showing that their lack of sensitivity was not specific to gemcitabine (Fig. 4.11).

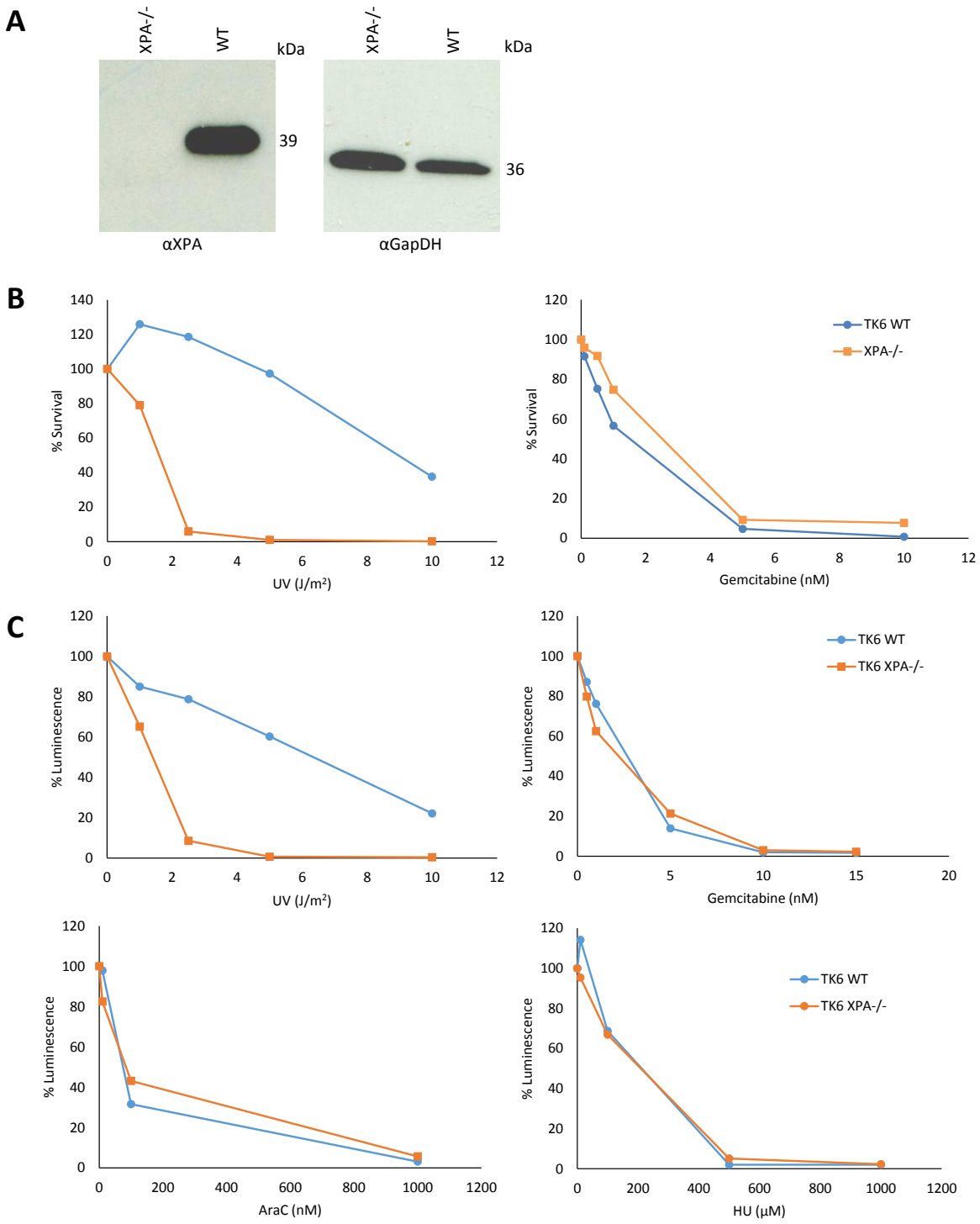


Figure 4.11 TK6 XPA-/- cells are sensitive to UV but not to gemcitabine or other replication inhibitors. (A) Western blot showing presence/ absence of XPA in TK6 XPA-/- cells and TK6 WT cells. Membrane stripped and probed for GapDH as a loading control. **(B)** Colony forming assay showing survival after UV-C irradiation or 24 hr gemcitabine treatment. **(C)** ATP based viability assay showing viability after cytarabine (AraC) and hydroxyurea (HU). Data are from single experiments.

4.4.1 Mirin sensitises both unmodified and XPA-/- TK6 cells to gemcitabine to the same extent

It was described above how knocking out *xpa* in TK6 cells had no effect on gemcitabine sensitivity, whereas in *S. pombe* mutating the *xpa* homologue (*rhp14*) sensitises cells. One reason for this discrepancy may be the presence of compensatory pathways in mammalian cells, making the role of XPA in gemcitabine resistance redundant. This was tested by inhibiting a candidate compensatory factor, MRE11. MRE11 is a nuclease that has been shown to facilitate gemcitabine resistance in *S. pombe* and MRE11 nuclease activity can be inhibited by the drug mirin (Dupré et al., 2008). Mirin has been shown to sensitise human cells to gemcitabine (Hartsuiker lab, unpublished data). If mirin sensitised TK6 XPA-/- to gemcitabine to a greater extent than the unmodified TK6 then it would indicate MRE11 activity was obscuring redundant XPA activity in mammalian cells. However, figure 4.12 shows this is not the case, as both XPA-/- and parent cell lines are equally sensitive to both mirin alone and gemcitabine plus mirin.

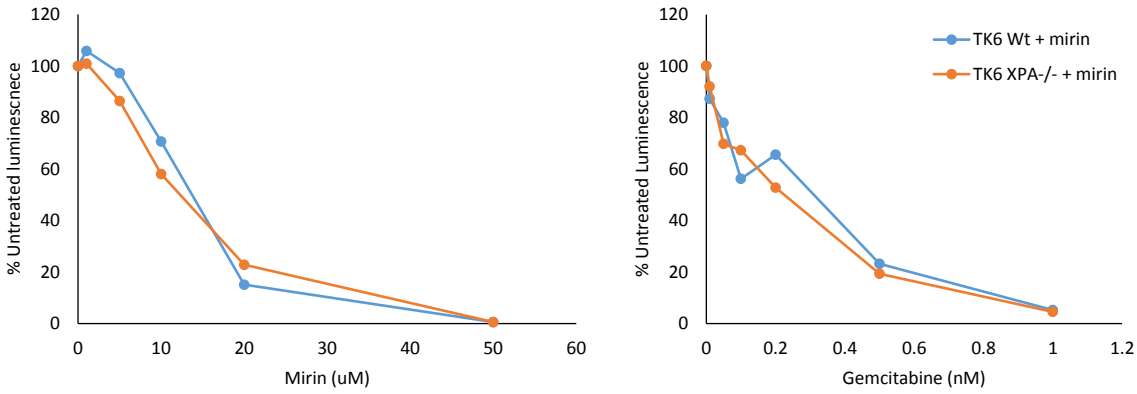


Figure 4.12 TK6 WT and TK6 XPA-/- are equally sensitised to gemcitabine by mirin. ATP based viability assays showing viability of TK6 WT and TK6 XPA-/- cells after 72 hrs treatment with mirin alone or gemcitabine plus 10 µM mirin. Data are from single experiments.

4.5 Chapter discussion

The previous chapter highlighted the problems with comparing cell lines from different individuals and the confounding effect of different genetic backgrounds when investigating gemcitabine resistance. In order to limit these effects, this chapter investigated the role of NER factors in gemcitabine resistance using approaches where controls were genetically matched except for the gene of interest. Firstly by 'rescue' of patient cell lines, where the deficient gene

is added back in and then by the use of CRISPR-Cas9 to knock a gene out of a cell line that is proficient for that gene. Work was focussed on the human homologues of genes which had been shown to have a role in gemcitabine resistance in *S. pombe*.

In the previous chapter all NER deficient lines tested appeared sensitive to gemcitabine when compared to MRC-5. In this chapter some of the same cell lines were tested (XP-A, XP-C and XP-G) after they had been stably transfected with the wild type gene, and this rescued UV sensitivity in all cases, but had a less sizeable effect on gemcitabine sensitivity. The XP-A cell line showed no alteration of gemcitabine sensitivity, the XP-C cell line showed a small rescue and the XP-G line showed a small sensitisation. This evidence suggests XPA has no role after gemcitabine, but that XPC and XPG may have protective and cytotoxic activities respectively, although the small effect size indicates the role of these factors may be minor. A major weakness of this experiment is the failure to check protein expression levels in XPC and XPG complemented cell lines, as this could have an important bearing on the effect size. These results contrast with the very high relative sensitivity of these cell lines detailed in the previous chapter and is further evidence to suggest their sensitivity compared with MRC-5 is not due to loss of NER factors. However, the complementation experiments had several limitations such as the addition of a large GFP tag to the *xpa* and *xpc* constructs and alterations of expression levels which may affected the function of the protein, and also the possibility that some functional protein may have remained in the patient lines. Therefore XPA-/- CRISPR-Cas9 knockout cells were tested for gemcitabine resistance against their *xpa* proficient parents. In agreement with the rescue experiment, the XPA-/- cells showed no additional sensitivity, adding weight to the conclusion that *xpa* has no role in gemcitabine resistance in human cells in contrast to the role of its homologue in *S. pombe*.

ERCC1-/- CRISPR-Cas9 cell lines were generated in two different cell lines, MRC-5 and HEK293 which surprisingly gave contrasting results with regards to gemcitabine resistance. Loss of ERCC1 in MRC-5 led to gemcitabine sensitivity whereas loss of ERCC1 in HEK293 did not. The difference between MRC-5 ERCC1-/- and HEK293 ERCC1-/- is puzzling and could have several different explanations. First, the effect of the loss of *ercc1* with respect to gemcitabine resistance could be cell type dependent and an alternative repair pathway may be compensating in HEK293 cells. Secondly, the HEK293 ERCC1 -/- knockout may be somehow imperfect and a functional *ercc1* product may be being produced from a downstream start codon. The antibody used for ERCC1 detection is raised against unspecified residues in the first 200 amino acids, it would be useful to also probe with an antibody raised against the C

terminus to check this. However, given the extreme MMC sensitivity this seems unlikely. Thirdly, the gemcitabine sensitivity of the MRC-5 ERCC1-/- line may be due to an off target effect of the CRISPR-Cas9 process or acquisition of a random mutation during selection. This possibility is investigated in the following chapter.

If the role of ERCC1 in gemcitabine resistance is indeed cell line dependent then this opens up the possibility that the role of *xpa*, *xpc* and *xpf* may be too, and this variation among cell types may explain the apparent lack of conservation between *S. pombe* and human cells. These factors may contribute to gemcitabine resistance in human cells, but only in the absence or presence of another pathway or factor that varies among cell types. This observation explains that it may be difficult to build a model of how the different NER factors contribute to gemcitabine resistance when the data is drawn from observations in different cell lines. Future work should concentrate both on investigating all the relevant factors in the same genetic background, and looking at how the role of an individual factor varies across a large number of cell lines.

5 THE ROLE OF ERCC1 IN GEMCITABINE RESISTANCE IN MRC-5

5.1 Introduction

The previous chapter interrogated the role of *xpa* and *ercc1* in gemcitabine resistance using CRISPR-Cas9 knockout cell lines. It was found that loss of ERCC1 in MRC-5 cells, but not HEK293 cells led to gemcitabine sensitivity demonstrating that ERCC1 may have a role in gemcitabine resistance in a cell type dependent manner. This finding is explored further in this chapter. Firstly *ercc1* cDNA was transfected back into the MRC-5 ERCC1-/- line to attempt to rescue the gemcitabine sensitivity of the cell line and thereby confirm the observed sensitivity is due to lack of ERCC1. Following this, several assays were employed to explore what resistance mechanism ERCC1 may be involved in. The sensitivity of the MRC-5 ERCC1-/- cell line to other replication inhibitors was tested to indicate whether ERCC1 is specifically involved in the response to nucleoside analogue stalled forks or a more general role in the replication stress response. Differences in ERCC1 localisation and foci formation after gemcitabine were investigated as this would be suggestive of participation in post gemcitabine repair. Finally, the aberrant segregation of chromosomes after gemcitabine treatment was measured as ERCC1 has been proposed to have a role in promoting proper chromosome segregation (Naim et al., 2013).

5.2 Rescue of gemcitabine sensitivity of MRC-5 ERCC1-/-

It was found in the previous chapter that loss of ERCC1 in MRC-5 cells caused sensitivity to gemcitabine, but loss of ERCC1 in HEK293 cells did not. Different repair processes or differences in gemcitabine metabolism may occur in HEK293 cells which may make the role of ERCC1 after gemcitabine redundant in these cells. However, there was still the possibility that the gemcitabine sensitivity of MRC-5 ERCC1-/- cells was due to an off-target mutation (Fu et al., 2013). For this reason it was important to complement these cells with *ercc1* cDNA

to see if this could rescue gemcitabine sensitivity, which would confirm the role of *ercc1* in gemcitabine resistance in these cells.

5.2.1 Transient transfection of MRC-5 ERCC1-/- with *ercc1* cDNA

Several studies have rescued the NER or ICL repair defects of ercc1 deficient cells by transient transfection with ercc1 cDNA (Friboulet et al., 2013; Jaspers et al., 2007; Rageul et al., 2011). The *ercc1* transcript undergoes alternative splicing to produce 4 distinct protein isoforms. Only one of these isoforms (isoform 1, Uniprot P07992) is known to bind XPF and function in ICL repair and NER (Friboulet et al., 2013). MRC-5 ERCC1-/- cells were transfected with a plasmid which carried the *ercc1* isoform 1 open reading frame (ORF). Western blotting confirmed the transfection was successful and the level of ERCC1 protein in MRC-5 ERCC1-/- was elevated to above that of the parent line for 24-72 hrs after transfection (Fig. 5.1). Transfected cells were plated in 96 well plates 24 hrs after transfection and tested for gemcitabine and MMC sensitivity by a luminescence based viability assay. Sensitivity was compared to MRC-5 and MRC-5 ERCC1-/- transfected with a control vector. Transfection with *ercc1* gave no rescue of gemcitabine or MMC sensitivity compared to MRC-5 ERCC1-/- transfected with a control vector. It has been reported previously that ERCC1 isoform 1 can rescue MMC sensitivity, so this result suggests there is a problem with the construct (Friboulet et al., 2013). A second indication that the construct is flawed was the failure to rescue levels of XPF protein which is discussed in the next section (Fig. 5.2). The issue with the ercc1 cDNA is unclear. The construct was sequenced and it did not contain any mutations, so should produce the correct protein. However, there is still the possibility that overexpression of ERCC1 interferes with its proper function or that the reports showing this isoform is functional are incorrect (Friboulet et al., 2013; Gibson et al., 2013). Figure 5.1 shows the transient transfection gives rise to much higher levels of ERCC1 than are present in the MRC-5 parent line and from 48hrs after transfection lower weight bands indicate a large amount of degradation products. To circumvent this problem a stably transfected line could be generated, which could be selected to exhibit levels of expression closer to the parent line.

5.2.2 Loss of ERCC1 destabilises XPF

ERCC1 is the binding partner of the nuclease XPF, which makes the incision 5’ of the lesion in NER. Many studies suggest these proteins are mutually stabilising and each protein will degrade in the absence of their binding partner (Arora et al., 2010; Gaillard and Wood, 2001; Jaspers et al., 2007). In light of these studies it was expected that XPF levels would be reduced

in the ERCC1-/- cell line and would be restored in the cell lines rescued with *ercc1* cDNA, as has been shown by other studies (Friboulet et al., 2013; Gaillard and Wood, 2001; Jaspers et al., 2007). Figure 5.2 shows that XPF expression is much reduced in MRC-5 ERCC1-/- but a small amount of XPF is detectable using x-ray film and a long exposure time. Contrary to expectations, very little rescue of XPF protein levels occurred after transfection with *ercc1* isoform 1 cDNA. As discussed above this indicates that there is a problem with the cDNA construct. The amount of XPF protein in the MRC-5 ERCC1-/- cell line appears much less in figure 5.2 B than in A. This is likely due to the lower amount of protein loaded and the digital imaging method which is better for comparison of expression levels, but less sensitive to very low levels of expression (Taylor et al., 2013). The interdependence between ERCC1 and XPF makes it difficult to attribute the gemcitabine sensitivity phenotype to an individual gene, but it would nonetheless be interesting to confirm that a MRC-5 XPF knockout would share the same phenotype.

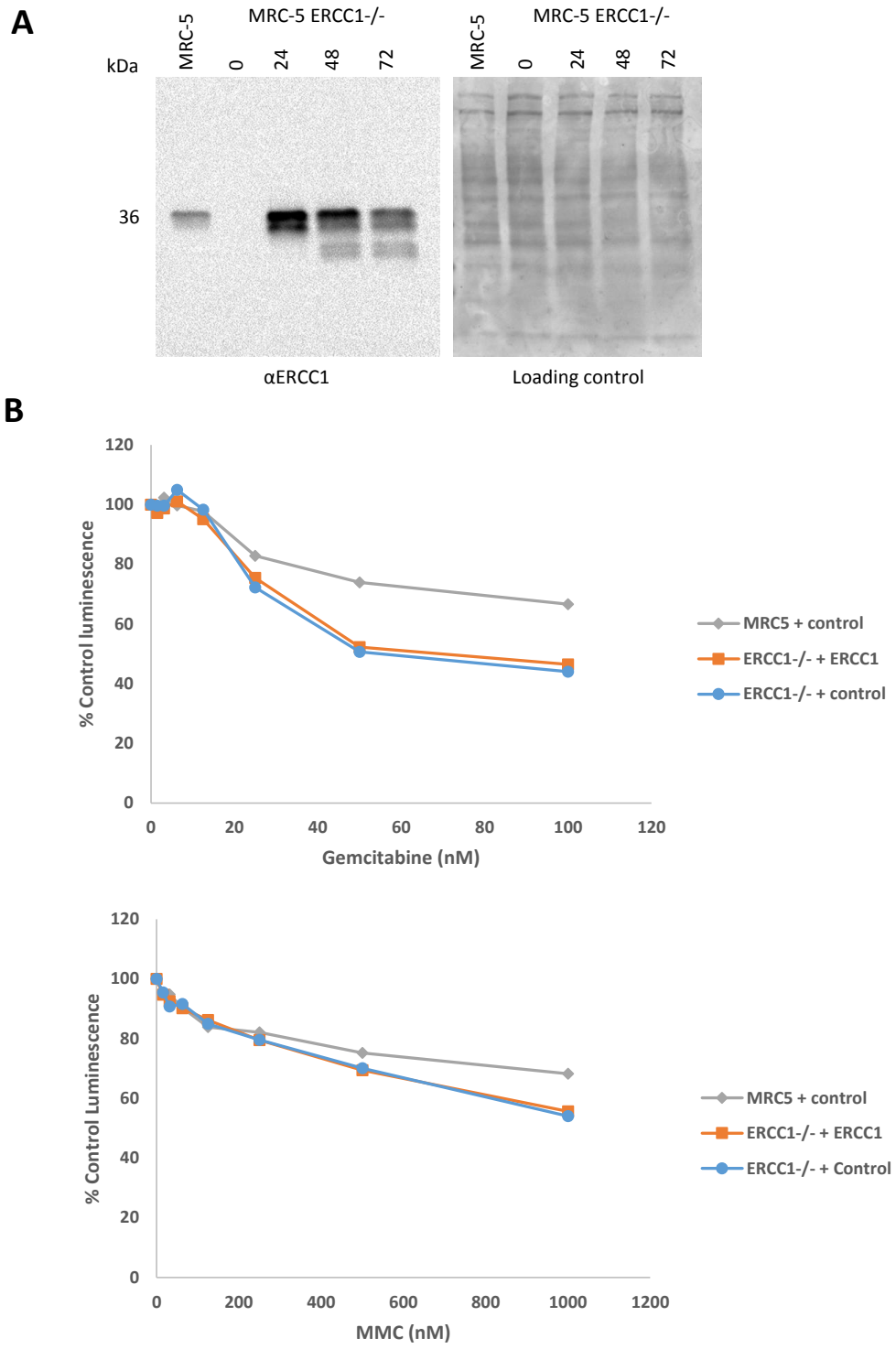


Figure 5.1. Transfection of MRC-5 ERCC1-/- with *ercc1* isoform 1 cDNA. (A) Western blot showing levels of ERCC1 protein in MRC-5 cells and MRC-5 ERCC1-/- cells at different time points after transfection with *ercc1* cDNA (left panel). Loading control displayed in the right panel. Imaged on Bio-rad ChemiDoc, 30 µg protein loaded. **(B)** ATP based assay showing viability after 72 hr treatment with gemcitabine (top) or MMC (bottom).

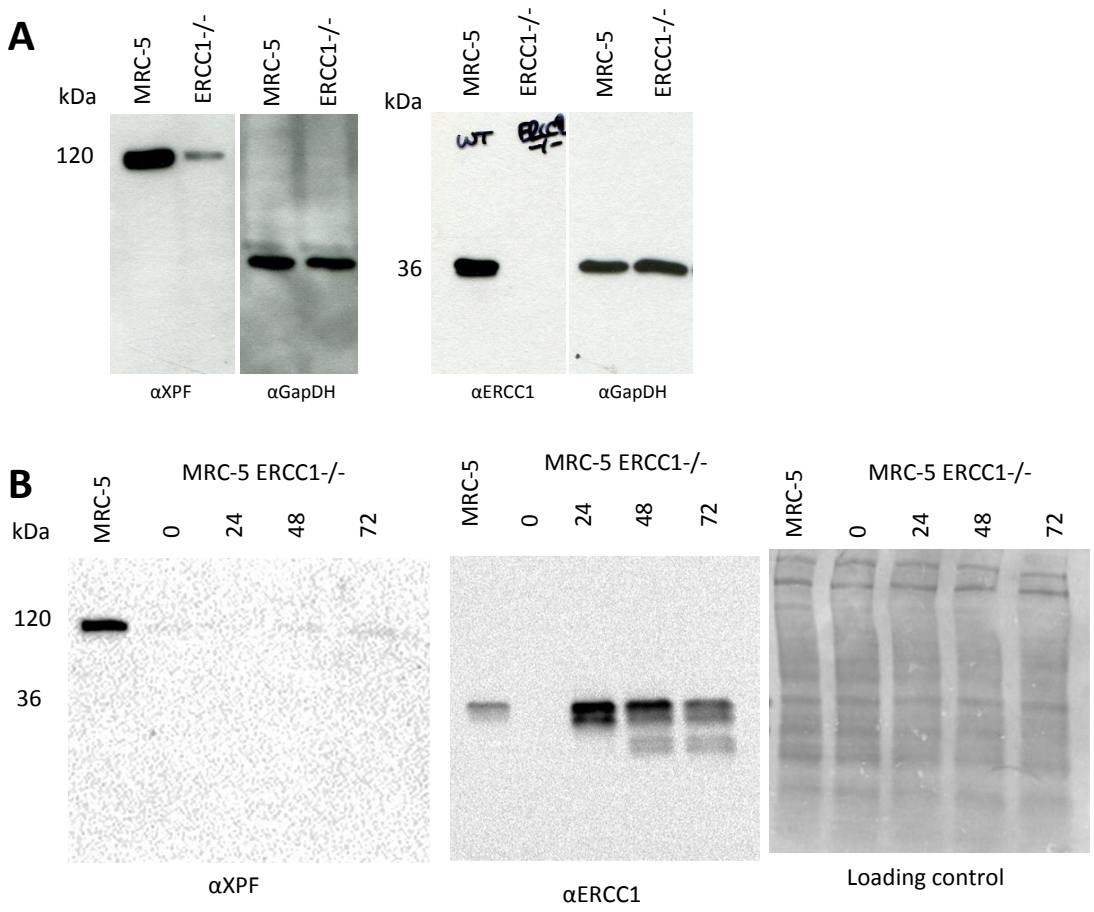


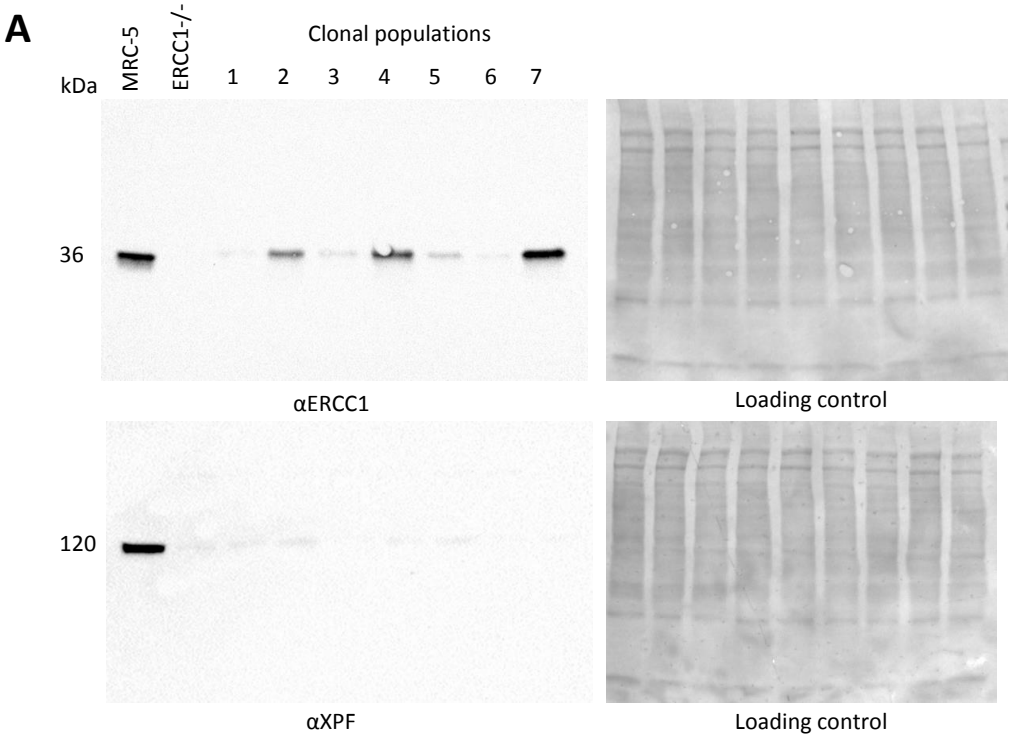
Figure 5.2 XPF protein levels in MRC-5 ERCC1-/- cells before and after transfection with *ercc1* isoform 1 cDNA. (A) Western blot showing levels of XPF and ERCC1 protein in MRC-5 (1) and MRC-5 ERCC1-/- (2). Imaged using X ray film, 50 µg protein loaded **(B)** Western blot showing levels of XPF and ERCC1 in MRC-5 cells and at different time points after transfection of MRC-5 ERCC1-/- cells with *ercc1* cDNA (αERCC1 image is the same as in 5.1). Imaged using Biorad ChemiDoc, 30 µg protein loaded.

5.2.3 ERCC1 isoform 3 does not bind to XPF or rescue gemcitabine sensitivity

The *ercc1* transcript undergoes alternative splicing to produce 4 distinct protein isoforms. Only isoform 1 (Uniprot P07992) is known to bind XPF and function in crosslink repair (Friboulet et al., 2013). MRC-5 ERCC1-/- cells stably expressing isoform 3 were generated by mistake, when aiming to stably express isoform 1, and tested for UV and gemcitabine sensitivity. The function of isoforms 2-4 is unknown, but they may have roles outside of the known functions of ERCC1 and therefore may have a role in gemcitabine resistance. Isoform 3 has an elongated

C terminus compared to isoform 1, which may interfere with XPF binding as this is mediated by the C terminal domain (Tripsianes et al., 2005).

The expression vector included a neomycin resistance marker, which enabled selection of cells which had incorporated the construct into their genomic DNA. Figure 5.3 shows several clonal populations (4, 6 and 9) were isolated which showed ERCC1 expression comparable with unmodified MRC-5. In agreement with previous reports that this isoform does not interact with XPF, XPF levels did not appear to be elevated in Clones 4 and 6 compared with clones which expressed little ERCC1. However Clone 9, which shows the highest expression of ERCC1 isoform 3, does appear to have slightly higher XPF levels thereby suggesting there is a small degree of interaction between isoform 3 and XPF (Fig. 5.3). Survival after UV irradiation is not rescued to the level of unmodified MRC-5 by expression of ERCC1 isoform 3, however there appears to be a modest rescue. Post UV survival of both clone 6 and clone 9 is elevated compared with MRC-5 ERCC1-/- after 2.5 ($P = 0.01, 0.03$) and 5 J/m2 ($P = 0.03, 0.01$) as calculated using a t test. These data suggest ERCC1 isoform 3 retains a small amount of functionality with regards to NER. Figure 5.3 shows gemcitabine sensitivity is also not rescued to the level of MRC-5, but there is a small rescue in one isoform 3 expressing clone at a single dose. Clone 9 shows a small increase in survival ($P = 0.02$) after treatment with 100 nM gemcitabine, while no significant differences are observed for clone 6. These results show ERCC1 isoform 3 does not make a large contribution to gemcitabine resistance in MRC-5 cells, but whether or not it makes a minor contribution is difficult to conclude from the small number of experiments presented here.


A
kDa
MRC-5
ERCC1-/-
Clonal populations
1
2
3
4
5
6
7
36
αERCC1
Loading control
120
αXPF
Loading control

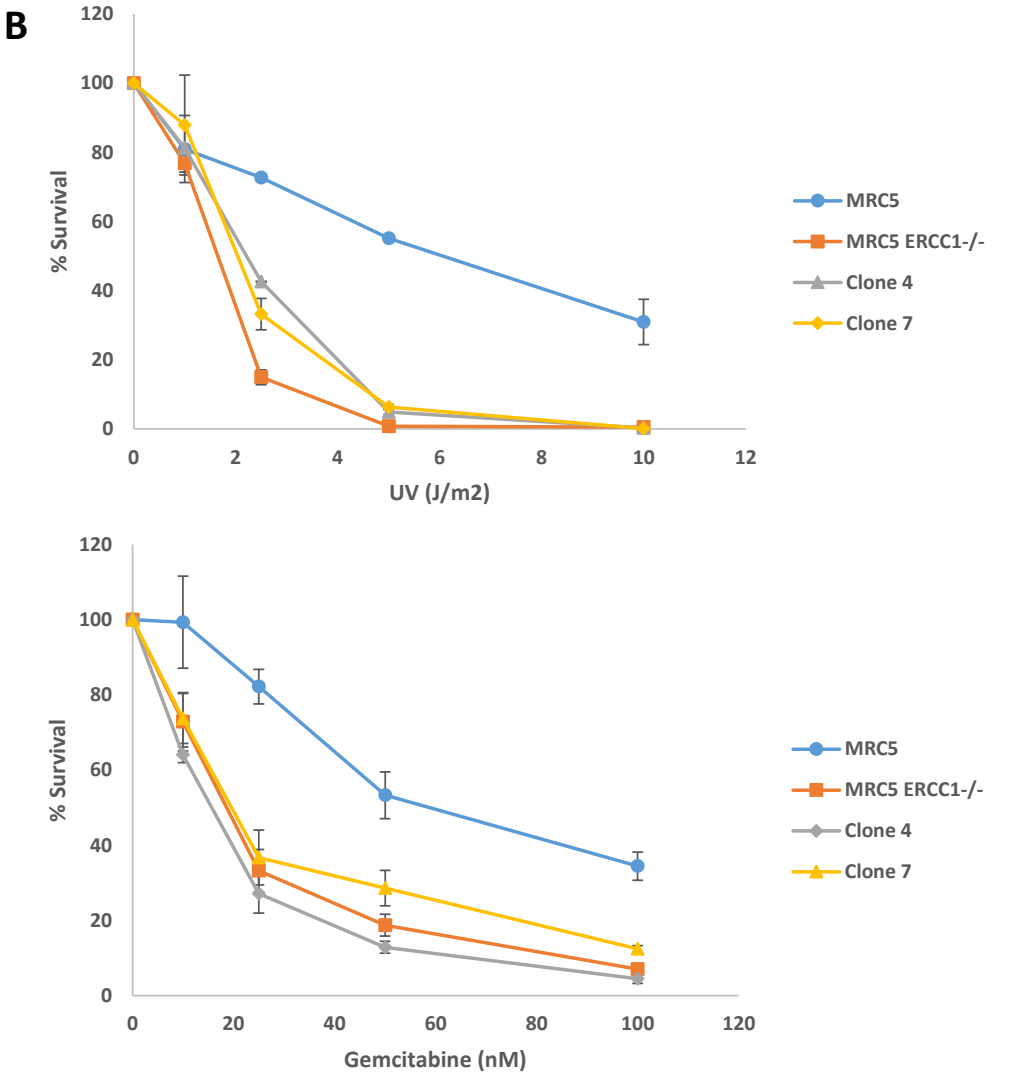

B
% Survival
UV (J/m2)
MRC5
MRC5 ERCC1-/-
Clone 4
Clone 7
% Survival
Gemcitabine (nM)
MRC5
MRC5 ERCC1-/-
Clone 4
Clone 7

Figure 5.3 Stable transfection of MRC-5 ERCC1-/- with *ercc1* isoform 3. (A) Western blots showing the presence of ERCC1 and XPF in MRC-5, MRC-5 ERCC1-/- and 7 different clonal populations of MRC-5 ERCC1-/- stably transfected with ERCC1 isoform 3. The same membranes were stripped and reprobed for GapDH as a loading control. **(B)** Colony forming survival assays showing survival after UV-C irradiation or 24 hr gemcitabine treatment. Data drawn from 2 independent experiments, apart from MRC-5 ERCC1-/- which is drawn from 3 independent experiments. Error bars show standard error.

5.3 Sensitivity to other replication inhibitors

The role of ERCC1 in NER as well as its role in crosslink repair requires ERCC1 to form a heterodimer with the nuclease XPF and it was hypothesised that the role of ERCC1-XPF in gemcitabine resistance was perhaps to excise gemcitabine from near the end of a nascent strand. If this was the predominant role of ERCC1 after gemcitabine treatment then it would be expected that MRC-5 ERCC1-/- cells would perhaps be more sensitive to nucleoside analogues than replication inhibitors with different mechanisms. For this reason sensitivity to cytarabine, an alternative nucleoside analogue and hydroxyurea, which works primarily by depleting dNTP pools and not inserting fraudulent bases, was tested. If MRC-5 ERCC1-/- cells showed greater sensitivity to gemcitabine and cytarabine than was seen for HU, it would indicate ERCC1 is involved in a repair process specific to nucleoside analogue stalled forks.

Nucleoside analogue specific sensitivity was not observed, with MRC-5 ERCC1-/- cells showing mild sensitivity to both HU and cytarabine (Fig. 5.4). This indicates ERCC1 is involved in a pathway which is important for survival after fork stalling, whether or not the fork stalling has been caused by chain terminating nucleotides or nucleotide depletion. However, sensitivity of the cells to gemcitabine is more extreme than to cytarabine or HU. The reason for this is unclear but gemcitabine may lead to more prolonged fork stalling due to the combined effect of dNTP depletion and chain extension inhibition and difficulty of gemcitabine removal may make fork restart more difficult. Prolonged fork stalling following gemcitabine treatment has been noted in other studies (Jones et al., 2014). This may require the use of different pathways for restart. It may be that whichever pathway ERCC1 is involved in is required to a greater extent after gemcitabine treatment, or it has a role in more than one pathway utilised in gemcitabine resistance.

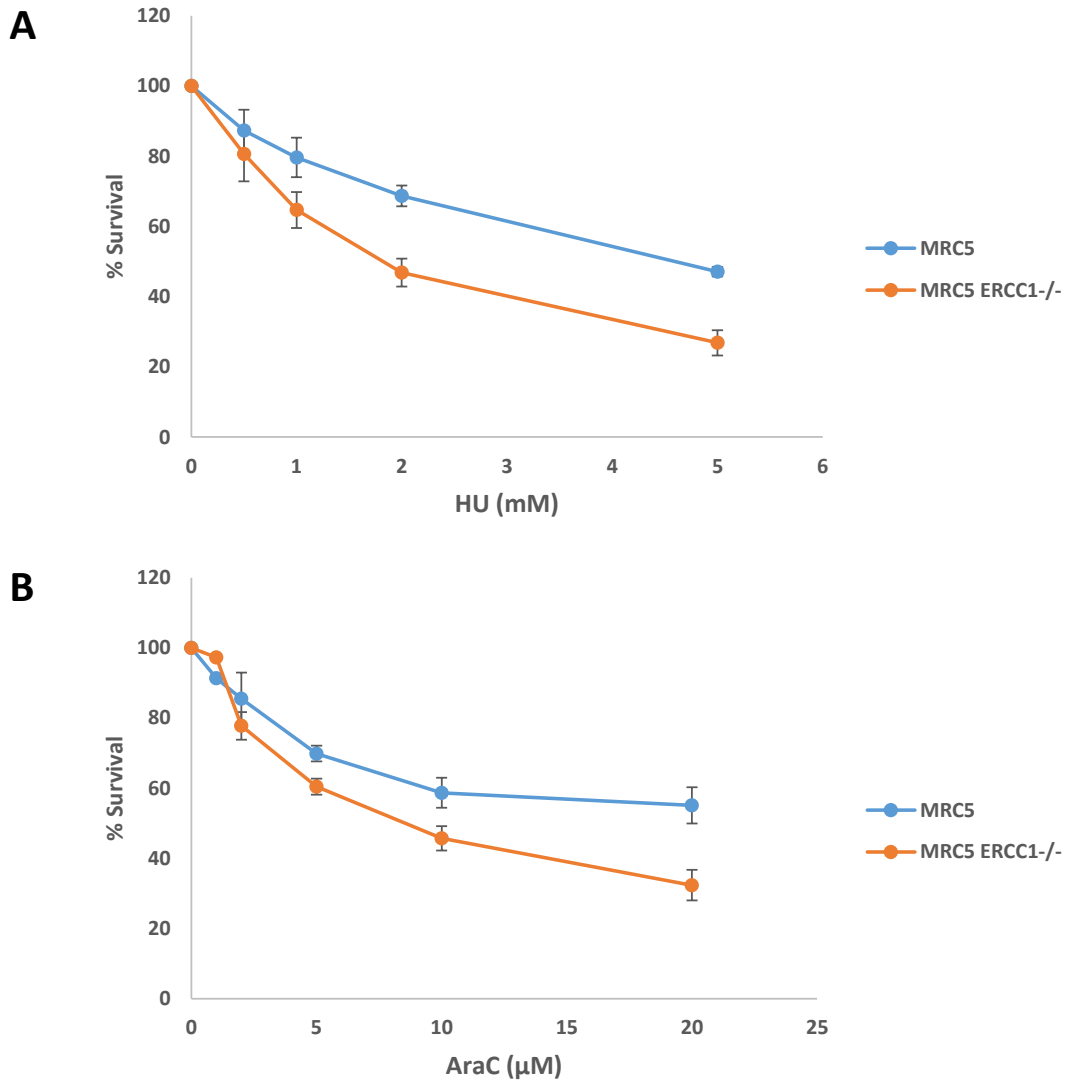


Figure 5.4 Sensitivity of MRC-5 ERCC1-/- to HU and cytarabine. Colony forming survival assays showing survival after 24 hr incubation with **(A)** Hydroxyurea (HU) or **(B)** Cytarabine (AraC). All data are from 3 independent experiments except from AraC 1 µM and 20 µM where n=1 and n=2 respectively.

5.4 ERCC1 localisation and foci formation

The localisation of ERCC1 in MRC-5 cells was investigated by confocal microscopy. In order to carry out any DNA processing role following replication inhibition it must be present in the nucleus and figure 5.5 shows this to be the case. It shows a fairly homogenous distribution across the nucleus, but is excluded from the nucleoli and is also present in the cytoplasm. This is in agreement with other studies which have looked at ERCC1 distribution (Friboulet et al., 2013; Houtsmuller, 1999; Naim et al., 2013; Volker et al., 2001). These images were captured

before the ERCC1 knockout line was made and therefore the XP-F cell line was used to check the specificity of the antibody and as expected shows greatly reduced expression and relative exclusion from the nucleus.

The same studies observed ERCC1 nuclear foci and the occurrence of nuclear foci with and without replication inhibition was investigated in this study. As was the case for XPA in chapter 1, the abundance of ERCC1 in the nucleus obscured nuclear foci so pre-extraction was used to remove unbound protein (Fig. 5.5). Foci occurred in both untreated cells and cells treated for 2hrs with 10 μM cytarabine. EdU was used as a marker of S phase and showed that ERCC1 foci occurred in both S phase and interphase cells. EdU is incorporated into replicating DNA and some ERCC1 foci showed colocalisation with EdU foci, suggesting ERCC1 is performing a role at replication forks. ERCC1 foci were also observed on condensed chromatin in prophase nuclei, which has been noted in a previous study (Naim et al., 2013) and suggests a role in processing DNA structures prior to mitosis.

Preliminary data was collected on the abundance of nuclear foci in S phase cells before and after a 2 hr treatment with the replication inhibitor cytarabine (Fig. 5.5). The percentage of S phase cells with ≥ 5 nuclear ERCC1 foci increases from 16% in untreated cells to 26% after cytarabine treatment. These data are from a single experiment, so must be treated with caution, however they indicate ERCC1 foci formation is an early response to replication inhibition in MRC-5 cells. This supports the conclusions of the survival experiments that ERCC1 mediates resistance to cytarabine in MRC-5 and suggests foci formation during S phase is functionally relevant. The colocalisation of some ERCC1 foci with EdU foci suggests a role at replication forks and the presence of foci in G2/M in the absence of EdU foci could either be foci formed at stalled forks in S phase and persisting or a second function in this phase of the cell cycle.

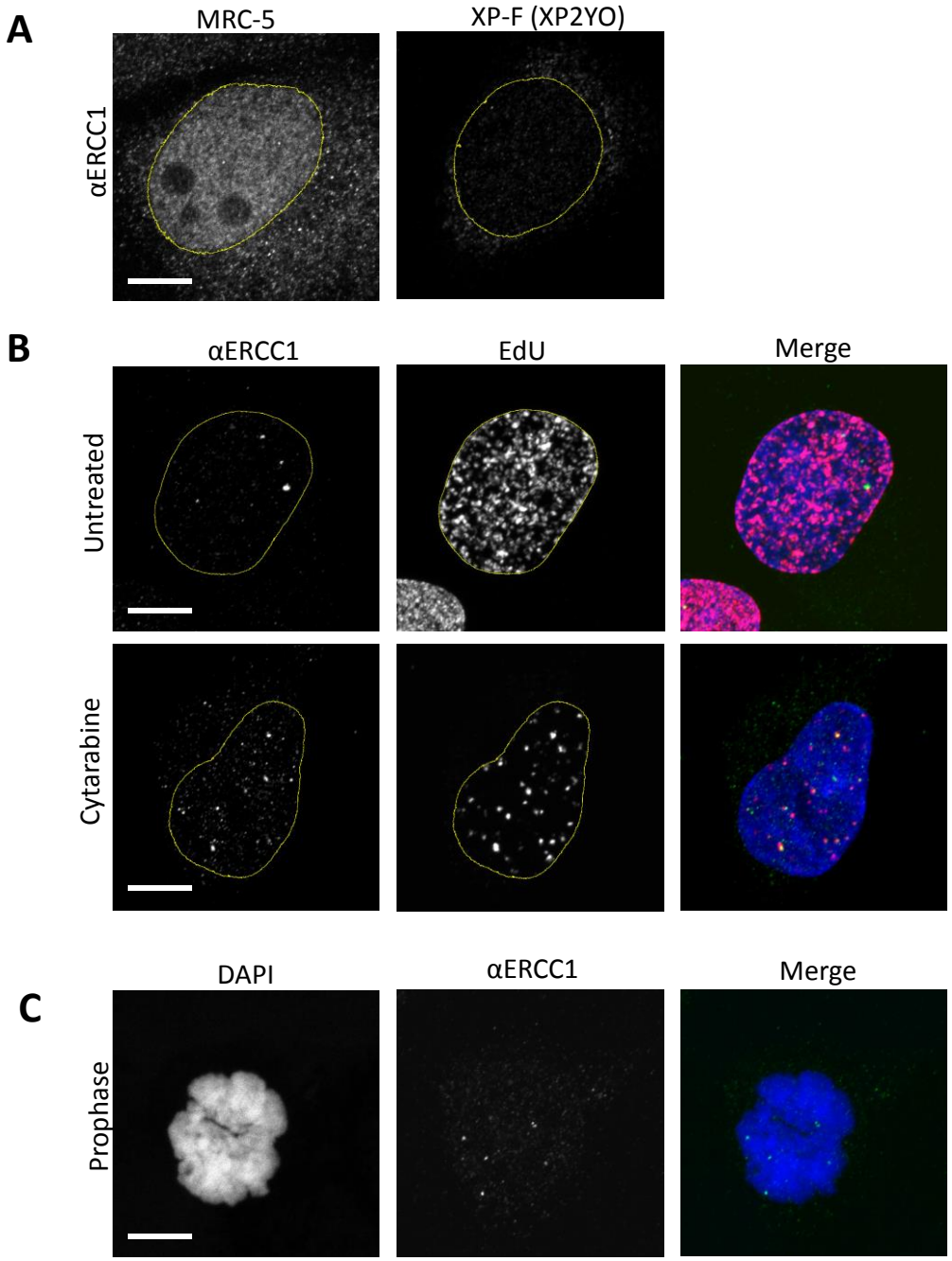

A
MRC-5
XP-F (XP2YO)
αERCC1
B
αERCC1
EdU
Merge
Untreated
Cytarabine
C
DAPI
αERCC1
Merge
Prophase
D
% S phase cells with ≥5 ERCC1 nuclear foci
30
25
20
15
10
5
0
Untreated
Cytarabine

Figure 5.5 Localisation of ERCC1. (A) Localisation of ERCC1 in S phase nucleus of MRC-5 and XP-F (XP2YO) cells. **(B)** Localisation of ERCC1 (green in merged image) and EdU (red in merged image) in MRC-5 cells (with pre-extraction) in untreated (upper panels) and cytarabine treated (10 µM, 2 hrs) cells (lower panels) Merged images show colocalisation. **(C)** Localisation of ERCC1 (green) in a prophase nucleus (DAPI, blue). Yellow outline shows nuclear area as marked by DAPI staining. Scale bars show 10 µm. **(D)** Chart showing quantification of nuclear ERCC1 foci in S phase cells with (n = 54) or without (n = 63) cytarabine treatment (10 µM, 2 hrs).

5.5 No increase in 53BP1 foci after gemcitabine treatment in MRC-5

The colocalisation of some ERCC1 and EdU foci suggests it is performing a role at the replication fork. There are many different permutations of fork recovery and restart which have been described that could involve ERCC1, including the processing of collapsed forks into DSBs. HR repair of persistently stalled forks can lead to intermediates which are processed by nucleases into cytotoxic double strand breaks (Jones et al., 2014). Two studies have demonstrated that gemcitabine treatment leads to an increase in DSBs and preventing DSB formation by depleting or mutating early HR factors can lead to gemcitabine resistance (Crul et al., 2003b; Jones et al., 2014). The nucleases MUS81 and XPF were postulated to be responsible for the conversion of stalled fork structures to DSBs, and indeed in one study depletion of either factor in U2OS cells led to a reduction in DSBs following gemcitabine treatment, as measured by 53BP1 foci (Jones et al., 2014). This would be expected to positively affect survival and lead to gemcitabine resistance of XPF depleted cells. However, this is at odds with the observed sensitivity of the MRC-5 ERCC1-/- mutant in this study, as ERCC1/XPF are expected to be working as a heterodimer and XPF is depleted in the absence of ERCC1 (Fig. 5.2). In order to explore this apparent conflict, the occurrence of DSBs, as measured by 53BP1 foci, was investigated after gemcitabine treatment in MRC-5 cells. 53BP1 is an established DSB repair factor and is considered a specific marker of DSBs (Schultz et al., 2000). The cells were treated with 2 µM gemcitabine for 2hrs, then washed and given fresh media, then fixed at various time points after washing. This was the same treatment regime used in (Jones et al., 2014) so a direct comparison of the results could be made.

Figure 5.6 shows the appearance of 53BP1 nuclear foci in MRC-5 cells with and without drug treatment. Phleomycin, a known DSB causing agent, is used as a positive control and elicits a

very high percentage of cells with ≥10 53BP1 foci (78%). Under normal conditions 18% of MRC-5 cells have 53BP1 foci, a percentage which reduces 24 hrs after gemcitabine treatment and returns to normal again by 72hrs after gemcitabine treatment. These results contrast with (Jones et al., 2014), where a very low percentage of U2OS cells show 53BP1 foci under normal conditions, which is increased dramatically 24 and 48 hrs after gemcitabine treatment. This suggests the formation of DSBs after gemcitabine is dependent on the cell line. Indeed, there are other studies using different cell lines which argue that the role of HR after gemcitabine is protective instead of cytotoxic, which may also be the case for MRC-5 (Choudhury et al., 2009; Tsai et al., 2010).

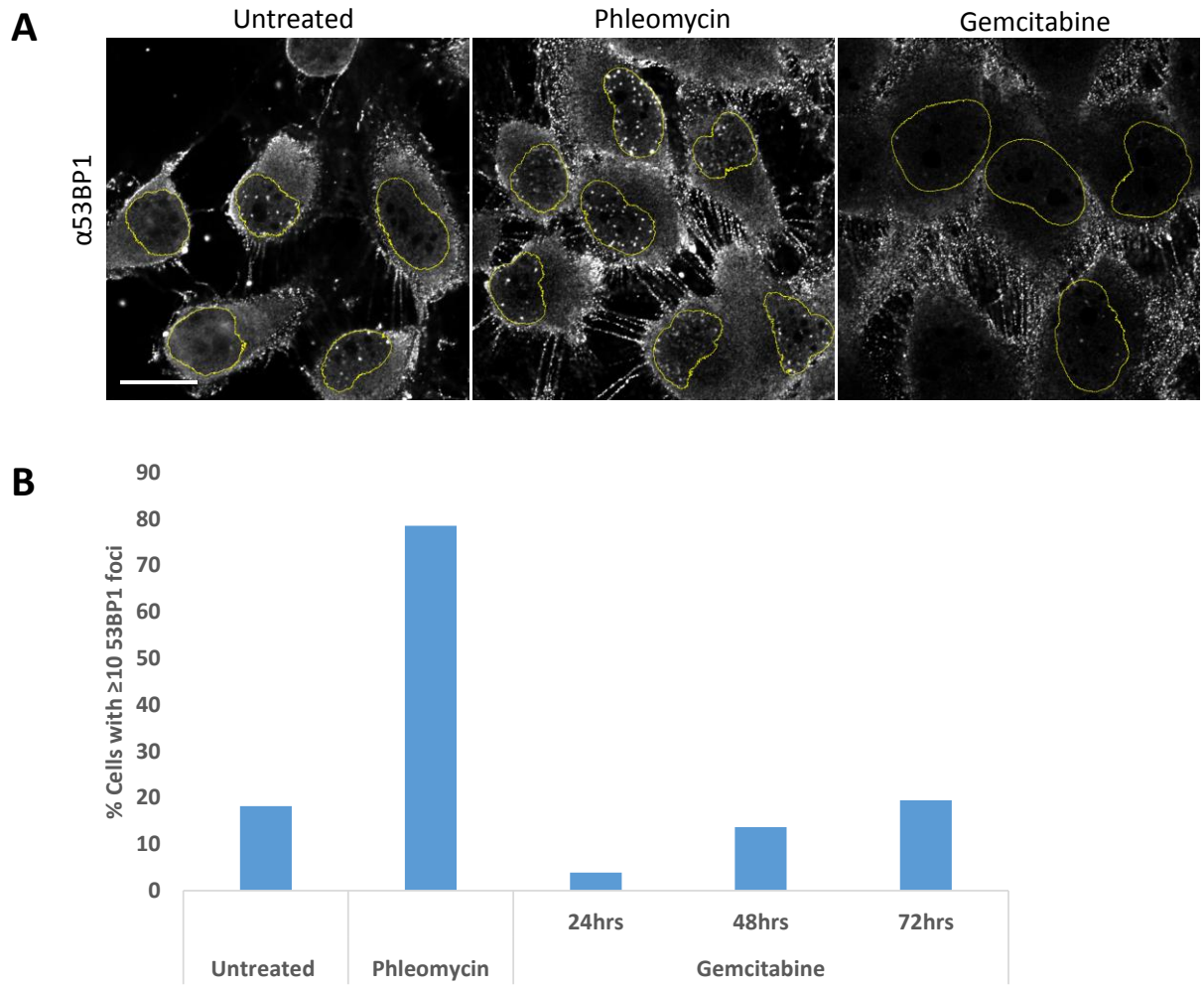


Figure 5.6 No increase in 53BP1 foci after treatment with gemcitabine. (A) Representative images showing 53BP1 foci in MRC-5 cells with no treatment or 24hrs after a 2 hr treatment with 30 µg/ml phleomycin or 2 µM gemcitabine. Scale bar shows 20 µm. **(B)** Bar chart displaying the percentage of cells with ≥ 10 nuclear 53BP1 foci. ≥ 70 nuclei were scored per condition. Data is from a single experiment.

5.6 Chromosome segregation following gemcitabine treatment

Failure to properly separate chromosomes during mitosis can lead to senescence and cell death and therefore an increase in segregation defects could be responsible for the sensitivity of MRC-5 ERCC1-/- cells to gemcitabine (Vitale et al., 2011). An investigation into chromosome segregation defects was prompted by a study proposing a role for ERCC1 in processing under-

replicated or interlinked DNA just prior to mitosis, to enable a successful division. The study showed increases in chromosome segregation defects, in primary MRC-5 cells after siRNA depletion of ERCC1 in both untreated cells and after treatment with the replication inhibitor aphidicolin (Naim et al., 2013). Moreover, in their study primary MRC-5 cells showed a particularly strong chromosome segregation defect after ERCC1 depletion, compared with HeLa cells. This has interesting parallels with this study where the results in chapter 2 suggest that the effect of ERCC1 loss is cell type dependent and that ERCC1 loss is particularly important for MRC-5 after replication inhibition. In addition ERCC1 foci were observed in prophase nuclei (Fig. 5.5), consistent with a role processing pre-mitotic chromosomes.

Therefore segregation defects, such as anaphase bridges, lagging chromosomes and multipolar mitoses, were quantified in MRC-5 and MRC-5 ERCC1-/-, with and without gemcitabine treatment (Fig. 5.7). A 24 hr treatment with 50 nM gemcitabine was used as there is a large difference in the survival of the two cell lines at this dose. The results showed MRC-5 and MRC-5 ERCC1-/- had similar levels of segregation defects under untreated conditions. However, following gemcitabine treatment the amount of segregation problems increases dramatically in both cell lines, and occur in 72% of mitoses in MRC-5 ERCC1-/- cells compared with 55% of MRC-5 mitoses, a difference which is significant when assessed with a t test ($P=0.002$, Fig. 5.7). This suggests ERCC1 does indeed have a role that promotes faithful chromosome segregation, under conditions of replication inhibition. However, these results do not show whether this role is the pre-mitotic processing of under-replicated or interlinked DNA described by Naim et al., (2013) or a function at replication forks during S phase.

Although increased numbers of segregation defects may lead to increased cell death after gemcitabine in MRC-5 ERCC1-/-, this is not demonstrated by this data and requires further investigation. Several studies have shown that p53 inactivation (such as that caused by SV40 transformation) allows the cell higher tolerance of aneuploidy and other abnormalities caused by segregation defects so this may not have a significant effect on cell death in these cells (Andreassen et al., 2001; Senovilla et al., 2009; Wright and Hayflick, 1972).

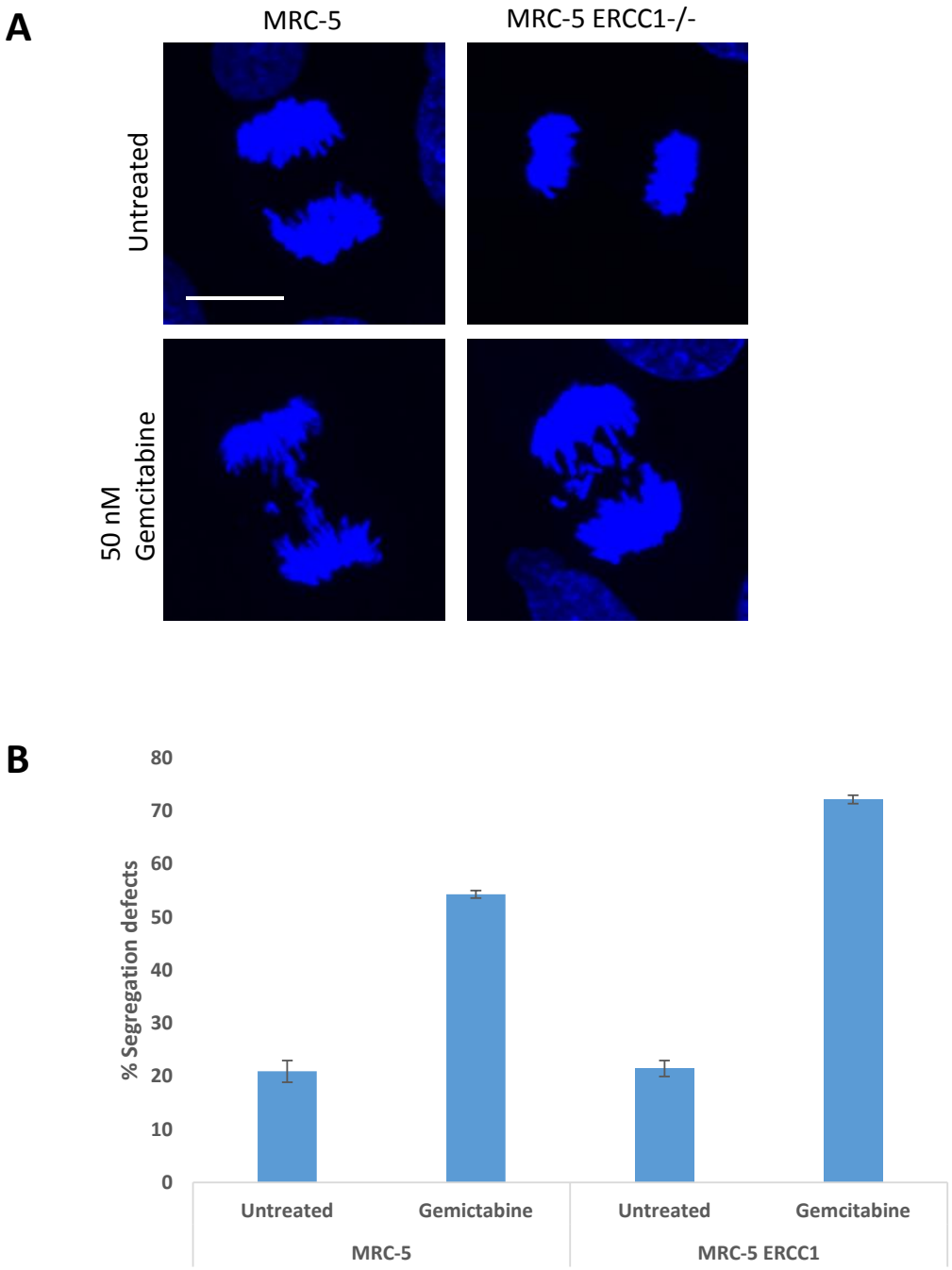


Figure 5.7 Chromosome segregation following gemcitabine treatment. (A) Representative images of MRC-5 and MRC-5 ERCC1-/- ana/telophases with and without gemcitabine treatment. Cells were treated with gemcitabine for 24 hr then washed and left in fresh media for 24 hrs before imaging. Scale bar shows 10 µm. **(B)** Bar chart showing the percentage of defective divisions. Anaphase bridges, lagging chromosomes and multipolar mitoses were scored as segregation defects. Approximately 50 ana/telophases were scored per condition for each experiment. N = 2, error bars show standard error.

5.7 Chapter discussion

This chapter began by attempting to confirm that the gemcitabine sensitivity of MRC-5 ERCC1-/- cells was due to the loss of ERCC1 and not an off target effect of the CRISPR-Cas9 process by transfection of the knockout line with isoform 1 *ercc1* cDNA. The results of this experiment were inconclusive as despite expression of the cDNA construct it failed to rescue MMC sensitivity or XPF protein levels. Sequencing showed the cDNA was the correct sequence, suggesting that it is maybe overexpression that is the problem or alternatively that this isoform is not functional which contradicts a previous study (Friboulet et al., 2013). The problem of overexpression could be solved by stable incorporation of the construct followed by selection of a clone with wild type expression levels. Many studies have demonstrated rescue of an ERCC1 phenotype with *ercc1* cDNA (for example; Friboulet et al., 2013; Jaspers et al., 2007; Rageul et al., 2011) and it would be useful to check with these authors which sequence they used and if possible obtain a construct from them. It is important that this validation is carried out before any further investigation of the gemcitabine sensitivity phenotype. If the role of ERCC1 in gemcitabine resistance is validated it has potential clinical applications. ERCC1 expression levels have already shown promise as a biomarker for resistance to the crosslinking agent cisplatin in non-small cell lung cancer. Establishing that ERCC1 has a role in gemcitabine resistance in MRC-5 will raise the possibility that it could be used in a similar way for gemcitabine (Bepler et al., 2011; Olaussen et al., 2006).

The remainder of the chapter explored the possible mechanisms by which ERCC1 could exert its effect. The possibility of a role specifically removing nucleoside analogues off the end of the nascent strand was considered but made less likely by MRC-5 ERCC1-/- showing approximately equal sensitivity to cytarabine and HU, a replication inhibitor which does not insert fraudulent bases into DNA. Therefore ERCC1 has a role in mitigation of replication stress whether it is caused by chain terminating nucleotides or nucleotide depletion. However, these results do not exclude ERCC1 having a role in removing gemcitabine from nascent DNA. This could be tested to some extent by the mass spectrometry assay described in chapter 3, which could be used to determine if there are higher amounts of incorporated gemcitabine in MRC-5 ERCC1-/- after treatment.

The localisation of ERCC1 into nuclear foci during S phase and prophase and the increase in numbers of foci after a short nucleoside analogue treatment, provides additional evidence for a replication associated function. Colocalisation of ERCC1 with EdU was an indication of presence at forks and this could be explored further by looking at other colocalising factors,

HR proteins for example. Several studies suggest a role for ERCC1-XPF in HR mediated repair of stalled forks following collision with a crosslink, and a similar role is conceivable after gemcitabine mediated stalling (Al-Minawi et al., 2009; Hanada et al., 2006; Niedernhofer et al., 2004).

With regards to the potential role of HR at gemcitabine stalled replication forks, reports in the literature are conflicting. Some studies show that depleting HR factors leads to gemcitabine sensitivity, suggesting HR has a positive effect on survival (Choudhury et al., 2009; Nakashima et al., 2015; Tsai et al., 2010). Others show the opposite, suggesting HR has a negative effect, perhaps through the creation of cytotoxic intermediates (Crul et al., 2003b; Jones et al., 2014). The latter study showed a large increase in the numbers of DSBs in U2OS cells following gemcitabine treatment and proposed these were the basis of gemcitabine cytotoxicity. Moreover, they showed depletion of the nuclease XPF decreased the numbers of DSBs and suggested this would lead to gemcitabine resistance. This seems to conflict with the sensitivity of the MRC5 ERCC1-/- cell line in this study (in which XPF levels are also depleted) and indeed in contrast to that study no increase in DSBs (as measured by 53BP1 foci) was observed in MRC-5 cells after gemcitabine treatment. These results concur with another study using OCI-AML3 cells which also showed no increase in DSB formation after a 2 hr 100 nM treatment with gemcitabine using pulse-field gel analysis (Ewald et al., 2008b). This suggests DSBs are not a major source of cytotoxicity after gemcitabine in MRC-5 cells, but this conclusion would benefit from confirmation via an assay that directly detects DSBs such as the pulse-field gel assay. It suggests a different fork recovery mechanism which avoids the formation of ercc1-xpf dependent DSBs is at play in MRC-5 cells and this could be responsible for the discrepancies in the literature as described above.

The slowing of replication by gemcitabine may lead to cells arriving in G2/M with under replicated DNA, in which case sister chromatids remain linked. The appearance of ERCC1 foci on prophase chromosomes echoed the findings of Naim et al., (2013) suggesting a role in processing these under replicated substrates just prior to mitosis. It is also consistent with the processing of late replication intermediates by the SMX trinuclease complex, which forms during G2/M and of which ERCC1-XPF is a part (Wyatt et al., 2017). In the cited study, the authors show an increase in segregation defects upon replication inhibition, which was greater in ERCC1 depleted cells. Results presented here are consistent with those findings, showing segregation defects increase upon gemcitabine treatment and *ercc1-/-* cells are more severely affected. Segregation defects can lead to cell death during or following mitosis and therefore it

is likely the increase in segregation defects contributes to the gemcitabine sensitivity of *ercc1-/-* cells (Vitale et al., 2011). The observed segregation defects do not necessarily represent a defect in the processing of late replication intermediates and could also be caused by earlier problems in S phase. Analyses of fork speed and stalled fork restart using DNA fibres could be used to examine the latter possibility.

The precise role of ERCC1 in gemcitabine resistance remains undefined by these observations but some conclusions can be drawn and new lines of enquiry opened. The sensitivity of MRC-5 ERCC1-/- to replication inhibitors was shown not to be exclusive to nucleoside analogues. Colocalisation with EdU suggests a role at replication forks. Given the reduction in numbers of DSBs after gemcitabine in MRC-5 cells, it seems unlikely ERCC1 processes stalled forks into DSBs in this cell line, as it is reported to do in U2OS cells. Finally, the data collected are consistent with, but do not prove a function in G2/M promoting successful chromosome segregation.

6 FINAL DISCUSSION

It is well established that DNA repair pathways contribute to resistance to the DNA damaging agents often used to treat cancer, but understanding the details of these interactions remains the subject of ongoing investigations. As established in the introduction, gemcitabine is a clinically important chemotherapy drug and the contribution of DNA repair pathways to gemcitabine resistance is poorly understood. Previous studies had demonstrated a potentially novel role of NER factors in gemcitabine resistance which appeared to be conserved between *S. pombe* and human cells (Beardmore, 2015; Gasasira, 2013). This project built upon those studies and aimed to arrive at a better understanding of this role.

Initially, as in the previous work, NER deficient XP patient cell lines were used to study the mechanism by which NER factors contributed to gemcitabine resistance. It is discussed here how two 'normal' fibroblast lines MRC-5 and GM637 were shown to have radically different sensitivities to gemcitabine. This rendered the comparison of XP patient fibroblasts with 'normal' fibroblast lines an unsuitable experimental design and necessitated different approaches such as using knockout cells lines generated by CRISPR-Cas9. This study characterises two ERCC1 knockout lines, generated by CRISPR-Cas9 from different parent cell lines. The protein ERCC1 forms a heterodimer with the 3' flap endonuclease XPF and is an essential NER factor, as well as participating in several other repair pathways (Manandhar et al., 2015). To our knowledge, there are currently no ERCC1 knockout lines published and these cell lines will be a useful tool to study the role of this multifunctional gene. The cell line MRC-5 ERCC1-/- revealed a role for ERCC1 in gemcitabine resistance although further work is required to validate and understand the details of that role. The cell line HEK293 ERCC1-/- was not sensitive to gemcitabine, which suggested the role of ERCC1 in gemcitabine resistance is cell line dependent. Additionally, this study found no evidence for a role of XPA in gemcitabine resistance, and expression of a rescue construct in XPC or XPG deficient fibroblasts elicited only very small changes in survival after gemcitabine treatment. It is discussed here how these findings add to our current understanding of gemcitabine resistance and NER and suggest how this work can be built upon in future.

6.1 The ‘normal’ human fibroblasts MRC-5 and GM637 have dramatically different sensitivities to gemcitabine

A widely used approach to investigate the function of NER factors in UV damage repair has been to use cell lines from XP patients and compare them to fibroblasts from unaffected individuals (for example Marti et al., 2006; Oh et al., 2011; Volker et al., 2001). This was the approach taken by the previous work investigating gemcitabine resistance in mammalian cells and the early part of this project. In agreement with the previous work all the XP patient fibroblast lines tested showed increased sensitivity to gemcitabine and UV when compared to MRC-5, a fibroblast line from an NER proficient individual. In addition to this XP-A and XP-G cell lines showed increased fork stalling and increased incorporation of gemcitabine into DNA when compared with MRC-5, as measured by DNA fibre and LC MS/MS assays respectively. This appeared to indicate a role for XPA and XPG in assisting replication fork progression under replication stress, and perhaps a role in removing gemcitabine from DNA, which would both be novel functions of these proteins. However, a second ‘normal’ human fibroblast line, GM637, was later tested and found to have a similar sensitivity to UV light but much greater sensitivity to gemcitabine than MRC-5. This confounds all the previous work, in this project and previously, in which XP patient fibroblasts were compared to MRC-5. As detailed in the introduction, nucleoside transporters and many enzymes are involved in gemcitabine metabolism and are known to influence gemcitabine resistance (Fig. 5, Bergman et al., 2002). That there are a great number of factors involved in gemcitabine metabolism may mean the potential for variation in gemcitabine sensitivity between cell lines is greater than for an agent that causes damage more directly such as UV light, a problem that was not anticipated at the outset of this project. This finding prompted a change in study design that enabled the effect of losing an NER gene to be measured while keeping the genetic background stable. This was achieved by two means, targeted knockout of a gene by CRISPR, or the use of XP patient cells stably transfected with a functional copy of the appropriate gene. The observation that ‘normal’ fibroblasts can have such profoundly different sensitivities to gemcitabine should be taken into consideration in future studies of this drug.

6.2 Characterisation of two ERCC1-/- knockout lines

CRISPR-Cas9 is a recently developed system which uses elements of the microbial immune system to allow targeted deletions and insertions to be made in many different organisms, including human cells (Mali et al., 2013; Ran et al., 2013a). ERCC1 was targeted for deletion

by CRISPR-Cas9 in two immortalised human cell lines, MRC-5 and HEK293, by R. Beardmore (2015). As part of this project, a homozygous clonal population was isolated and characterised to produce MRC-5 ERCC1-/- and HEK293 ERCC1-/- cell lines. The CRISPR-Cas9 guide sequences had been targeted to exon 1 as this is common to all 4 known ERCC1 isoforms (UniProt, P07992). The loss of ERCC1 was established by both testing for the protein and detecting the sequence alterations. Both cell lines tested negative for ERCC1 protein by western blotting and in both cases PCR analysis detected one *ercc1* allele had incorporated the homology directed repair sequence in place of exon 1, which eliminated the start codon. The second allele for each cell line was sequenced. MRC-5 ERCC1-/- contained a 19 nucleotide deletion in exon 1 resulting in a frameshift after 26 amino acids and premature stop codon after 85. The second allele of HEK293 ERCC1-/- contained a larger deletion of 228 nucleotides, which included the start codon. MRC-5 ERCC1-/- demonstrated increased UV sensitivity which was an expected phenotype of ERCC1 loss. Likewise, HEK293 ERCC1-/- demonstrated increased sensitivity to the crosslinking agent MMC. Taken together these results allow the two cell lines to be considered as lacking ERCC1 with reasonable confidence. However, the validation could be improved by western blotting with a second antibody raised against the C terminus of the protein, as it is still possible that a truncated protein could be produced from a downstream start codon.

To our knowledge, there are currently no human ERCC1 knockout lines published so the generation of these two cell lines provides a novel tool for studying the function of this gene in relation to gemcitabine resistance and more widely. For example, MRC-5 ERCC1-/- has since been used by Dr. Khobta (Mainz) to investigate the repair of different types of lesion by ERCC1, using a plasmid containing a reporter construct which is disrupted by a specific lesion. Generating knockout lines in this way provides advantages over other common approaches such as using cell lines from patients with mutations and depletion by RNA interference. For some genes such as ERCC1, patients deficient in the gene are very rare as loss of the gene causes severe problems early in development. Patient cells lines can also often still produce some protein from the affected gene that has a residual function (Jaspers et al., 2007). Many studies investigating ERCC1 function have used transient knockdown by RNA interference to deplete the gene, but this may still allow low levels of expression and the transfection procedure itself is cytotoxic (Liccardi et al., 2014; Naim et al., 2013; Rageul et al., 2011). By contrast, the knockout cell lines should produce no protein and the lack of expression is stable, making it possible to perform experiments with a longer timecourse.

6.3 The role of ERCC1 in gemcitabine resistance is cell line dependent

To our surprise the effect of ERCC1 loss on gemcitabine resistance was different in the two cell lines. MRC-5 ERCC1-/- showed considerable sensitivity to gemcitabine compared to the parent line while HEK293 ERCC1-/- showed no change in sensitivity in a colony forming assay and displayed resistance in the ATP based viability assay. This indicates that the role of ERCC1 in gemcitabine resistance varies according to cell line. This cell line dependency of the role of repair factors is not without precedent. The role of HR factors such as RAD51 and BRCA2 in gemcitabine resistance has been the subject of several published studies with contrasting results. Some studies showing the loss of HR factors causes sensitivity and others that it causes resistance (Choudhury et al., 2009; Crul et al., 2003a; Jones et al., 2014; Tsai et al., 2010). These studies were carried out using different cell lines which is a possible explanation for the contradictory results. To investigate the role of ERCC1 in different cell lines further, it would be interesting to delete or deplete ERCC1 in a panel of cell lines in order to see how frequently among cell lines a gemcitabine sensitivity phenotype occurs and if there is any connection between the types of cell lines it occurs in. Such a screen could be carried out by CRISPR-Cas9 or more quickly using siRNA depletion.

The basis for this difference between cell lines may lie in differences in the pathways used to protect and restart stalled forks. Recent studies have found forks have a variety of ways in which they can respond to replication inhibition, but the factors which govern choice of these pathways, how these choices influence survival and how different agents influence pathway choice are poorly understood. Some cell lines may use pathways in which ERCC1 can play a key role and other cell lines may activate a response where ERCC1 is not needed. For example, results in this study suggest a difference in the response to gemcitabine between MRC-5 and U2OS cells. A recent study showed that gemcitabine treatment led to the rapid accumulation of cytotoxic double strand breaks in U2OS cells, as measured by 53BP1 foci and pulse field gel analysis (Jones et al., 2014). Under exactly the same experimental conditions results presented here showed there was a slight decrease in 53BP1 foci after gemcitabine treatment in MRC-5 cells. The quantification of 53BP1 foci is an indirect method for measuring DSBs and it would be interesting to confirm these results using a direct detection method such as pulse field gel or comet assays. Jones et al (2014) proposed the DSBs observed in the U2OS cells in their study were a result of XPF and MUS81 cleaving HR intermediates. The results

presented here suggest this does not happen in MRC-5 cells, or else does not result in an accumulation of DSBs.

There remains a possibility that the differences observed between the two cell lines is not due to a cell line dependent role of ERCC1, but is instead due to an off target effect of the CRISPR-Cas9 procedure in MRC5 ERCC1-/-. The sensitivity of MRC-5 to gemcitabine might not be due to loss of ERCC1 but instead be due to another change elsewhere in the genome. Off target effects are a known complication with the CRISPR-Cas9 procedure (Fu et al., 2013). The Cas9 nickase enzyme, which involves the use of two adjacent guide sequences to improve specificity, was used to generate the cell lines to minimize the occurrence of off-target effects but even still they remain a possible alternative explanation for the gemcitabine sensitivity of MRC-5 ERCC1-/- cells. (Ran et al., 2013b).

Validating that the sensitivity of the MRC-5 ERCC1-/- cell line is due to loss of ERCC1 could be achieved by rescuing the phenotype with *ercc1* cDNA. Several studies have shown rescue of ERCC1 phenotypes by transfection with *ercc1* cDNA (Friboulet et al., 2013; Jaspers et al., 2007). This was attempted in the present study but it failed to produce a successful rescue. As previously mentioned ERCC1 has 4 known isoforms but only isoform 1 (UniProt, P07992) is known to be able to bind to XPF and function in NER and ICL repair (Friboulet et al., 2013). Therefore the MRC-5 ERCC1-/- cells were transfected with cDNA encoding isoform 1. Expression of the construct was successful as ERCC1 protein levels were elevated to above that of the parent MRC-5 cells for over 72hrs post transfection, however it did not alter the sensitivity of MRC-5 ERCC1-/- cells to gemcitabine or the crosslinking agent MMC as judged by a viability assay. The observation that expression of the cDNA did not correct the sensitivity to a crosslinking agent, which is a known phenotype of ERCC1 deficiency, suggests there is a problem with the construct and the protein it produces is deficient in at least one of its known functions (De Silva et al., 2000). The binding of ERCC1 to XPF is important for the role of ERCC1 in NER and ICL repair and is a mutually stabilising interaction (Arora et al., 2010; Biggerstaff et al., 1993; Gaillard and Wood, 2001). In accordance with these studies, it was shown here that XPF levels are much reduced in the MRC-5 ERCC1-/- cell line. XPF levels are not rescued by the expression of *ercc1* isoform 1 cDNA which provides a mechanistic explanation for the failure of the construct to rescue MMC sensitivity. The reason for the construct failing to produce functional ERCC1 is unclear. There was no time left in the current project but it would be useful to sequence the construct to rule out mutations and then if this is uninformative obtain the cDNA off another group that has successfully performed a rescue.

Whatever the cause, the failure to express a functional ERCC1 protein renders the results of the validation inconclusive and it cannot be ruled out that the gemcitabine sensitivity of MRC-5 ERCC1-/- is due to an off target effect.

6.4 The role of ERCC1 in providing gemcitabine resistance to MRC-5 cells

Assuming that the gemcitabine sensitivity of MRC-5 ERCC1-/- cells is a true consequence of ERCC1 loss and can be validated in future, investigations were made into the nature of this putative role. The cell line showed sensitivity to two other replication inhibitors, hydroxyurea and cytarabine. Cytarabine, like gemcitabine, is a cytidine analogue which enters the cell via the same nucleoside transporters and shares many aspects of intracellular metabolism. Again like gemcitabine it is incorporated into nascent DNA where it serves as a more potent chain terminator than gemcitabine and unlike gemcitabine it has little effect on dNTP pools (Ewald et al., 2008a). Conversely hydroxyurea is not a nucleoside analogue, enters the cell via diffusion and inhibits replication solely through the lowering of dNTP pools. Like gemcitabine it does this through inhibition of RNR although unlike gemcitabine the inhibition is reversible (Gwilt and Tracewell, 1998). The similar relative sensitivity of MRC-5 ERCC1-/- to hydroxyurea and cytarabine suggests ERCC1 plays a role in relation to fork stalling that is not specific to nucleoside analogue induced stalling. However, the results do not rule out that ERCC1 may be performing different roles in response to hydroxyurea and cytarabine. For completeness, it would also be interesting to also test MRC-5 ERCC1-/- for sensitivity to aphidicolin, a replication inhibitor that neither lowers nucleotide pools nor inserts fraudulent bases and so has a mechanism of fork stalling completely independent from that of gemcitabine. A recent study showed ERCC1 depleted MRC-5 cells exhibited an increase in segregation defects after aphidicolin treatment but did not test whether ERCC1 depletion had a negative effect on survival (Naim et al., 2013).

Despite MRC-5 ERCC1-/- exhibiting sensitivity to HU and cytarabine relative to the parent line, this relative sensitivity is less than that seen after gemcitabine. This suggests that different replication inhibitors will require different responses from DNA repair factors. Whichever pathway ERCC1 functions in after gemcitabine treatment in MRC-5 cells may be needed less after treatment with HU or cytarabine. A possible reason for this could be that fork restart may be more difficult and stalling more prolonged after gemcitabine than the other agents. Prolonged stalling of forks has been noted after even a short incubation with gemcitabine

(Jones et al., 2014). Gemcitabine may cause more prolonged fork stalling than the other agents firstly because it causes both nucleotide depletion and inhibition of chain extension. Which requires the cell to both replete nucleotide pools and remove gemcitabine or somehow else circumvent chain extension inhibition. With regards to the second point it has been shown that gemcitabine is excised less efficiently than cytarabine by the proofreading activity of polymerases, and is extended more efficiently which may mean more extensive resection is required to remove it (Gandhi et al., 1996). This difficulty in restart could in theory lead to the increased accumulation and persistence of abnormal fork structures and under-replicated DNA after gemcitabine, which may require ERCC1 to rectify.

As described in the introduction ERCC1 has well characterised roles in NER and replication coupled ICL repair that are dependent on the nuclease activity of XPF. The results presented here do not address this question but it would be interesting to determine in future whether the role in gemcitabine resistance is also dependent on XPF catalytic activity. For this reason it would be useful to generate an XPF CRISPR-Cas9 knockout in MRC-5 to firstly see if it shows the same phenotype. Such a line is published but the authors were unwilling to share it. It is expected that MRC-5 XPF-/- would show the same phenotype, even if the phenotype is not due to the catalytic activity of XPF, as without XPF it is reported that ERCC1 cannot enter the nucleus (Lehmann et al., 2017). However, complementing MRC-5 XPF-/- with a 'nuclease dead' *xpf* construct, which allows the mutually stabilising ERCC1-XPF interaction to take place but has a point mutation which prevents nuclease activity, would overcome these problems. XPF nuclease dead constructs have been used in several studies investigating the role of XPF in different contexts (Enzlin and Schärer, 2002; Staresincic et al., 2009; Wyatt et al., 2017). There is some evidence to suggest the role of ERCC1 in gemcitabine resistance could be XPF or XPF nuclease independent. Stable complementation of an XP-F patient cell line (XP2YO) with wild type XPF led to rescue of UV sensitivity but no change in gemcitabine sensitivity, however residual XPF function is reported for this cell line, which may mask the effects of complementation and expression of the introduced construct was weak (Beardmore, 2015; Yagi et al., 1997). A group of recent studies have described a role of ERCC1 in pre-mitotic cells that appears to be independent of XPF. Naim et al (2013) observed ERCC1 foci on prophase and prometaphase chromosomes and observed an increase in chromosome segregation defects upon knockdown of ERCC1 or MUS81, but knockdown of XPF failed to produce the same phenotype. Knockdown of either ERCC1 or MUS81 followed by aphidicolin treatment led to a decrease in breaks at metaphase, but an increase in breaks in the following

G1. Under the same conditions there was no change in the frequency of breaks in G2. Once again, knockdown of XPF did not produce the same phenotype. This lead the authors to conclude cleavage of under-replicated or interlinked DNA by a MUS81 or ERCC1 dependent, but XPF independent mechanism occurred at metaphase and prevented detrimental chromosome breakage during mitosis. Similarly, a second study found knockdown of ERCC1, but not knockdown of XPF, in HeLa and C5RO cells resulted in multi-nucleation and abnormal mitoses in the absence of replication stress (Rageul et al., 2011).

A mechanism for an XPF nuclease independent pre-mitotic function of ERCC1 has recently been suggested by work on the SMX tri-nuclease complex (Wyatt et al., 2017). It was shown that ERCC1-XPF is associated with SLX4-SLX1 throughout the cell cycle, but MUS81-EME1 only joins the complex at G2/M, whereupon the complex was shown in biochemical assays to efficiently cleave a range of DNA substrates such as Holliday junctions and replication forks via the nuclease action of MUS81. The nuclease action of XPF was not required for efficient cleavage of any of the substrates tested, but the presence of ERCC1-XPF in the complex resulted in a large increase in the efficiency of Holliday junction cleavage, suggesting ERCC1-XPF has a structural role. Whether ERCC1 could bind and stimulate the complex on its own was not tested, but is unlikely as XPF is the subunit responsible for binding to SLX4 (Fekairi et al., 2009).

None of the above studies tested how knockdown of ERCC1 affected survival after replication inhibition but mitotic defects can lead to cell death, so increased abnormal mitoses in MRC-5 ERCC1-/- could contribute to the gemcitabine sensitivity of this cell line. Consistent with this idea, it was presented here that MRC-5 ERCC1-/- showed an increase in chromosome segregation defects after gemcitabine treatment and ERCC1 foci were visible in MRC-5 prophase nuclei. However, the segregation defects could equally be attributable to increased fork slowing or stalling earlier in S phase and the foci may have formed earlier in S phase and persisted, so these results are not conclusive as to the role of ERCC1 in pre-mitotic processing after gemcitabine treatment.

It was presented here that ERCC1 formed nuclear foci in untreated and cytarabine treated S phase cells, which in some cases colocalised with EdU labelled nascent DNA. Additionally, the incidence of foci formation increased after a 2hr treatment with cytarabine, which suggests ERCC1 has a role at stalled forks during S phase after treatment with this agent. However, this experiment was only performed once so these conclusions must be treated with caution. These experiments were performed before the knockout cell lines were generated and it would be

interesting to repeat the experiment with gemcitabine, given the finding that MRC-5 ERCC1-/- is more sensitive to gemcitabine than it is to cytarabine and it would therefore be expected that foci formation would show a larger increase. A role of ERCC1 at the replication fork could be investigated further by DNA fibre analysis, which would allow the identification of a role in progression or restart of stalled forks.

The role that ERCC1 might play during S phase can currently only be speculated upon, but it could be related to its well characterised roles in NER and replication coupled ICL repair. These are both pathways which are active during S phase but it is unclear how they would function in relation to gemcitabine induced replication stress. It was found in *S. pombe* that mutations in *xpa*, *xpc* or *xpf* homologues caused gemcitabine sensitivity, although mutations in the *xpg* homologue did not (Gasasira, 2013). This suggested an NER-like mechanism, perhaps excising gemcitabine from the end of the nascent strand which may not require an XPG incision. In support of this hypothesis iPOND experiments found XPA, XPF and XPD binding to DNA in close proximity to replication forks (Gilljam et al., 2012). ERCC1-XPF is recruited to the NER pre-incision complex by interaction with XPA and therefore it would be interesting to test whether an MRC-5 XPA-/- mutant is sensitive to gemcitabine, which would support the idea that the role of ERCC1-XPF in gemcitabine resistance is reliant on the same interaction as its role in NER (Orelli et al., 2010). The results presented in this project suggest this is not the case as TK6 XPA-/- is not sensitive to gemcitabine and an XP-A patient fibroblast line was not rescued by complementation with *xpa* cDNA, however as the role of ERCC1 appears to be cell line dependent it is important to test the role of XPA specifically in MRC-5 cells. The next section discusses results related to the role of other NER factors in more detail.

Whether NER is capable of gemcitabine removal may also be testable by biochemical assays. Constructs containing labelled gemcitabine within duplex DNA, or within a replication fork like substrate could be incubated with NER factors to ascertain whether they can recognise and remove gemcitabine. The optimisation of a mass spectrometry method for quantifying gemcitabine incorporated into DNA was described in chapter 3. This could be used to investigate whether incorporated gemcitabine levels are altered in MRC-5 ERCC1-/- compared to the parent line. Larger amounts of incorporated gemcitabine would indicate ERCC1 is either somehow removing gemcitabine or preventing its incorporation, but would not point to a specific mechanism.

It was described in the introduction how ERCC1-XPF participates in replication coupled ICL repair by making one or both unhooking incisions at the stalled fork. It can also incise splayed

arm substrates, which resemble replication forks, in the absence of an ICL (Abdullah et al., 2017; De Laat et al., 1998b; Wyatt et al., 2017). Although it is unclear how incising the template strand would be beneficial to the cell in the context of gemcitabine treatment, it is possible that it somehow stimulates stabilisation or restart of the fork. Again, assessing whether the gemcitabine sensitivity of MRC-5 ERCC1-/- cells is dependent on XPF or XPF nuclease activity would be informative. There are residues in the nuclease domain of XPF that are specific to making ICL incisions and still allow the nuclease to cleave NER substrates (Klein Douwel et al., 2017). If a MRC-5 XPF-/- line was sensitive to gemcitabine and complementing it with an *xpf* construct carrying these mutated residues did not rescue the sensitivity, this would indicate it is making an incision at gemcitabine stalled forks similar or the same as in ICL repair. ERCC1-XPF is recruited to replication forks stalled at ICLs by SLX4. There have also been residues identified which are important for the binding of XPF to SLX4, which when mutated prevent to localisation of ERCC1-XPF to ICLs, but do not effect recruitment to NER substrates (Klein Douwel et al., 2017). As described above, complementing an XPF knockout line with this separation of function mutant would show whether the same interaction with SLX4 was important for gemcitabine resistance. Replication coupled ICL repair also requires the activation of the fanconi anaemia pathway. Many components of this pathway are implicated in replication stress independent of ICL formation, such as after hydroxyurea and aphidicolin, but it is unclear whether fanconi anaemia factors lead to the recruitment and nucleolytic activity of ERCC1-XPF in these contexts (Michl et al., 2016).

A better understanding of how ERCC1 may be mediating gemcitabine resistance and which other factors this role depends upon could have clinical applications. ERCC1 protein expression levels have shown promise as a biomarker of resistance to the crosslinking agent. cisplatin in non-small-cell lung cancer patients and to oxaliplatin in malignant melanoma patients (Bepler et al., 2011; Hatch et al., 2014; Olaussen et al., 2006). Additionally, a small molecule inhibitor targeting the interaction between ERCC1 and XPF showed a synergistic effect with cisplatin demonstrating the potential of ERCC1-XPF as a drug target (Jordheim et al., 2013). The results presented here suggest that ERCC1 expression may be worth investigating as a clinical biomarker of gemcitabine resistance.

6.5 The contribution of other NER factors to gemcitabine resistance

As discussed in the previous section ERCC1-XPF has an essential role in NER but it is also known to participate in several other repair pathways, independent of the other NER factors. It would therefore not be too surprising if its putative role in gemcitabine resistance is also independent of other NER factors, except for the observation that in *S. pombe* the homologues of *xpa* and *xpc* also contribute to gemcitabine resistance (Gasasira, 2013). It would be informative to do further work in *S. pombe* to see if the *xpa* and *xpc* homologues participate in the same pathway as the *ercc1* homologue. This could be achieved by assessing whether the sensitivity of the double mutants is greater than that of the single mutant. The current study investigated the role of XPA, XPC and XPG in human cells and these results are discussed below.

6.5.1 XPA

Several lines of evidence recommended that XPA may have a role in gemcitabine resistance. The evidence from *S. pombe* as discussed above, the observation that XPA is associated with nascent DNA close to the replication fork in human cells, and XPA is the protein responsible for recruitment of ERCC1-XPF in NER through an interaction with ERCC1 (Gilljam et al., 2012; Orelli et al., 2010). Despite this, little evidence was found in this study to support a role of XPA in gemcitabine resistance. Stable expression of GFP-XPA in the XPA deficient patient cell line XP2OS rescued UV sensitivity but had no effect on gemcitabine sensitivity. This is relatively strong evidence against XPA having a role in gemcitabine resistance in this cell line, however it is compromised by a couple of factors. The large GFP tag nearly doubles the size of the protein and whilst this does not seem to affect its role in NER, it could affect a hypothetical new role in gemcitabine resistance. The patient cell line XP2OS has a splicing mutation in the xpa gene but small amounts of abnormal xpa mRNA can still be detected. Although no XPA protein could be detected by western blot, small amounts of protein could still be present that could function in gemcitabine resistance. In the lymphoblast cell line TK6, a TK6 XPA-/- CRISPR mutant was not sensitive to gemcitabine compared to the parent line which strongly suggests XPA has no role in gemcitabine resistance in this cell line. However, it is still possible XPA has a redundant role, although with our current level of knowledge it is difficult to predict what pathway or factors it may be redundant with. It has been shown that the nuclease Mre11, provides resistance to gemcitabine and treatment with the Mre11 inhibitor mirin enhances gemcitabine sensitivity (Beardmore, 2015; Ewald et al., 2008b, E. Hartsuiker,

unpublished). However treatment with mirin sensitised TK6 XPA-/- cells and the parent line to the same extent making redundancy with an Mre11 mediated pathway of gemcitabine resistance unlikely.

It is unfortunate that this work was carried out prior to learning that MRC-5 ERCC1-/- was sensitive to gemcitabine and that the role of ERCC1 appears to be cell line dependent. This makes it quite possible that a potential role of XPA (and other candidate repair factors) is also cell line dependent, which could be an explanation for the failure to detect a role for XPA in the experiments described above. Given that ERCC1 provides gemcitabine resistance in MRC-5, and XPA could be functioning in the same pathway, it would be interesting in future to test the role of XPA in this cell line. It was shown in chapter 1 that XPA forms nuclear foci in MRC-5 cells, some of which colocalise with EdU labelled nascent DNA, which supports the idea that it may play a role at replication forks in this cell line, although the occurrence of foci increased only very slightly after gemcitabine treatment.

6.5.2 XPC and XPG

The contributions of XPC and XPG to gemcitabine resistance or replication stress in general have not been the subject of many investigations. The UV sensitivity of XPC (XP4PA) and XPG (XPCS1RO) deficient patient cells was rescued by stable expression of XPC-GFP and XPG constructs respectively and expression of these constructs also had a small effect on gemcitabine resistance in both cases. XPC is responsible for lesion detection in GG-NER (Volker et al., 2001). A small rescue of gemcitabine sensitivity was elicited by expression of XPC-GFP in the XPC deficient cell line. Once again, the extent of the rescue could be limited by the GFP tag interfering with protein function or the endogenous protein retaining some function, as it is reported to do in XP4PA (Li et al., 1993). Taken together, the small rescue observed here and the role of the *xpc* homologue in *S. pombe* means the role of XPC in gemcitabine resistance in human cells merits further investigation.

XPG is the 5’ flap endonuclease that makes the incision 3’ of the lesion in the NER reaction and also has an important function in transcription independent of its nuclease activity (Schärer, 2008). Counter to the results for XPC, expression of XPG in the XPG deficient cell line appeared to slightly aggravate the gemcitabine sensitivity of the cells. Sensitisation upon XPG expression suggests XPG makes a cytotoxic repair intermediate in response to gemcitabine treatment, likely through its nuclease activity. This result suggests XPG is not operating in the same pathway as XPC and ERCC1, which is similar to the situation in *S. pombe*. However, it

is difficult to draw general conclusions and suggest a model for the roles of different factors when the data for each factor is drawn from a different cell line. Especially when there is evidence to suggest that cell lines differ in the DNA repair factors that they use to combat gemcitabine damage. It would therefore be more informative to generate knockouts for all the NER factors of interest in the MRC-5 cell line.

6.6 Conclusion

This project set out to understand the contribution of NER factors to gemcitabine resistance in human cells. Results presented in this thesis provide evidence for a previously uncharacterised role for ERCC1 in gemcitabine resistance in the immortalised human fibroblast line MRC-5. Conversely, ERCC1 is shown not to fulfil the same role in a second immortalised human cell line, HEK293. This suggests the role of ERCC1 is cell line dependent and that the DNA repair response to gemcitabine differs between cell lines. A second nucleotide excision repair factor, XPA, was found not to have a role in gemcitabine resistance in the human lymphoblast cell line TK6 and could not rescue gemcitabine sensitivity in an XPA deficient fibroblast line. Data collected from the rescue of NER deficient cell lines suggested XPC has a small role in gemcitabine resistance and XPG has a small sensitising effect. These findings suggest that some NER factors do have a role in gemcitabine resistance in some human cells, and that may be clinically relevant. The results suggest two avenues of inquiry for further work. Firstly, gaining a deeper understanding of the role of ERCC1 in gemcitabine resistance in MRC-5 cells and understanding whether this role is related to the known functions of ERCC1 in NER or ICL repair. Secondly, screening different cell lines to see how widespread this role of ERCC1 is and attempting to understand the basis of differences between cell lines.

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