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The Role of Nucleotide Excision Repair Factors and the Mre11 Nuclease in Nucleoside Analogue Sensitivity

Thesis by Richard Beardmore

Supervisors: Dr Oliver Fleck and Dr Edgar Hartsuiker

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Abbreviations

- 4NQO – 4-nitroquinoline-1-oxide
- APE1 - apurinic/apyrimidinic endonuclease 1
- ARA-C – cytarabine (Cytosine β-D-arabinofuranoside)
- AT – ataxia telangiectasia
- ATLD – ataxia telangiectasia like disease
- ATM – ataxia teleangiectasia mutated
- ATR – ATM and Rad3 related
- BER – base excision repair
- BrdU – bromodeoxyuridine
- CFA – colony forming assay
- CHK1 – checkpoint kinase 1
- CHK2 – checkpoint kinase 2
- COFS – cerebrooculofacioskeletal syndrome
- CPDs – cyclobutane pyrimidine dimers
- CPT - camptothecin
- CS – Cockayne syndrome
- dFdC - gemcitabine
- DSB – double strand breaks
- esiRNA – endoribonuclease prepared siRNA
- FA – fanconi anemia
- GG-NER – global genome nucleotide excision repair
- HR – homologous recombination
- HU – hydroxyurea
- IDLs – insertion/deletion loops
- LC-MS/MS – liquid chromatography tandem mass spectrometry
- MMC – mitomycin C
- MMR – mis-match repair

- MTS – (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
- NA – nucleoside analogues
- NER – nucleotide excision repair
- NHEJ – non homologous end joining
- NBS – Nijmegen breakage syndrome
- PARP – poly ADP ribose polymerase
- RNR – ribonucleotide reductase
- SCAN1 – spinocerebellar ataxia with axonal neuropathy 1
- shRNA – short hairpin RNA
- siRNA – small interfering RNA
- ssDNA - single strand DNA
- SS – Seckel syndrome
- TC-NER – transcription coupled nucleotide excision repair
- TDP1 – tyrosyl DNA phosphodiesterase 1
- TOP1 – topoisomerase 1
- TTD - trichothiodystrophy
- UDS – unscheduled DNA synthesis
- XP – xeroderma pigmentosum

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Summary

The Nucleoside Analogue (NA) gemcitabine (2', 2'-difluorodeoxycytidine; dFdC) is a clinically important cancer drug that is used in the treatment of solid tumours including pancreatic, metastatic breast, ovarian and non-small cell lung cancer (Bergman et al., 2002). Gemcitabine is similar to deoxycytidine (the naturally occurring nucleotide), in that after phosphorylation to its triphosphate form, dFdCTP competes with dCTP for integration into DNA during DNA replication and acts as a chain terminator, inhibiting further DNA synthesis (Prakasha Gowda et al., 2010). For a cell to survive treatment with this drug, the modified nucleotide needs to be removed from the DNA to allow replication restart. It remains largely unknown which DNA repair pathways are responsible for the removal of NAs from DNA. Based on previous data from the Hartsuiker lab using the model organism *Schizosaccharomyces pombe* (*S. pombe)*, it was hypothesised that the Nucleotide Excision Repair (NER) pathway may be one mechanism that is used for gemcitabine removal from the genome.

NER is responsible for removing bulky adducts from the DNA strand, particularly those caused by UV radiation, such as 6-4 photoproducts, or cyclopyrimidine dimers (Vermeulen, 2011). NER is an orchestrated process which is carried out by no less than 30 proteins, with a core set of 7 proteins, termed XPA to G, that are responsible for the main mechanism. The whole NER mechanism can be broken down into the steps of recognition of damage, DNA unwinding, confirmation of damage, excision of the lesion, followed by repair and ligation of the strand. NER can be further categorised into global NER (GG-NER) where damage is recognised by surveillance of the whole genome, or transcription coupled NER (TC-NER) where the damage is detected by the stalling of RNA polymerase, in transcription (Kamileri et al., 2012).

Genetic deficiency in NER causes the condition termed Xeroderma Pigmentosum (XP). XP patients have extreme sensitivity to UV light and have a 1000 fold increased incidence of skin cancers if exposed to sunlight (Ahmad and Hanaoka, 2008; Rezvani et al., 2010). Work here uses fibroblasts from XP patients to investigate the mechanisms within NER, in both structural and enzymatic requirements of each NER protein, to determine their role in the

removal of nucleoside analogues from the DNA. The resistance of some cancers to chemotherapeutic attack is often a result of the work done by such DNA repair pathways, therefore understanding the repair pathway that offers resistance to various drugs is essential for optimising treatment for individual cancers.

Results presented in this thesis show that NER factors are required for cell survival following nucleoside analogue treatment, particularly XPA, XPC and XPG. Phenotypes shown following nucleoside analogue treatments in various NER deficient cell lines from different XP complementation groups differ from the classical reported sensitivity to UV and the UV mimetic drug 4NQO as measured by colony formation and unscheduled DNA synthesis (UDS). Results reported here further suggest that NER deficient XP cell lines are sensitive to both ATR and CHK1 kinase inhibition, and when combined with nucleoside analogues, this inhibition increases cytotoxicity compared to wild type controls.

Furthermore, the requirement of the Mre11 nuclease in the resistance to nucleoside analogues, as well as the topoisomerase I poison, camptothecin was also studied. Topoisomerases that are responsible for resolving topological stress within the genome during replication and transcription are targets for chemotherapeutic attack in cancers by camptothecin and etoposide. Work here builds on published data from *S .pombe* which showed that the nuclease activity of Mre11 is required in camptothecin resistance in higher eukaryotes (Hartsuiker et al., 2009).

Overall this work indicates a potential role of NER factors in repair and stabilisation of nucleoside analogue induced stalled replication forks, that has a dependence on the ATR-CHK1-Cdc25A S-phase checkpoint cascade. Ultimately this may reveal several potential targets for increasing gemcitabine and nucleoside analogue efficacy in cancers which carry NER factor mutations.

Chapter 1 – Introduction

Introduction

The integrity of the genome within a cell is vital for its survival. DNA is constantly damaged due to a variety of assaults from endogenously occurring oxidative damage, exogenous damaging compounds or by errors during DNA replication and transcription. Without constant repair from the cell, mutations can arise in key genes that ultimately lead to cancer or cell death. Cancer can evolve from cells that lose functionality of important tumour suppressor genes that either prevent cells from inappropriately dividing, or prevent cells from dying when damaged. Moreover, gain of function mutations can occur in genes that have a pro-active role in cell division. If the delicate balance between resting and replication signals is altered, mutations in more and more essential genes can occur, eventually lapsing into cancer. This forms the basis of the gate keeper model of cancer initiation (Hanahan and Weinberg, 2011; Hanahan et al., 2000). Several distinct pathways have evolved in order to protect the genome from a variety of damage types. These DNA repair pathways seek to maintain the integrity of genetic information within the cell and prevent mutations occurring. The major DNA repair subtypes are discussed below.

Pathways of DNA Repair and Genome Stability

Base Excision Repair (BER)

Base excision repair (BER) is the main repair mechanism for single base damage caused by reactive oxygen species which cause single-strand nicks, as well as base modifications such as deaminations (Madhusudan et al., 2005). Superoxide radical ions occur endogenously from cellular metabolism and are able to oxidise DNA bases resulting in lesions such as 8-oxoguanine. BER is also responsible for removing uracil bases from genomic DNA as well as a varied array of methylated base substrates (Kassam and Rainbow, 2007). BER is initiated by one of a family of DNA glycosylases which recognise their respective damage substrate and cleave the N-glycosidic bond between the deoxyribose backbone and the damaged base (Fig.1.1). The removal of the damaged DNA base creates an apurinic or apyrmidinic site (AP

site). The DNA backbone is then cleaved 5' to the AP site by a DNA AP endonuclease enzyme or AP lyase activity of bifunctional DNA glycosylases. DNA AP endonuclease family enzymes create a 3'-OH and a 5'-phosphate at the site of the newly created single-strand break which allows for the re-synthesis of the DNA strand by polymerase β and re-ligation by ligase III/XRCC1 complex (Christmann et al., 2003) (Fig.1.1). An additional sub-pathway of BER occurs when larger sections of the damaged DNA strand are removed, termed long-patch BER. Long patch BER is initiated when the enzyme APEX1 (DNA-apurinic or apyrimidinic site lyase 1) produces a 5'- DNA single strand nick at the abasic site which leads to the recruitment of polymerase β, polymerase δ and PCNA (Christmann et al., 2003; Robertson et al., 2009). These polymerases begin re-synthesising DNA in the 3' direction which leads to the formation of a DNA flap substrate which can be cleaved by FEN1 (flap structure specfic endnonuclease 1) (Hohl et al., 2003; Klungland et al., 1999; Madhusudan et al., 2005) (Fig.1.1).

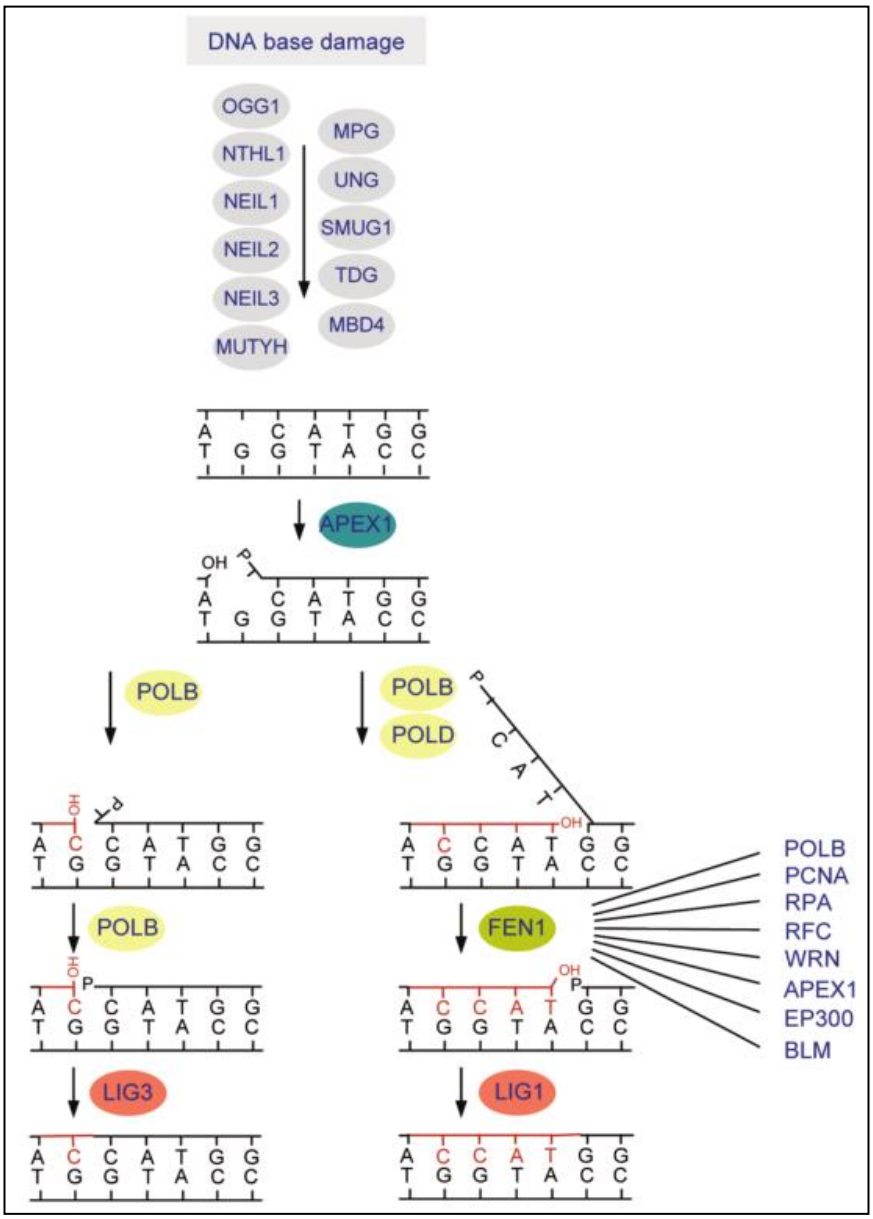

Figure 1.1: Base Excision Repair (BER) Molecular Mechanism. Damaged DNA base removed by various glycosylase enzymes, before backbone cleavage and single base replacement in short patch BER (left) or re-synthesis of several DNA bases in long patch BER (right). Some DNA glycosylases have AP lyase activity and are classed as bifunctional. (Robertson et al., 2009).

DNA Double Strand Break Repair

DNA double Strand-Breaks (DSBs) are the most disastrous type of DNA damage. DSBs can occur as a result of short wavelength radiation such as X-rays, or collapsed replication intermediates where single stranded DNA (ssDNA) branch structures are converted into DSBs. Accurate repair of DSBs is essential to maintain the function of genes, and prevent the loss of genetic information, which occurs via two main pathways (Fig.1.2). Most DSBs in mammalian cells are repaired by non-homologous end joining (NHEJ). This DNA repair pathway involves the recognition of a DSB by the heterodimer KU70/80 which recruits the signalling PI3-kinase, DNA-PKcs to from a complex known as DNA-PK. This heterotrimer tethers the two broken strands together followed by the gap sealing by XRCC4/Ligase IV dimer (Fig.1.2) (Burma et al., 2006; Christmann et al., 2003; Iliakis, 2009; Sanchez et al., 2014). The DNA-PKcs subunit of DNA-PK is responsible for the damage signalling role of the complex which phosphorylates several other substrate proteins (Chapter 5) that ultimately control the cell cycle arrest that allows time for efficient DNA repair to occur (Pommier, 2003). NHEJ is a highly mutational type of DNA repair because single base pairs are often removed from DSB ends during processing, which can result in frameshift mutations and gene deletions. NHEJ is predominantly used in G1 phase of the cell cycle.

DSBs can also be repaired by homologous recombination (HR) which occurs when cells are in G2 and S-phase where condensed chromosomes are present. HR double-strand break repair uses sister chromatids as templates to repair DSBs. HR is initiated by the Mre11-Rad50-NBS1 (MRN) complex (discussed in Chapter 7), which binds to ends of DSBs, followed by long single strand resection by EXO1 of the broken DNA end. The MRN complex tethers the ends of DSBs via the DNA binding subunit Rad50, before nucleolytic end processing by the Mre11 nuclease which has both exo and endonuclease activity (Kanaar and Wyman, 2008). The third subunit of the MRN complex termed NBS1, is responsible for the recruitment of the MRN complex to sites of DSBS in the repair pathway via phosphorylation by the ATM kinase (Furuta et al., 2003).

Immediately following MRN binding to ends of double strand breaks, the Mre11 subunit initiates single-stranded end resection with the aid of CTiP which produces single-stranded

DNA overhangs on both sides of the DSB. This single-stranded DNA is then further resected by the 5'-3' exonuclease termed EXO1 (Langerak et al., 2011). The long single-stranded DNA created by the action of EXO1 following MRN end processing is then coated in RPA, before being replaced by Rad51 subunits. Rad51 filament formation is the major event of the pre-synapsis stage of HR (Krejci et al., 2012). The Synapsis phase of HR then occurs as the Rad51 filament invades the neighbouring chromatid to find the homologous DNA sequence, which produces the heteroduplex DNA structure or D-loop. Rad51 mediated D-loop formation allows for the invading 3' DNA strand to be used as a primer for DNA re-synthesis which in turn removes the Rad51 subunits (Jasin and Rothstein, 2013; San Filippo et al., 2008). The two double strands of DNA linked together following D-loop formation form a complex quaternary structure known as a Holliday Junction (Fig.1.2) (Wilson and Thompson, 2007). Holliday junctions formed by HR can then be dissolved by a complex of RecQ helicases such as WRN and BLM (Brabant et al., 2000; Jasin and Rothstein, 2013). Following dissolution of the Holliday junction, both DNA double strands are in-tact and replication continues. HR is much less mutagenic than NHEJ because it uses specific sequence homology from the sister chromatid to repair the break (Christmann et al., 2003; Takata et al., 2009; Truong et al., 2013).

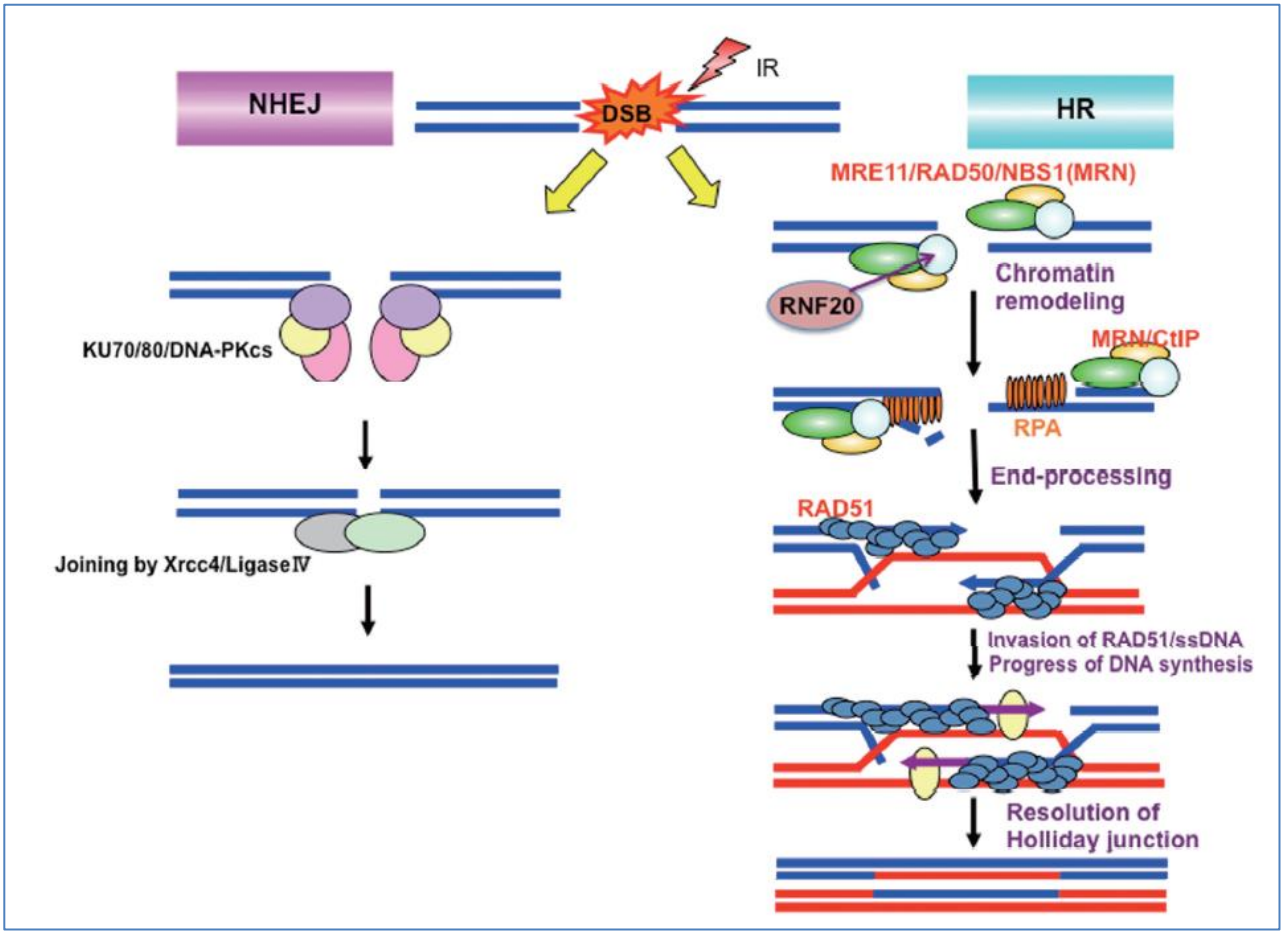

Figure 1.2: Double Strand Break (DSB) Repair. Double strand breaks are primarily repaired by NHEJ performed by Ku70/80 in higher eukaryotes, whereas HR occurs when homologous chromatids are available in G2 phase (Saito et al., 2013).

The sites of DNA DSBs are marked by the phosphorylation of the H2AX histone subunit at position Ser139 which is termed yH2AX (Chapter 4). This phosphorylation occurs in the chromatin immediately surrounding the DSB and is thought to act as a marker for DNA damage which attracts the recruitment of DNA repair factors (Podhorecka et al., 2011). The phosphorylation of yH2AX in response to DSBs is thought to occurs via the action of the ATM kinase (Ewald et al., 2007).

Mismatch Repair (MMR)

Mismatch repair (MMR) is the main mechanism that repairs mis-paired bases in newly synthesised DNA, as well as small loop structures produced by slippage of the two DNA strands, termed insertion/deletion loops (IDLs) (Jiricny, 2013) (Fig.1.3). In higher eukaryotes, mis-paired bases are recognised by the MutS heterodimer, which is made up of either MSH2/MSH6, or MSH2/MSH3, which then recruits the heterodimer MutL which can be made up of one MLH1 protein bound to either PMS1, PMS2 or MLH3 (Hays et al., 2005; Stojic et al., 2004). Where a base mismatch occurs in the lagging strand, EXO1 removes the Okazaki fragment from free 5' end (Zhao et al., 2009) However if a DNA base mismatch occurs in the leading strand, the endonuclease activity of the MutLα is required to create a single-strand nick, which provides EXO1 with a free 5' end that allows from long 5'-3' resection before re-synthesis by polymerase δ and re-ligation by ligase I (Iyama and Wilson, 2013; Zhao et al., 2009). IDLs occur frequently at sites in the genome where sequence tandem repeats are found termed microsatellites. Sequence tandem repeats are prone to strand slippage during replication which results in IDL formation.

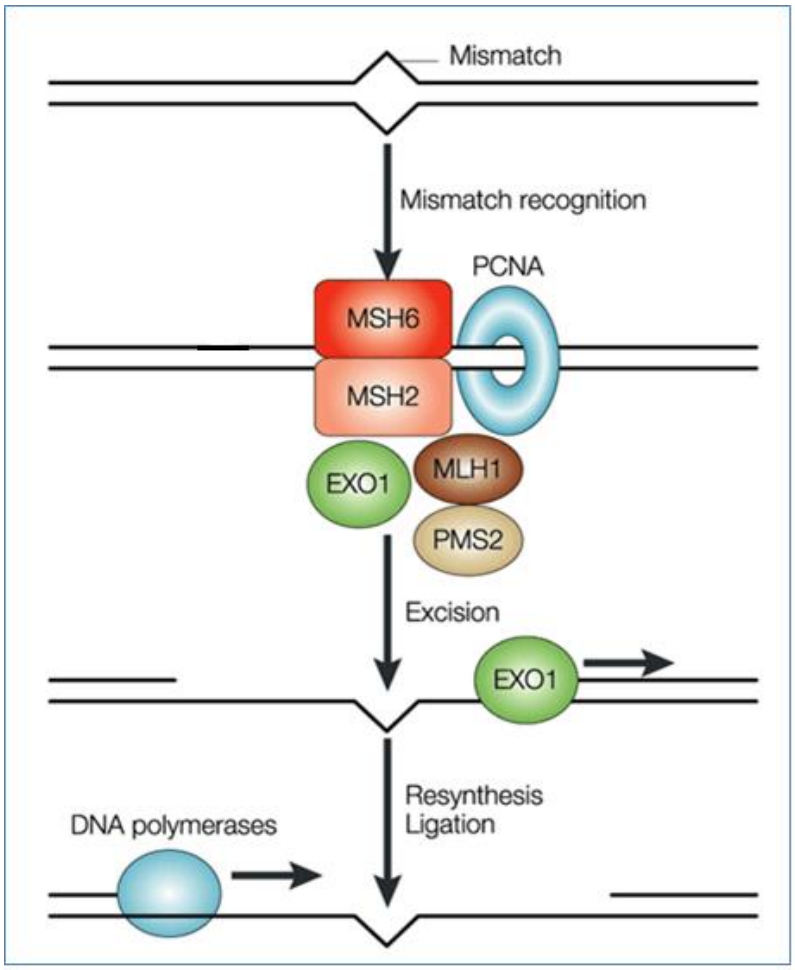

Figure 1.3: DNA Mismatch Repair Mechanism. MSH6/MSH2 dimer binds to base mismatches and brings about the recruitment of MutLα and EXO1 to remove a large section of newly synthesised DNA before resynthesis and religation by DNA polymerase (Martin and Scharff, 2002).

Cell Cycle Checkpoints and Apoptosis

To prevent the passage of DNA damage from the affected cell to daughter cells, several cell cycle checkpoints have evolved. These checkpoints halt the replication process at key points or halt the transition to the next stage of cell division, to prevent further accumulation of DNA damage which allows time for DNA repair processes to act. Persistent cell cycle checkpoint activation can bring about permanent cellular senescence or programmed cell death (Houtgraaf et al., 2006). Firstly, in humans entry into the cell cycle from G0 to G1 phase is controlled by hyper-phosphorylation of the pRb protein, which controls the release of E2F family transcription factors that initiate G1 and cell division (Ali and DeCaprio, 2001). Following commitment to cell division, the cell cycle can be halted or slowed by activation of several parallel pathways at the G1/S transition, intra-S-phase or at the G2/M transition (Kastan and Bartek, 2004). The activities of most cell cycle checkpoint proteins can be separated into DNA damage sensors, signal transducers, and effectors (Fig.1.4).

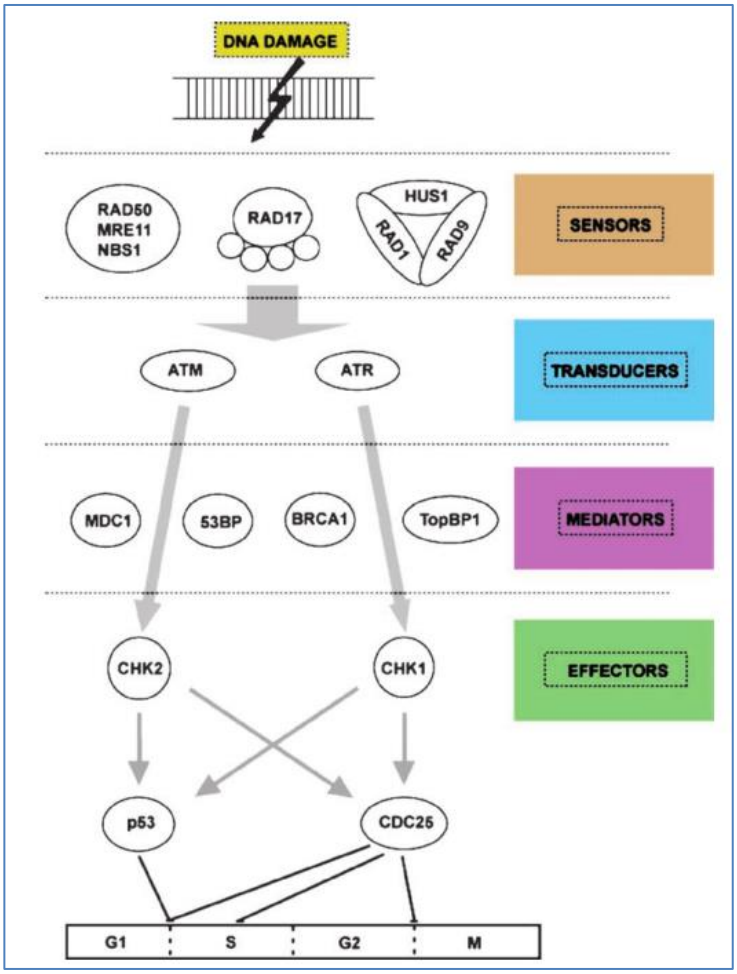

Figure 1.4: Cell Cycle Checkpoint Pathways. DNA damage sensor proteins promote ATM or ATR phosphorylation that activate either CHK2 or CHK1 kinases, which ultimately control checkpoint activation and cell cycle arrest via several mediator proteins such as TopBP1, 53BP and BRCA1 (Houtgraaf et al., 2006).

DNA DSBs are primarily detected by the MRN complex (Chapter 7), whereas single-stranded DNA sections or DNA nicks are detected by RAD17 or the 9-1-1 complex (Fig.1.4) in association with the ssDNA binding protein RPA (Vermeulen et al., 2003). Following the detection of DNA damage, the signal transducing phosphatidylinostilol 3-kinase like (PIKK) family proteins ATM or ATR are activated. ATM is predominately activated by MRN in response to double-strand breaks, whereas ATR is activated by single-stranded DNA sections or stalled replication forks (Trenz et al., 2006). ATM and ATR kinases phosphorylate a vast number of protein targets, many of which are directly involved in DNA repair, or the recruitment of relevant machinery that stabilises damaged DNA such as BRCA1 and Rad51. However, ATR and ATM mainly activate the kinases CHK1 and CHK2, respectively, in order to activate cell cycle arrest (Chapter 4) (Goto et al., 2015; Thompson, 2012). CHK1 and CHK2 kinases transfer the DNA damage signal to the Cdc25 phosphatase family proteins via phosphorylation, which are responsible for dephosphorylate cyclin dependent kinases (CDKs) that prevents entry into the following cell cycle phase (Goto et al., 2015; Houtgraaf et al., 2006; Robinson et al., 2006). The phosphorylation of Cdc25 family proteins by CHK1 and CHK2 prevents their proteosomal degradation leading to their accumulation which aids in the prevention of CDK mediated origin firing (Parsels et al., 2011a) CHK1 and CHK2 kinases also phosphorylate p53, which prevents its proteosomal degradation (Muller and Vousden, 2013). Activated p53 prevents entry into S-phase and further activates the transcription of many proteins involved in DNA repair, as well as pro-apoptotic proteins such as Bax (Tomicic and Kaina, 2013). When DNA damage signalling persists, p53 signalling brings about the activation of apoptosis via the permeabilisation of the mitochondrial membrane which releases caspase proteins that assemble and begin the process of programmed cell death (Houtgraaf et al., 2006; Muller and Vousden, 2013; Robertson and Plunkett, 1995).

Nucleotide Excision Repair (NER)

As the main focus of this thesis, the pathway of nucleotide excision repair is discussed in greater detail below.

Nucleotide excision repair (NER) is the main mechanism by which UV-induced DNA damage is repaired. UV radiation causes the formation of 6-4 photoproducts and cyclobutane prymidine dimers (CPDs), which occur when DNA bases absorb energy and form covalent bonds that prevent DNA transcription (Staresincic et al., 2009). UV induced lesions that cause helical distortions in the DNA are recognised by one of two sub-pathways of NER (Fig.1.5). Global genomic nucleotide excision repair (GG-NER) repairs the majority of damage in low transcription areas of the genome. The DNA damage recognition factor XPC, along with its binding partner HR23B, bind opposite helical distortions within the DNA duplex in GG-NER and initiate the recruitment of NER factors. In higher eukaryotes, two other factors involved in GG-NER known as DDB1 and DDB2 have been shown to increase the affinity of XPC-HR23B to several damage substrates, as well as recognise substrates on their own (Wakasugi et al., 2007; Yeh et al., 2012) (Fig.1.5).

In areas of high transcription, many helical distortions are recognised by the second NER subtype termed transcription coupled NER or TC-NER. Here, recognition by RNA Pol II occurs when the enzyme encounters DNA damage. This causes the unloading and activation of two proteins termed CSA and CSB, which bring about the recruitment of the NER machinery (van Cuijk et al., 2014; Fousteri and Mullenders, 2008). It has been shown that cyclobutane pyrimidine dimers (CPDs) only cause minor helical distortions that are not well recognised by GG-NER leading to them predominately being repaired during transcription. Conversely 6-4 photoproducts cause serious kinks in DNA which are easily recognised by XPC leading to them preferentially being repaired in GG-NER (de Lima-Bessa et al., 2008).

The TFIIH complex is recruited after DNA damage recognition, either by GG-NER or TC-NER. The TFIIH complex consists of 10 subunits and contains two helicases with opposing polarity. The XPD helicase subunit unwinds the DNA in the 5’-3’ direction, whereas the XPB subunit unwinds 3’-5’ direction (Rudolf et al., 2009). This action of the TFIIH complex opens the damaged DNA section allowing the damage verification protein XPA and single strand

binding protein RPA to enter and bind the damaged DNA section (Saijo et al., 1996). XPA has been shown to be involved in the confirmation of DNA damage, as well as the positioning of the 5' endonuclease heterodimer XPF-ERCC1 (Saijo et al., 1996; Su et al., 2012). The conformational change in XPF-ERCC1 brought about by its interaction with XPA allows for the 5' incision of the damaged DNA section. The recruitment of the XPG 3' endonuclease is essential for the stabilisation of the NER pre-incision complex, especially the stability of the TFIIH complex. The binding of the XPG endonuclease is required for dual incision both 3' and 5' of the DNA damage (Hohl et al., 2007; Staresincic et al., 2009; Tsodikov et al., 2007; Zotter et al., 2006). The 25-31 base section of damaged DNA is then removed and the gap is refilled by DNA polymerase δ, κ or ε in the presence of PCNA and RPA, followed by the re-ligation by DNA ligase I (Boer and Hoeijmakers, 2000).

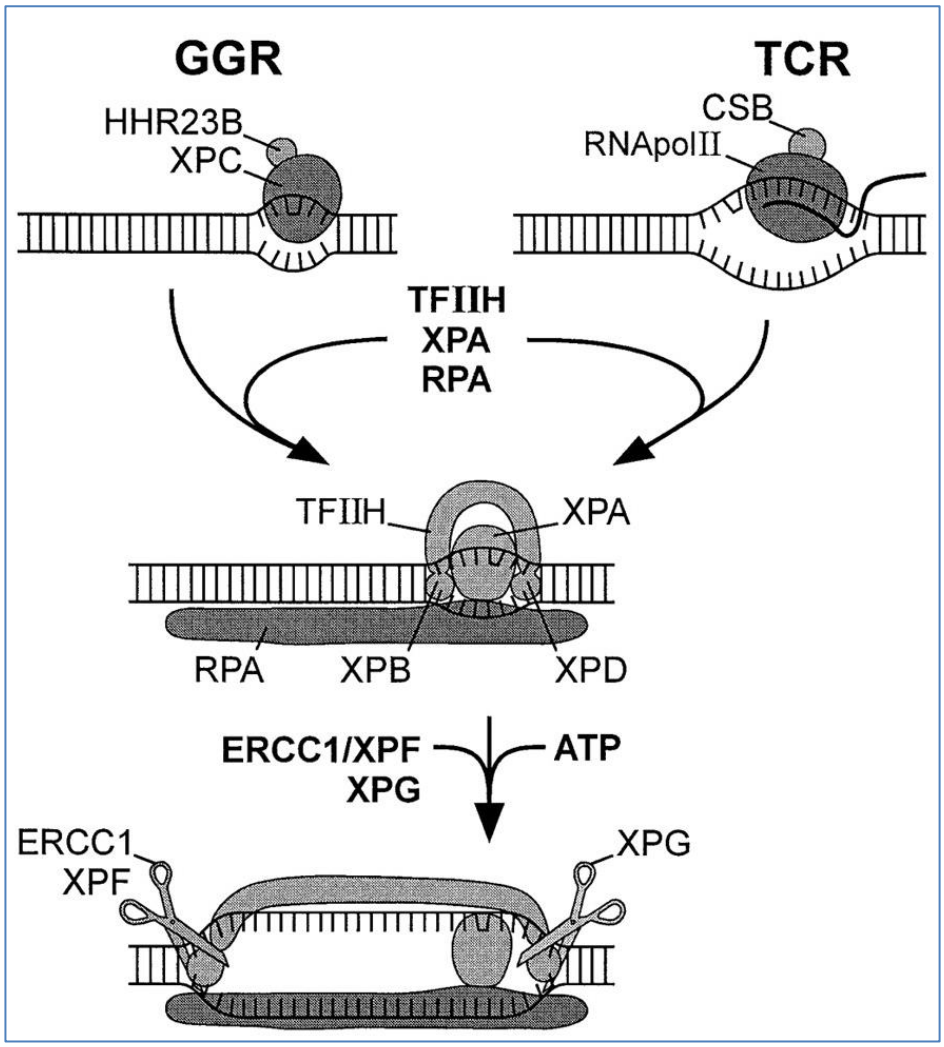

Figure 1.5: Molecular Schematic of NER. DNA damage recognition by either GG-NER or TC-NER, followed by the recruitment of the TFIIH complex and the XPA protein. Once the XPD and XPB helicases have unwound the DNA structure, XPF and XPG incision occurs (Boer and Hoeijmakers, 2000 modified).

XPA

The *XPA* gene located on chromosome 9 (9q34.1) encodes for the 273 amino acid XPA protein (Mcdoweu et al., 1993). The 31KDa XPA protein has a zinc finger DNA binding domain and has been shown to be involved in the confirmation of damage following helicase unwinding at sites of NER repair (Maria Berra et al., 2013). The DNA binding affinity of XPA is dramatically increased by its interaction with RPA, at sites of DNA damage (Li et al., 1995). Once bound at the centre of the NER pre-incision complex, XPA interacts with ERCC1 to correctly position the 5'endonuclease XPF in order to make the incision and in the absence of XPA, the ERCC1-XPF hetero-dimer is not recruited (Saijo et al., 1996; Tsodikov et al., 2007).

Due to its role in the early stages of both GG-NER and TC-NER, XPA has been extensively studied in UV related DNA repair. Studies have shown that normal human fibroblasts contain approximately 150,000-200,000 copies of the XPA protein, and that cells expressing only 10% of this level are equally resistant to UV as wild type cells, indicating that only small amounts of XPA are required for successful NER (Muotri et al., 2002; Roginskaya and Wood, 2005). The total lack of XPA protein produced by gene deletion in mice models results in cancerous tumour formation in response to both UV irradiation and the oral delivery of the UV mimetic drug 4NQO (Ide et al., 2001).

XPA has recently been reported to have roles in cell cycle checkpoint activation, as well as novel interactions with the replication machinery. For example, XPA has been shown to interact with the ATR-activating protein ATRIP, and in the absence of XPA, ATR activation and CHK1 phosphorylation is decreased following UV irradiation (Bomgarden et al., 2006; Li et al., 2011; Wu et al., 2007). XPA therefore has a role in DNA damage signalling following UV irradiation which is separate from its role the classical NER pathway. In addition, XPA has been shown to interact with PCNA via its APIM domain (AlkB homolog 2 PCNA interacting domain) (Gilljam et al., 2012). This XPA-PCNA interaction results in co-localisation at replication foci which is also the case for XPC, XPD, XPF, and XPG, therefore potentially indicating replicative roles for NER factors. Similarly, XPA has been shown to interact with poly(ADP-ribose) polymerase-1 (PARP-1), and inhibition of PARP-1 or siRNA knockdown, results in delayed repair of UV related DNA lesions (King et al., 2012).

XPB

The XPB protein is a 3'-5' helicase that forms one of the subunits of the larger TFIIH complex. The *XPB* helicase gene is located at chromosome 2q21 and encodes for the 782 amino acid protein, which is also known as ERCC3 (Fuss and Tainer, 2011). Following the recognition of DNA damage by XPC, or by RNA Pol II in TC-NER, the TFIIH complex is then recruited to the site where XPB unwinds the DNA in the 3'-5' direction. The precise mechanism that regulates the role of the TFIIH complex between NER and transcription has not been reported, however the phosphorylation of XPB at Serine 751 has been shown to inhibit XPB helicase activity in NER, but this phosphorylation switch does not affect the transcriptional activity of the TFIIH complex (Coin et al., 2004).

XPC

The XPC protein forms the core of the DNA damage recognition step in low expression areas of the genome which initiates the NER sub-pathway termed global genome nucleotide excision repair (GG-NER). Together with its binding partner HR23B, XPC binds major helical distortions in the DNA structure leading to the recruitment of the TFIIH complex, which opens the DNA helix (Sugasawa, 2008a). HR23B and the additional protein Centrin 2 bind XPC to form a heterotrimer that dramatically increases its affinity to various DNA substrates. Following DNA damage, XPC has been shown to bind on the opposite strand to the lesion which allows for the vast number of different damage substrates to which XPC can bind (Melis, 2012; Sugasawa, 2008a). The binding of XPC induces a 45 degree kink in the DNA which facilitates the recruitment of the TFIIH complex which interacts with XPC via its XPB and p62 subunits (Yokoi and Sugasawa, 2000; Zhu et al., 2012). The *XPC* gene is located on chromosome 3p25.1 has been shown to have a p53 response element within the promotor sequence which powerfully increases *XPC* expression following DNA damage by UV and ionising radiation (Adimoolam and Ford, 2002). Outside of GG-NER, XPC has been shown to interact with the BER proteins thymine DNA glycosylase (TDG) and hOGG1, which remove incorporated uracil bases or mis-paired T/G bases and oxidised guanine bases (8-oxogaunine) respectively (Chavanne et al., 2000; Sugasawa, 2008a). This interaction has been shown in co-localisation experiments where oxidative DNA damage was repaired far slower in the absence of XPC (Menoni et al., 2012). Additionally, XPC knockdown has been

shown to reduce NHEJ double-strand break repair efficiency following etoposide treatment (Zhang et al., 2009).

XPD

The ATP-dependent 5'-3' DNA helicase XPD forms one of the components of the TFIIH complex. This helicase with opposite polarity to XPB is responsible for unwinding DNA in the 5'-3' direction following TFIIH recruitment by XPC (Johnson and Squires, 1992). The *XPD* gene, also known as ERCC2, is located on chromosome 19q.13.2. The XPD protein has been shown to have very low helicase activity *in vitro* but within the TFIIH complex, when interacting with the p44 subunit, its helicase activity is 10x higher (Lehmann, 2001). XPD and XPB have both been shown to physically interact with p53, which helps to control apoptosis following DNA damage (Robles et al., 1999; Wang et al., 1996). Similarly, XPD has been shown to control the switching between phosphorylation events on histone H2A (serine 139, tyrosine 142) that controls the decision between DNA repair and apoptosis following DNA damage (Kaushik Tiwari and Rogers, 2013).

XPE

UV-DDB (UV-damaged DNA-binding protein) is a heterodimer of DDB1 and DDB2 (Kazantsev et al., 1998). The UV-DDB complex has been shown to have high affinity to UV damaged DNA and is thought to aid in the initiation of GG-NER by facilitating XPC binding (Sugasawa, 2008a). The DDB2 subunit possess the DNA binding ability of the complex, whereas DDB1 forms complexes with many other proteins leading to a great many different functions (Sugasawa, 2009). It has been shown that the UV-DDB complex has extremely high binding affinity to 6-4 photoproducts, as well as CPDs which are not well recognised by XPC (Sugasawa, 2008a; Sugasawa et al., 1998). The ability of UV-DDB to bind CPDs increases the repair capacity of GG-NER following UV damage by enabling XPC to recognise additional damage lesions that it has low affinity for alone. the UV-DDB interacts with the ubiquitin ligase complex consisting of the subunits cullin 4A and Roc1, which polyubiquitinates DDB2 and XPC which alters their affinity to damaged DNA as well as mediates the switching from DNA damage detected by UV-DDB, to XPC which allows for the initiation of the NER process (Rapić-Otrin et al., 2002; Ray et al., 2013; Sugasawa et al., 2005) (Fig.1.6)

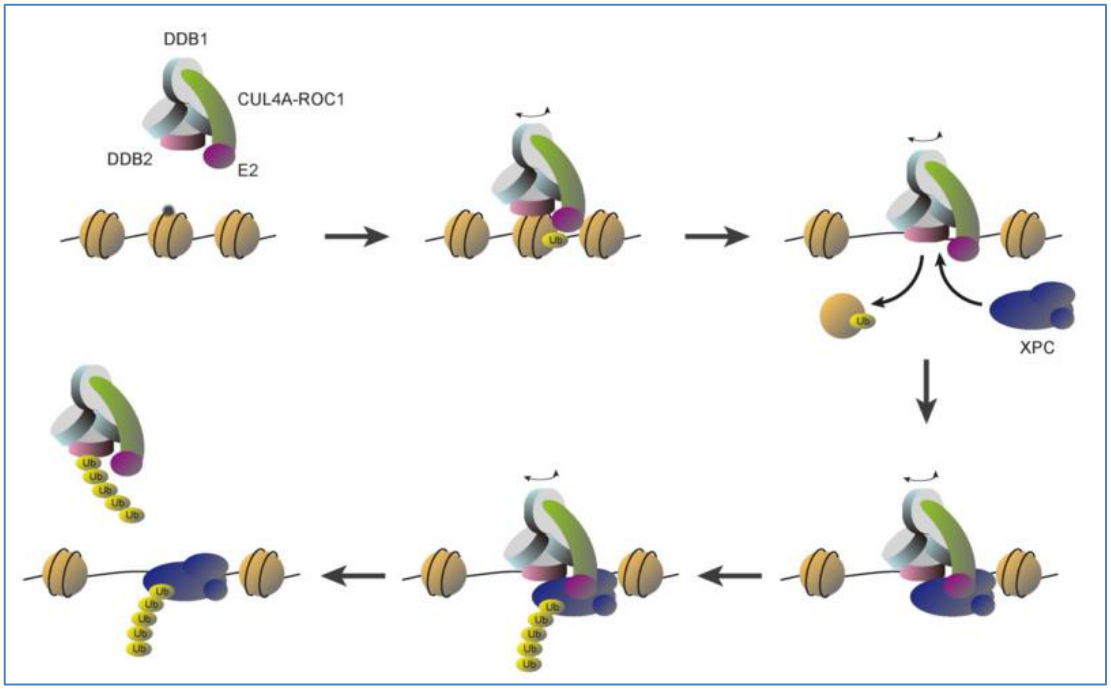

Figure 1.6: Model of UV-DDB/XPC Interaction. UV-DDB in association with Cullin 4A binds to UV damaged DNA and ubquitinates the histone within the nucleosome leading to its removal. Histone removal allows XPC recognition to occur. Cullin 4A in complex with UV-DDB also ubiquitinates XPC increasing its affinity to the lesion, which allows for the initiation of GG-NER (Sugasawa, 2009).

XPF/ERCC1

The XPF-ERCC1 heterodimer is responsible for the 5' incision which is among the last steps of the NER mechanism. The extremely tight interaction between XPF and ERCC1 has been shown to be essential for its nuclear localisation (Ahmad et al., 2010). The XPF-ERCC1 heterodimer also interacts with XPA which aids in its correct positioning before 5' incision takes place (Tsodikov et al., 2007). Once the 5' incision by XPF takes place, a conformational change in the NER complex occurs which allows for the 3' incision to take place (Staresincic et al., 2009; Tsodikov et al., 2007). The XPF-ERCC1 complex has non-NER roles in inter-strand crosslink repair which has been shown to remove DNA damage caused by mitomycin C and cisplatin (Gregg et al., 2011).

XPG

The *XPG* endonuclease gene is located on chromosome 13q32 and is responsible for the 3' incision within the NER pathway (Clarkson, 2003). The XPG endonuclease complexes with the TFIIH transcription factor and aids in its stabilisation (Emmert et al., 2001). The correct positioning of the XPG endonuclease has been shown to be important in the recruitment

and incision of the XPF-ERCC1 complex, and that the correct positioning of the XPG endonuclease is required for the unwinding of the DNA helix (Clarkson, 2003). It has also been shown that XPG has non-catalytic roles outside of NER in acting as a co-factor for the BER DNA glycosylase NTH1 which shows higher affinity to damaged DNA in the presence of XPG, and that this interaction does not require XPG catalytic activity (Bessho, 1999).

CSA/CSB

CSA and CSB proteins bind to RNA Pol II and form the basis of TC-NER. Following UV irradiation, CSA as part of the larger CRL4 E3 ubiquitin ligase, ubiquitinates the transcription complex including RNA Pol II and facilitates its controlled degradation, allowing the later stages of NER to occur (Nardo et al., 2009). The CSB subunit has ATPase activity and also facilitates the ubiquitination of the transcription complex (Horibata et al., 2004).

XPV/Polη

UV damaged DNA can be bypassed by translesion polymerases which have lower fidelity than other replicative DNA polymerases (Bomgarden et al., 2006). Polymerase H or Polη is able to bypass UV damaged DNA that contains a variety of lesions such as 6-4 photoproducts and cyclopyrmidine dimers (Fassihi, 2012). Translesion polymerases allow for continued replication in cells with damaged DNA, however this error prone DNA repair is often highly mutagenic (Fassihi, 2012; Sugasawa, 2008b).

Pathologies Associated With Deficient DNA Repair

There are several congenital pathologies associated with deficient or absent processes in the repair or signalling of DNA damage. Many of these diseases result in greatly increased risk of cancers, as well as physical and mental abnormalities which highlight the complexity of the pathways involved in DNA damage response. Xeroderma Pigmentosum (XP) is the most widely studied and relevant disorder here and is discussed below.

Xeroderma Pigmentosum (XP)

The hereditary genetic condition Xeroderma Pigmentosum (XP) was first described by Moriz Kaposi in 1874 in Vienna, however the condition was not accurately defined until 1968 by Jim Cleaver who first defined the mechanism of NER related to XP (Fassihi, 2012; Lehmann et al., 2011). XP is most common in the Japanese population affecting 1:20,000 people, the disease is less common in western countries affecting 1:250,000 people (Kraemer and DiGiovanna, 2014). XP is defined as a loss of function mutation in any one of 8 of the core NER factors involved in the repair of UV damaged DNA. Based on which one of the core NER factors a mutation occurs in, XP can present with a wide range of severities. Patients are separated in to complementation groups depending on their gene mutation, XPA-G with the 8th group being patients that have a mutation in a translesion polymerase (*POLH*), referred to as XP-V (Table 1.1) (Lehmann et al., 2011; Sugasawa, 2008b).

The main characteristic of XP is extreme sensitivity to UV light resulting in extensive sunburn, erythema, blistering and freckle-like pigmentation from which XP is named (Fassihi, 2012). The absence of UV induced DNA repair in XP patients results in an overwhelming increase in risk of skin cancers, specifically melanoma, basal cell carcinoma and squamous cell carcinoma. The increased risk of skin cancers in XP patients is estimated to be 10,000 fold with a 50x increased risk of internal cancers (Cleaver, 1975; Kraemer and DiGiovanna, 2014). 20-30% of XP patients suffer from progressive neurological degeneration with mental retardation, mostly seen in patients that belong to the XP-A, XP-D and XP-G complementation groups (Anttinen et al., 2008). Neurological symptoms are thought to be caused by the progressive accumulation of oxidative DNA damage which has

not been adequately repaired (Fassihi, 2012). Other common complications of XP include macular and ophthalmic degeneration, hearing loss, and oral cavity swelling with mucosal cancers (Cleaver, 1975; Lehmann et al., 2011; Wayli, 2015). The median survival for a XP patient with neurological symptoms is 29 years, and 37 for patients without neurological involvement.

Several mutations identified in XP Complementation groups can have cross over symptoms with other diseases caused by defects in DNA repair, specifically Cockayne syndrome (CS), Trichothiodystrophy (TTD) and Cerebrooculofacioskeletal syndrome (COFS). These are discussed below. In these incidences where XP symptoms are considerably worse, or cross over with other DNA repair diseases, it has been shown that altered function of the transcription factor TFIIH is likely involved (Ito et al., 2007; Vermeulen et al., 1993; Zhu et al., 2012).

Table 1.1: Frequencies and Phenotypes of XP Complementation Groups.

Complementation Group	Gene	Phenotype **	Percentage of Patients in Western Countries/Japan (%) *	Neurological Symptoms	Cutaneous Skin Cancers
A	*XPA*	XP	9 / 55	+	+
B	*XPB*	XP XP/CS TTD	1 / 0	Mild + +	+ + -
C	*XPC*	XP	43 / 3	-	+
D	*XPD*	XP XP/CS XP-TTD COFS TTD	28 / 3	Varied + + + -	+ + + - -
E	*DDB2*	XP	3 / 3	-	+
F	*XPF*	XP	0 / 7	-	+
G	*XPG*	XP XP/CS	3 / 1	- +	+ +
V	*POLH*	XP	7 / 12	-	+

*data extrapolated from (Bradford et al., 2011; Fassihi, 2012; Kraemer and DiGiovanna, 2014; Lehmann, 2003, 2008). ** phenotypes relate to position and nature of mutations.

Cockayne Syndrome (CS)

Cockayne syndrome, first described in 1936 is also an inherited disease caused by defects in NER, specifically the transcription coupled or TC-NER recognition subtype (Ridley et al., 2005). Although also a NER deficiency disease, Cockayne syndrome has very little in common with XP. CS patients have no increased risk of skin cancers, or any skin pigmentation. The main characteristics of CS are severe mental and physical retardation, bird like facial features with sunken eyes, microcephaly and long limbs (Ito et al., 2007; Okinaka et al., 1997). CS occurs due to mutations in either *CSA* or *CSB* genes which form proteins that interact with RNA Pol II and help to initiate NER at sites of stalled transcription (Cooper, 1997). CS is rare and patients do not often survive past 6-7 years.

Trichothiodystrophy (TTD)

Trichothiodystrophy is the rarest of the NER associated diseases of DNA repair. Patients with TTD exhibit a range of neurological symptoms of varying severity, and have extremely brittle, sulphur depleted hair and nails (Theil et al., 2014). Patients with TTD have been shown to have mutations in 3 different genes. Most other patients with TTD were found to carry mutations in XPB or XPD which are also subunits of the TFIIH complex forming the 5-3' and 3-5' helicase function, as well as the TTDA protein subunit (Schärer, 2008a; Taylor et al., 1997). Patients with TTD have low levels of the TFIIH complex leading to the hypothesis that mutations in complex subunits which destabilise the overall structure leads to a reduction in TFIIH which results in the TTD phenotype (Morice-Picard et al., 2009).

Cerebo-oculofacio-Skeletal Syndrome (COFS)

COFS, first described in 1971, is characterised by severe pre and postnatal skeletal abnormalities, microcephaly, and neurodegeneration. Patients also suffer from cataracts, optic atrophy, and progressive joint contractures (Lowry et al., 1971). Patients diagnosed with COFS have been found to carry mutations in *CSB*, *XPG* and *XPD* genes (Gregg et al., 2011). However, one additional COFS patient has been identified with a truncation mutation in the ERCC1 gene. Patient 165TOR was found to have a C>T substitution resulting in a stop codon at position Gln158 (Jaspers et al., 2007). This truncation mutation has been shown to prevent the XPF-ERCC1 interaction, and an immortalised fibroblast cell line generated from

patient 165TOR was found to be sensitive to UV and mitomycin C (Gregg et al., 2011; Jaspers et al., 2007).

Ataxia Telangiectasia (AT)

Ataxia telangiectasia (AT) is a congenital disorder characterised by the loss of functional ATM (Ataxia Telangiectasia Mutated) (Lavin and Shiloh, 1996). The ATM protein is a critical signalling factor in DSB repair which functions to recruit other repair factors and induce cell cycle arrest (Lee et al., 2013). AT syndrome was first described in 1926 but was not properly characterised until 1957 (Biemond, 1957). Patients with AT present with immunodeficiency, neurological abnormalities and extreme ionising radiation sensitivity. Predisposition to lymphoid and myeloid cancer also occurs (Lavin and Shiloh, 1996; Lee et al., 2013). The term telangiectasia refers to angiogenesis seen on the surface of the cornea of the eye (Opeskin et al., 1998). Most of the identified mutations in the *ATM* gene arise from frameshift truncations and in frame deletions. The incidence of AT is thought to be 1:100,000 births worldwide with the maximum reported life expectancy of 34 years.

Ataxia Telangiectasia-Like Disease (ATLD)

ATLD syndrome is so named due to the high similarity of the condition to AT. However whereas AT is caused by mutations in *ATM*, ATLD is caused by mutations in *MRE11,* which contains the catalytic nuclease activity of the MRN complex (Chapter 7). ATLD is extraordinarily rare, as of 2004, only 6 ATLD patients have ever been identified all of which showed either a N117S or R633-stop mutated MRE11 protein which resulted from point mutations. Like classical AT patients, ATLD patients show progressive mental retardation and radiation sensitivity, however, no cranial facial abnormalities were seen nor any immunological deficiency (Schiler and Hopfner, 2012; Taylor et al., 2004).

Nijmegen Breakage Syndrome (NBS)

Nijmegen breakage syndrome (NBS) is a hereditary condition resulting from the absence of functional NBS1. NBS1 is part of the MRN complex which is involved in DSB repair. NBS1 is also able to be phosphorylated by ATM to enable the recruitment of the MRN complex to the sites of DNA damage. NBS syndrome was first described in 1981 and predominately

seen in patients of Polish or Czech descent (Digweed and Sperling, 2004). 90% of all NBS patients identified are homozygous for a 5 base pair deletion that results in a frame shift truncation mutation (657Δ5) (Digweed and Sperling, 2004). This truncation mutation results in extreme radiation sensitivity, immunodeficiency, microcephaly, mental retardation, and a characteristic facial appearance with a high forehead, prominent midface and reseeding mandible (Digweed and Sperling, 2004).

Seckel Syndrome (SS)

Seckel Syndrome is defined as heterogeneous form of primordial dwarfism (Shanske and Marion, 1998). Several inherited genetic mutations have been found in Seckel syndrome patients, including the DNA damage signalling kinase ATR. The ATR kinase is the main signalling molecule for single-stranded DNA and stalled replication forks, which when activated, phosphorylates a great many substrates that bring about cell cycle arrest and DNA repair machinery recruitment (Faivre and Cormier-daire, 2005; Mohni et al., 2014). Patients with Seckel syndrome exhibit severe microcephaly and mental retardation with dwarfism and bird like head and facial features (Qvist et al., 2011).

Fanconi Anemia (FA)

Fanconi anemia (FA) is a hereditary disease carrying a mutation in one of many of the proteins that are involved in the Fanconi DNA repair pathway. The Fanconi pathway is the main mechanism by which naturally occurring DNA crosslinks and chemotherapeutic inter-strand crosslinking agents such as MMC are repaired. 13 core *Fanc* genes have been identified which either stabilise crosslinked base lesions, recruit other repair factors to the lesion or interact with nucleases to resolve lesions (Takata et al., 2009). 66% of Fanconi patients belong to the FANCA group which is one of the core scaffolding proteins involved in inter-strand crosslink repair (Kee and Andrea, 2012). Unlike many other DNA repair related pathologies, FA patients do not always present with obvious physical deformities. 25-40% of FA patients are physically normal, except for a short stature (de Winter and Joenje, 2009). The main feature of FA is higher sensitivity to inter-strand crosslinking agents such as mitomycin C, as well as bone marrow failure and hyperinsulinemia (Kee and Andrea, 2012).

FA patients have an 800x increased risk of developing haematological cancers such as myeloid leukaemia.

Table 1.2: Fanconi Anemia Protein Mutational Prevalence and Functions

FA Protein	% of Patients with Mutation*	Function
FANCA	66	Core complex – required for FANCD2 – I ubiquitination
FANCB	2	Core complex – required for FANCD2 – I ubiquitination
FANCC	10	Core complex – required for FANCD2 – I ubiquitination
FANCD1 (BRCA2)	2	HR mediator- downstream of FANCD2 activation
FANCD2	2	Ubiquitinated following DNA damage
FANCE	2	Core complex – required for FANCD2 – I ubiquitination
FANCF	2	Core complex – required for FANCD2 – I ubiquitination
FANCG	9	Core complex – required for FANCD2 – I ubiquitination
FANCI	2	Ubiquitinated following DNA damage
FANCJ	2	Helicase downstream of FANCD2 activation
FANCL	0.2	Core complex – required for FANCD2 – I ubiquitination
FANCM	0.2	Helicase within core complex, required for FANCD2 ubiquitination
FANCN	2	FANCD1 interactor

*data extrapolated from (Moldovan and D'Andrea, 2009)

The Fanconi anemia repair pathway is initiated by FANCM and FAAP24 which binds single strand to double stranded DNA junctions which occur at stalled replications forks (Kee and Andrea, 2012). The FA core complex is then recruited and assembled which contains the majority of the FANC proteins (Fig.1.7). The activated FA core complex then precedes to mono-ubiquitinate FANCD2 and FANCI which forms the critical step of the FA repair pathway that the majority of FA patient mutations prevent (Patel and Joenje, 2007). Following the successful recruitment and activation of the FA core complex and FANCD2

mono-ubiquitination, several downstream proteins such as FANCD1 facilitate Rad51 filament formation which leads to further lesion repair by HR.

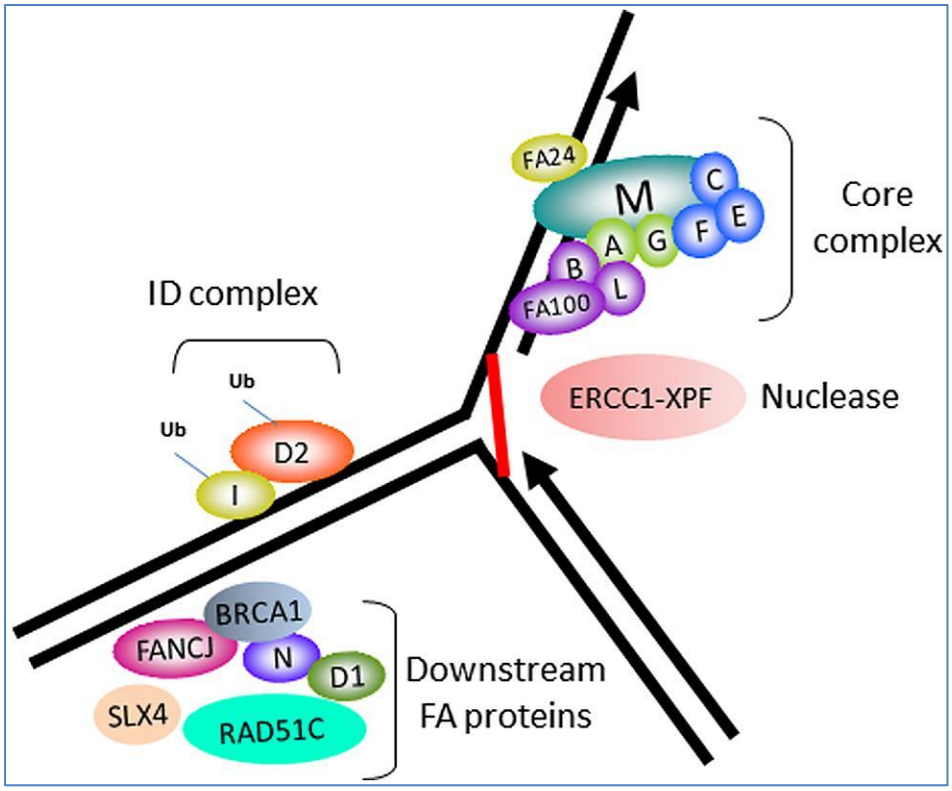

Figure 1.7: Molecular Schematic of the Fanconi Anemia Pathway. DNA strand crosslinks at replication forks (red bar) are detected by FANCM which leads to the FA core assembly and FANCD2 and FANCI mono-ubiquitination (Brosh and Cantor, 2014).

Lynch Syndrome (LS/HNPCC)

Lynch syndrome is an autosomal dominant genetic disorder of the mismatch repair pathway. One of four key mismatch repair genes (*MLH1, MSH2, MSH6 or PMS2*) may be mutated in Lynch syndrome where mis-paired DNA bases that occasionally occur after DNA replication are not recognised or appropriately repaired. Failure to repair small loop structures that occur after DNA strand slippage (IDLs) are particularly mutagenic in Lynch syndrome patients, where genes that contain microsatellites are lost due to the introduction of frameshift mutations (Hays et al., 2005; Martin and Scharff, 2002). This effect at sites of tandem repeats is termed microsatellite instability and has been shown to introduce cancer causing mutations. Lynch syndrome affects 1:500 people and is thought to causes 3-4% of all colorectal cancers (Steinke et al., 2013). Colorectal cancers linked to hereditary

mutations in mismatch repair proteins are often termed HNPCC (hereditary nonpolyposis colorectal cancer).

Bloom Syndrome (BS)

Bloom syndrome is an autosomal recessive condition that is defined by the absence of a functional *BLM* gene product which is a RecQ helicase responsible for dissolving complex DNA structures such as Holliday junctions (Arora et al., 2014). The BLM RecQ helicase has been shown to prevent replication fork arrest associated recombination events by stabilising and helping to restart DNA replication forks (Brabant et al., 2000). Bloom syndrome occurs most commonly in people of Ashkenazi jewish descent, and patients have an 150-200 times increased chance of acquiring malignancies such as B and T cell lymphomas, uro-genital cancers, and cancers of the upper and lower gastrointestinal tract (Arora et al., 2014).

Werner Syndrome (WS)

Werner syndrome, similarly to Bloom syndrome, is an inherited mutation disorder of a replicative helicase. The WRN helicase or RecQ3 is required for stable cell division and resolution of Holliday junction-like structures (Goto et al., 2013). 75% of Werner syndrome patients are Japanese, and have adolescent onset growth failure with premature ageing, neural and ophthalmic degeneration and immunodeficiency (Christmann et al., 2008). Werner syndrome patients have a broad spectrum of cancer predisposition including immunological, dermatological and meningeal neoplasia (Goto et al., 2013).

Nucleoside Analogues

Nucleoside analogues are a subclass of antimetabolite chemotherapeutic drugs used in the treatment of cancer and chronic viral conditions. The action of nucleoside analogues is to mimic the structure of natural nucleosides/nucleotides and precursors which ultimately leads to decreased replicative ability due to physical hindrance of polymerases or depletion of replicative substrates such as nucleotide precursors (Jordheim et al., 2013). For most nucleoside analogues, their incorporation into newly synthesised DNA causes chain termination due to steric hindrance of the replicative polymerase, which causes replication stalling, checkpoint activation and ultimately slows cell division in cancerous cells (Achiwa et al., 2004). Other nucleoside analogues inhibit enzymes which are involved in the biosynthesis of nucleotides required for DNA replication. Two main groups of nucleoside analogues have been developed for the treatment of various cancers, purine analogues and pyrimidine analogues, the majority of which mimic the structure of the deoxyribose sugar within the DNA nucleosides adenosine or cytidine.

Pyrimidine Nucleoside Analogues

The first cytidine analogue cytarabine (1-β-D-arabinosylcytosine ((ARA-C) was approved for the treatment of acute myeloid leukaemia in 1969 (Iwasaki et al., 1997). Cytarabine is nearly identical to the structure of cytidine except for the presence of a hydroxyl group at the 2' position on the deoxyribose ring (Ewald et al., 2008a). Once internalised, cytidine analogues such as cytarabine are first phosphorylated to their monophosphate form by the enzyme deoxycytidine kinase (dCK) to ara-dCMP, before being further phosphorylated to their di-phosho and triphosphorylated form (ara-dCTP) (Mini et al., 2006). The triphosphate forms of cytidine analogues are substrates for DNA polymerase enzymes and become incorporated into newly synthesised DNA where consecutive base additions are inhibited. Following the development of cytarabine in treatment of haematological malignancies, further cytidine analogues have been developed which have stronger pharmacokinetics and can be used in solid tumour malignancies.

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) was developed as a second generation cytidine analogue in the late 1980's. Gemcitabine, similarly to cytarabine, mimics the

structure of cytidine except for the di-fluorination of the 2' carbon of the deoxyribose ring. Gemcitabine also strongly inhibits chain elongation after incorporation by polymerases during DNA replication following its phosphorylation by dCK to dFdCMP and subsequently dFdCDP and dFdCTP. However, unlike cytarabine, gemcitabine has an additional mode of action in inhibiting the enzyme ribonucleotide reductase (RNR) which is responsible for generating deoxynucleotides required for DNA replication (Mini et al., 2006). This inhibition of RNR decreases the amount of endogenous deoxynucleotides available for replication as well as potentiate the intra-cellular half-life of dFdCTP which results in increasing the likelihood of gemcitabine incorporation, therefore dramatically increasing its effectiveness compared to cytarabine (Achiwa et al., 2004; Mini et al., 2006). Gemcitabine is used in the treatment of solid tumours such as pancreatic cancers, and in combination regimes for the treatment of non-small cell lung carcinoma, bladder and breast cancers (Huang et al., 1991; Plunkett et al., 1995).

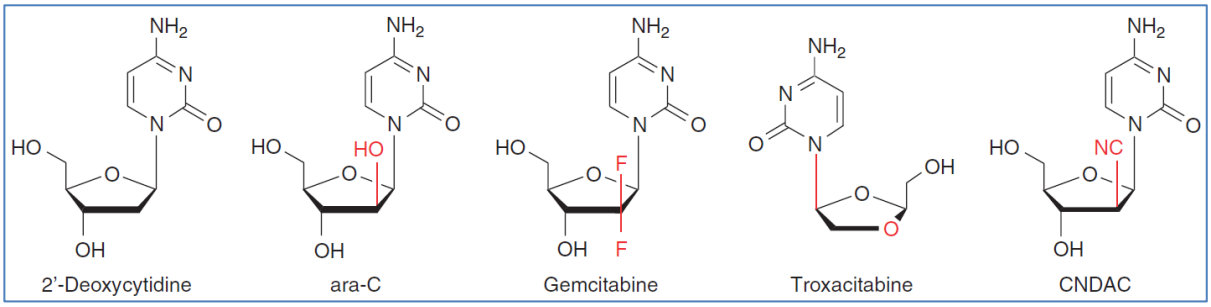

Figure 1.8: Structure of Cytidine Analogues. Nucleoside analogues structures with chemical modifications shown in red (Ewald et al., 2008 modified)

Troxacitabine (L-1,3-dioxolane-cytididne (L-OddC) was originally developed as an anti-viral cytidine analogue, however after successfully being tested for anti-tumour activity, it is now used in conjunction with cytarabine in haematological malignancies (Wang et al., 2008). Troxacitabine is the only one of the cytidine analogues that is in the L- optical isomer orientation compared to the β-D orientation of cytarabine and gemcitabine (Ewald et al., 2008a). Although troxacitabine does not have the RNR inhibitor effect of gemcitabine, it has still been shown to be highly effective in replication inhibition due to the lack of 3'-OH required for nucleotide polymerisation which results in chain termination (Kukhanova et al., 1995).

The analogue 2'-C-cyano-2'deoxy-1-β-D-arabino-pentofuranosylcytosine (CNDAC) is unique among the cytidine analogues. Unlike gemcitabine or cytarabine which cause immediate

replication stalling following their incorporation, CNDAC is incorporated into newly synthesised DNA then undergoes β-elimination which collapses the 3'-OH bond between itself and the next incorporated nucleotide which creates a single strand DNA nick (Wang et al., 2012, 2007).

Resistance to Pyrimidine Nucleoside Analogues

Cells are able to scavenge free nucleosides from their environment via two classes of nucleoside importers. Concentrative nucleoside protein pumps are able to import nucleoside using the sodium gradient across the cell membrane, whereas equilibrative nucleoside importers passively internalise free nucleosides via diffusion (Achiwa et al., 2004). Nucleoside analogues are substrates for cellular internalisation via these transporters termed hENT1 and hCNT1 which form the rate limiting factor in the effectiveness of nucleoside analogue treatment (Fig.1.9) (Fang et al., 1996). Cell lines from various cancer types deficient in hENT1 expression and the use of the transporter inhibitor BIBW22BS have shown a 30-100 fold decrease in nucleoside analogue sensitivity (Mackey et al., 1999).

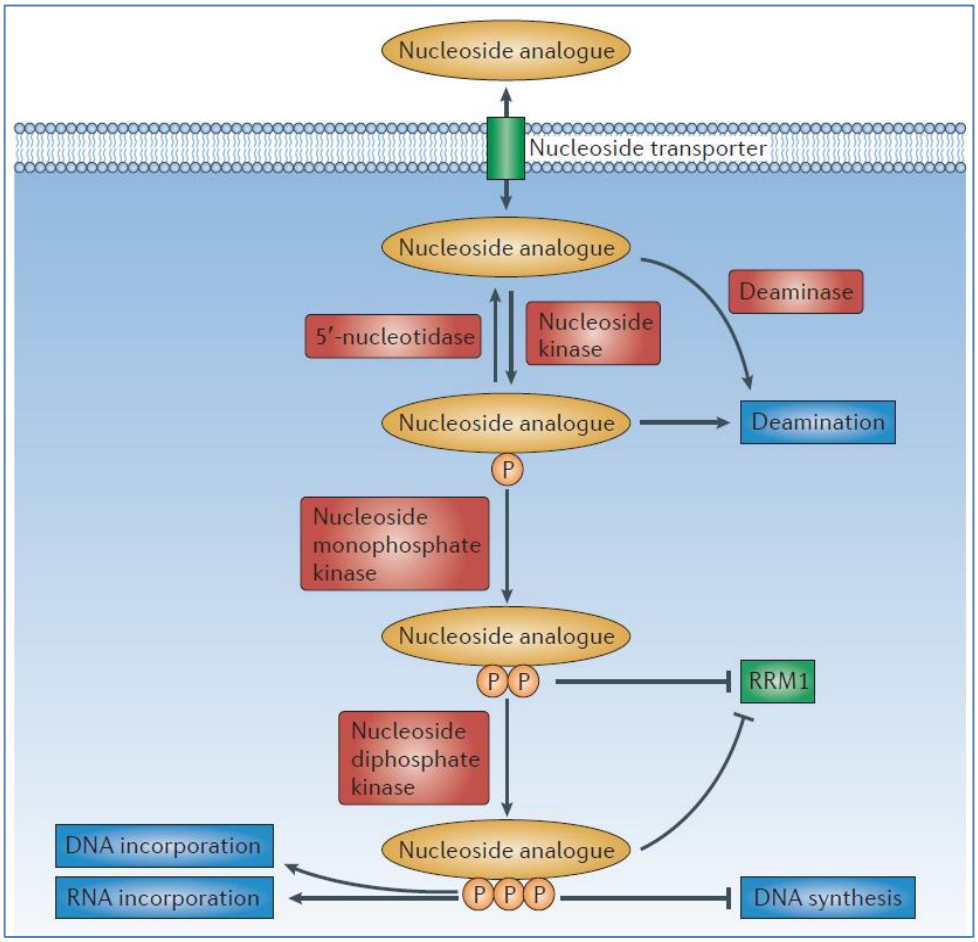

Figure 1.9: Nucleoside analogue internalisation and activation. Model of nucleoside analogues cellular internalisation showing consecutive phosphorylation leading to DNA incorporation as well as deactivation by deaminase enzymes or 5'nucleotidase, and inhibition of the RRM1 subunit of the enzyme ribonucleotide reductase (Jordheim et al., 2013 modified)

Both Cytarabine and Gemcitabine can be deactivated by the enzyme deoxycytidine deaminase which converts excess deoxycytidine to deoxyuridine. Cells over producing this enzyme have been shown to be 3.7 times more resistant to gemcitabine which can be reversed using the deoxycytidine deaminase inhibitor tetrahydrouridine (Ruiz van Haperen et al., 1993). Conversely, low levels of deoxycytidine deaminase have been associated with increased gemcitabine toxicity (Jordheim et al., 2013). Nucleoside analogues can still be deactivated once mono-phosphorylated by 5'nucleotidase which removes the phosphorylation and converts the analogue back to its pro-drug state. Experiments in HEK293 cells show that over production of 5'nucleotidase made cells 22 times less sensitive to gemcitabine (Hunsucker et al., 2001). Similarly, chronic low dose exposure to gemcitabine decreased deoxycytidine kinase expression and increased 5'nucleotidase activity resulting in a 30,000x increase in gemcitabine resistance (Ruiz van Haperen et al., 1993). The over production of anti-apoptotic proteins, and under expression of pro-apoptotic proteins such as BAX have also been shown to reduce cytarabine and gemcitabine efficacy (Achiwa et al., 2004; Rathos et al., 2012).

Once replicative polymerases become stalled on DNA by gemcitabine, cytarabine and other nucleoside analogues, it is unclear how the aberrant DNA base is removed from the genome to allow replication to continue. Several reports indicate that the proof-reading capacity of polymerases causes the reversal of the enzyme which allows for 3'-5' exonuclease removal of the last 2-3 incorporated bases, before restarting in the 5'-3' direction (Achiwa et al., 2004; Ewald et al., 2008a; Gandhi et al., 1996). However, this slow process is unlikely to be the only mechanism which allows for the removal of nucleoside analogues due to the slow enzyme kinetics.

Purine Nucleoside Analogues

The deoxyadenosine analogues fludarabine, cladribine and clofarabine (Fig.1.9) are used in the treatment of B cell malignancies (Ewald et al., 2008a). All three of these analogues also inhibit the activity of RNR similarly to gemcitabine, however the deoxyadenosine analogues more readily incorporate into RNA which has been shown to reduce the expression of anti-apoptotic proteins such as XIAP and Mcl-1 which increases their effectiveness (Huang et al., 2000). Deoxyadenosine analogues have also been shown to stimulate apoptosis directly by

destabilising the mitochondrial membrane which cause the release of cytochrome C due to the interference with normal ATP activity within the mitochrondria (Ewald et al., 2008a).

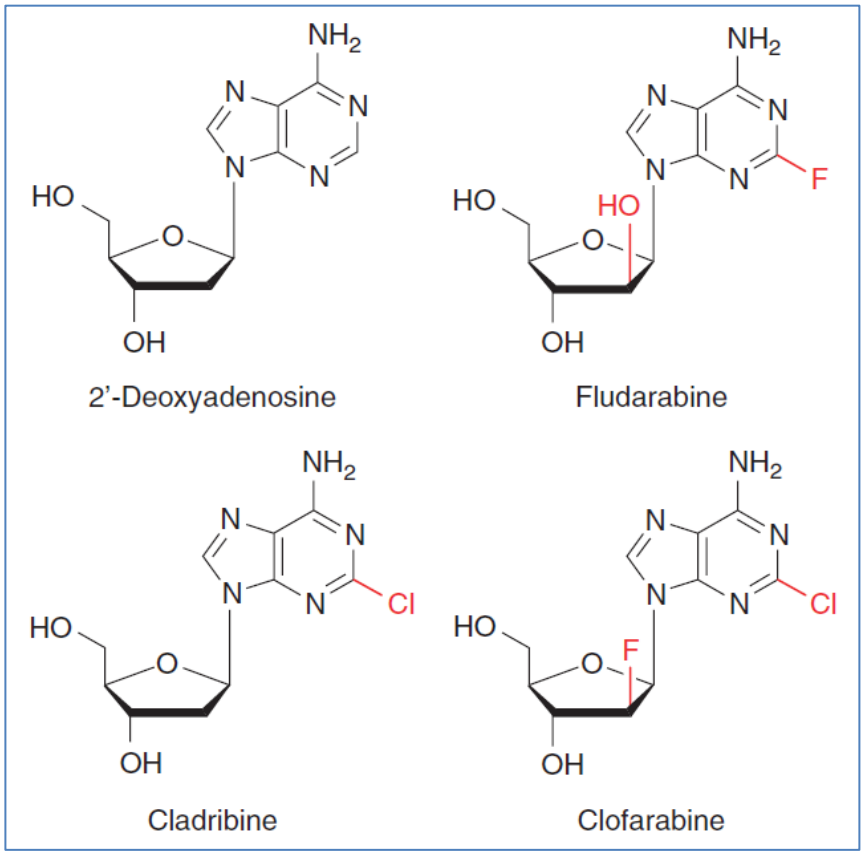

Figure 1.10 : Structure of Deoxyadenosine Analogues. Adenosine analogues chemical structure with modifications shown in red (Galmarini et al., 2001).

Additional Roles of Nucleoside Analogues

In cancers, epigenetic gene silencing often occurs due to methylation of cytosine bases or histone modification. DNA methyltransferase enzymes are able to methylate 5' carbons of cytosine bases, when this occurs in gene promotor regions, expression of the specific gene is dramatically reduced. In cancers regions of hypermethylation are common, specifically around the promotor regions of tumour suppressor genes such as p15 and p16 (Herman et al., 1997). The nucleoside analogues azacytidine and decitabine have been shown to covalently trap DNA methyltransferases to DNA following their incorporation leading to the proteolytic degradation of the methyltransferase enzyme, which re-activates the transcription of several tumour suppressor genes (Stresemann and Lyko, 2008). Several classes of nucleoside analogues are used in anti-viral therapies to treat chronic HIV and

hepatitis which specifically inhibit viral DNA polymerases. However due to the continual mutational evolution of DNA viruses, the active site of viral polymerases is highly changeable leading to decreased drug activity (Jordheim et al., 2013).

Project Rationale

Previous results from our lab have shown that in the model organism *S. Pombe* cells lacking several core NER factors are sensitive to the nucleoside analogues gemcitabine and cytarabine. The *S. pombe* cells with NER factor deletions in homologs Rhp14^{XPA}, Swi10^{ERCC1} and Rhp41/42^{XPC} showed sensitivity to nucleoside analogues, whereas cells lacking the 3' and 5' endonucleases Rad13^{XPG} and Rad16^{XPF} showed no sensitivity to gemcitabine or cytarabine. These unpublished finds might suggest that some, but not all NER factors are required for resistance to nucleoside analogue treatment. Furthermore, results in the lab using these *S. pombe* mutant strains have shown that Rhp14^{XPA}, Swi10^{ERCC1} and Rhp41/42^{XPC} are required for the removal of nucleoside analogues from DNA. Following incubation with both gemcitabine and a cytidine isotope ($^{15}N_3$dC), extracted DNA was isolated and hydrolysed before being measured via liquid chromatography tandem mass spectrometry (LC-MS/MS) where the ratio of gemcitabine to $^{15}N_3$dC was calculated and compared to wild-type. Consistent with the sensitivity data, *S. pombe* mutants Rhp41/42^{XPC}, Rhp14^{XPA} and Swi10^{ERCC1} were found to have an increased gemcitabine to 15N3dC ratio compared to wild-type, indicating a reduced gemcitabine removal efficiency.

The aim of the current project is to attempt to replicate these findings in higher eukaryotic models using human fibroblast cell lines from patients with XP. Using a collected library of cell lines from patients with various XP complementation group mutations, the requirement of several NER factors to resistance to nucleoside analogues, as well as the UV mimetic drug 4NQO will be measured.

Chapter 2: Materials and Methods

Cell Culture

All human XP immortalised skin fibroblasts and the respective wild type cell line control, MRC5 were grown in Dulbecco's modified eagles medium (DMEM) (Sigma) supplemented with 10% foetal bovine serum (FBS) (Gibco), with 2 mM L-glutamine (Sigma) and 100 U/mL penicillin/streptomycin (Sigma). Cultures were maintained at 37°C under humidified 5% CO_2 incubation. The cell lines XP12RO (XP-A), XP2YO (XP-F) and XPCS1RO (XP-G) were a kind gift from Alan Lehmann from the University of Sussex, XP4PA (XP-C), XP6BE (XP-D) and CS3BE (CS-A) cell lines were purchased from the Coriell Institute (New Jersey, USA). All XP cell lines used are summarised in Chapter 3, Table 3.1.

DT40 cell cultures were maintained in RPMI 1640 medium (Sigma), supplemented with 10% foetal bovine serum (Gibco), 2% chicken serum (Sigma) with 2 mM L-glutamine (Sigma) and 100 U/mL penicillin/streptomycin (Sigma). Cells were grown at 39°C under humidified 5% CO_2 conditions. MRE11 D60N and H129N were generated by targeted integration by Dr Ellen Vernon, University of Bangor.

Drugs and Chemicals

All drugs were diluted from stock concentration aliquots prepared as shown in Table 2.1.

Table 2.1: Stock Drug Concentrations and Suppliers

Drug	Supplier	Cat#	Stock Concentration Solution/Solvent
Gemcitabine	Discovery Fine Chemicals	122111-03-9	100 mM (PBS)
Cytarabine	Sigma	C1768	100 mM (PBS)
4NQO	Sigma	N8141	100 mM (DMSO)
VE-821	Selleck Chemicals	S8007	27 mM (DMSO)
MK-8776	Selleck Chemicals	S2737	13 mM (DMSO)
Camptothecin	Sigma	C9911	10 mM (DMSO)
5-Flurouracil	Sigma	F6627	100 mM (DMSO)
Etoposide	Sigma	E1383	10 mM (DMSO)

Colony Forming Assay

1000-2000 cells were seeded on 100 mm diameter tissue culture dishes (Corning) in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 20% foetal bovine serum, with 2 mM L-Glutamine and 10 U/mL Penicillin/Streptomycin in 5 mL volume and allowed to attach overnight. Drug treatments were applied for 24 hours, before washing once with 1x DPBS (Sigma) and suspending in 10 mL of 20% FBS complete DMEM media. Plates were then incubated for 10-14 days, depending on drug treatments and cell line, before media aspiration and staining with 0.4% Methylene Blue (Sigma) in 50% ethanol (w.v) for 20 minutes, followed by 1x PBS wash. Plates were then scanned in a flatbed scanner at 1200 dpi and colonies counted using ImageJ software. Survival was expressed as percentage of untreated controls.

For DT40 colony survivals semi solid RPMI 1640 media was made as follows. 6 g of methylcellulose powder (Sigma) was autoclaved in a 1 L bottle with stirring flea, before adding 500 mL of pre-warmed RPMI 1640 media and heated to 70°C for 40 minutes. The media was then stirred overnight at 4°C until clear. 100 mL of foetal bovine serum and 10mL of heat inactivated chicken serum was added before 5 mL penicillin/strepmycin solution and 5mL L-Glutamine, final concentration 0.1 U/mL and 2mM respectively. The media was stirred for an additional 30 minutes before warming to 37°C. 5 mL of media was then added to each well of a 6 well plate (Corning), 500 DT40 Cells were added per well, plus relevant drug dilutions, before incubation for 7-10 days. Plates were then scanned and colonies counted using ImageJ software. Survival was expressed as percentage of untreated control.

MTS Assay

Cells were seeded in 96 well plates (Corning) at 20,000 per mL in 100 µL volume in complete phenol red free DMEM media (Sigma) and allowed to attach overnight. Drug dilutions were then applied in 2x concentration in 100 µL of media to achieve a 1x solution per well. Plates were incubated for 72 hours before addition of 20µL MTS reagent (Promega). Plates were then returned to the incubator for 4 hours before being read for absorbance at 492nm using a Victor X3 microplate reader. Survival was expressed as a percentage of untreated controls minus an a-cellular control well absorbance.

Direct and Indirect Antibody Flow cytometry

For flow cytometric analysis $1x10^6$ cells were seeded in 100 mm plates in 10 mL complete DMEM media and allowed to attach overnight. Drug dilutions were applied for indicated times before trypsinisation and fixation in 2% formaldehyde (methanol free) (Life Technologies) at room temperature for 15 minutes before being held overnight at 4°C. Cells were then washed 1x in Dulbecco's phosphate-buffered saline (DPBS) and washed again in antibody binding buffer (1xDPBS with 0.1% Tween 20 and 2% BSA). Cells were then incubated with 0.5 µL of diluted fluorescent primary antibody (Table.2.2) in 50 µL antibody binding buffer for 30 minutes in the dark, followed by a 1x PBS wash and counter staining in 2 mL antibody binding buffer with 2.5 µg/mL propidium iodide (Sigma) and 50 µg/mL RNase (Sigma), before analysis by flow cytometry.

For indirect antibody flow cytometry, cells were washed twice in PBS after primary antibody incubation then incubated in 50 µL antibody binding buffer with 0.5 µL of the relevant secondary conjugate antibody for 30 minutes in the dark at room temperature. Cells were then washed twice in PBS and counter stained with 2.5 µg/mL propidium iodide and 50 µg/mL RNase. Antibody dilutions used in flow cytometry experiments are summarised in Table 2.2. Samples were run on a Partec Cube8 flow cytometer, data was analysed using flowing software 2 (www.flowingsoftware.com).

Table 2.2: Flow Cytometry Antibody Dilutions Used.

Antibody	Supplier/Catalogue Number	Dilution
Anti-Rabbit ALEXA488	Abcam AB150077	1:100
Anti-Mouse ALEXA488	Abcam AB150105	1:100
Anti-Cleaved PARP Dylight488	Abcam AB139850	1:100
Anti-yH2AX (Mouse)	Abcam AB26350	1:1000
Anti-Phospho-ATM s1981 (Rabbit)	Abcam AB81292	1:500
Anti-BrdU ALEXA488	Abcam AB171061	1:100
Anti-Annexin V FIT-C	Abcam AB14085	1:100

Annexin V Apoptosis Assay

$5x10^5$ cells were seeded in 6-well plates and allowed to attach overnight, before incubation in gemcitabine at indicated concentrations for 30 hours. Cells were then collected by trypsinisation (including floating debris) and washed 1x in DPBS. Cell pellets were resuspended in 50 µL annexin binding buffer (Abcam) with 0.5 µL annexin V-FIT-C conjugate antibody and 0.5 µL propidium iodide solution, and then incubated in the dark for 15 minutes, followed by addition of 1.5 mL annexin binding buffer and analysis by flow cytometry.

Cell Cycle Analysis

For cell cycle analysis $1X10^6$ cell were seeded in 100 mm plates and allowed to attach overnight before incubation with gemcitabine at indicated concentrations and time points. Cells were then trypsinised and pelleted before fixation in ice cold 70% ethanol and held at -20°C overnight. Samples were then washed twice in cold PBS and stained with 2.5 µg/mL propidium iodide and 50 µg/mL RNase for 30 minutes at room temperature before analysis by flow cytometry.

Bromodeoxyuridine Incorporation Assay

$1x10^6$ Cells were seeded in 100 mm plates in 10 mL complete DMEM media and allowed to attach overnight. 100 nM gemcitabine was added 1 hour before 30 µM bromodeoxyuridine (Sigma). Cells were then trypsinised at indicated time points and fixed in ice cold 70% ethanol and stored at -20°C overnight. Cells were washed once in DPBS, before DNA denaturing in 500 µL 2M HCl (Sigma) for 30 minutes in the dark. Cells were next washed once in DPBS and washed again in antibody binding buffer (1xDPBS with 0.1% Tween 20 and 2% BSA). Cells were then incubated with 0.5 µL mouse anti BrdU ALEXA 488 conjugate antibody (Abcam) in 50 µL antibody binding buffer for 30minutes in the dark, followed by 1x PBS wash and counter staining in 2 mL antibody binding buffer with 2.5 µg/mL propidium iodide and 50 µg/mL RNase, before analysis by flow cytometry.

NER protein and γH2AX Western Blotting

For NER protein detection, whole cell extracts were prepared from $2x10^6$ cells using M-PER lysis buffer (Life Technologies) with 1:100 protease inhibitor cocktail (Melford). 40 µg of

protein was then run on 4-15% precast protein gels (Bio-Rad) at 200V for 45minutes before transfer to methanol activated PVDF membranes using the trans-blot turbo system (Bio-Rad). Blots were then blocked in 1xPBS with 0.5% Tween20 and 3% milk powder (PBST) for 3 hours before overnight incubation with primary antibodies at 1:20,000. Blots were then washed 3x in PBST for 15 minutes and incubated with 1:20,000 HRP conjugate secondary antibodies in PBST for 2 hours before 3x washing and addition of ECL substrate according to manufacturer guidelines (Amersham) and developing onto Hyperfilm ECL (GE Healthcare). Antibodies used in western blot experiments are summarised in Table 2.3.

For yH2AX activation detection western blots, $1x10^6$ cells were plated in 100 mm plates and allowed to attach overnight before incubation with indicated drug concentrations. Protein extracts were prepared as above and blots were probed for yH2AX activation. All western blots were re-probed with α-tubulin antibody for use as a loading control (1:30,000).

Table 2:3: Western Blotting Antibody Dilutions Used

Antibody	Supplier/Catalogue Number	Dilution
Anti-MRE11 (Rabbit)	Abcam AB190179	1:20,000
Anti-XPG (Rabbit)	Abcam AB76390	1:10,000
Anti-XPD (Mouse)	Abcam AB54676	1:30,000
Anti-XPF (Rabbit)	Abcam AB76948	1:20,000
Anti-XPA (Mouse)	Abcam AB65963	1:20,000
Anti-ERCC1 (Rabbit)	Abcam AB129093	1:20,000
Anti-XPC (Rabbit)	Abcam AB78064	1:10,000
Anti- Alpha Tubulin (Mouse)	Abcam AB10637	1:30,000
Anti-Mouse HRP (Rabbit)	Abcam AB97046	1:20,000
Anti-Rabbit HRP (Goat)	Abcam AB6721	1:20,000

XPG and XPF cDNA Construct PCR Detection

Detection of cDNA constructs in XPF D676A and XPG E791A cell lines was performed by PCR. For XPG cell line XPCS1RO exon 9 forward primer 5'-AACGGATCGCTGCTACTGTCA-3' and exon 11 reverse primer 5'-TTCGGTATAATCACTTCCAAG-3' were used. These primers span

1807 base pairs of genomic DNA at the XPG locus; however in the absence of introns (cDNA), a 317 base pair fragment is amplified. For XPF, exon 9 forward 5'-CGCTATCTCACTGCTTTGG-3', and exon 10 reverse 5'-GAGTGTCAGTGGAAACATCTG-3' primers were used to detect a 157 base pair XPF cDNA fragment. All PCR reactions were performed using myTAQ red mix (Bio-line) with an initial denaturation of 95°C for 5 minutes, before 30 cycles of 30 seconds denaturing, 15 seconds annealing (61°C XPF, 60°C XPG) and 60 seconds extension at 71°C, finally followed by a final extension at 71°C for 5 minutes. Following PCR reactions, samples were loaded into wells on 2% TBE agarose and run at 110v for 45 minutes. Following gel imaging, the bands of predicted size were cut out and gel extraction was performed. Samples were then mixed with forward primer and sent for sequencing by Eurofins MWG.

esiRNA Knockdown

Mission esiRNA targeting XPC was purchased from Sigma and diluted into DNAse/RNase free TE Buffer (Life Technologies). 5x10^{5} cells were seeded into 6 well plates and allowed to attach overnight. Cells were then washed once in PBS and the media replaced with OptiMEM transfection media (Gibco) with 5% FBS and 2 mM L-glutamine. 50 ng of esiRNA was then diluted into 50 µL OptiMEM media before the addition of 6 µL Lipofectamine RNAiMax (Life Technologies). RNA-transfection reagent complexes were allowed to form at room temperature for 15 minutes before addition to each well. Cells were then returned to the incubator for 72 hours with protein extracts prepared every 24 hours. Extracts were then probed for XPC via western blotting as above.

shRNA Knockdown

Lentiviral packaged shRNA particles targeting XPG were purchased from Santa Cruz. 5x10^{5} cells were seeded in 6 well plates and allowed to attach overnight. 5 µL of lentiviral particles and 5 µL of polybrene (Santa Cruz) was then added to each well and incubated for 48 hours. Cells were then washed once in PBS and the media replaced with complete DMEN with 1 ug/mL Puromycin. Puromycin resistant colonies were isolated and probed for XPG expression via western blotting and GFP expression via flow cytometry.

CRISPR/CAS9 Knockout

ERCC1 CRISPR/CAS9 knockout reagents and constructs were purchased from Origene (San Diego, USA). A 1 µg mixture of two guide gRNA targeting plasmids (sequences 5'-ATTTGTGATACCCCTCGACG-3' and 3'-TTGCTGGCGGCCCTGAGGGC-5') and a homology directed repair (HDR) construct containing GFP and a puromycin resistance cassette were combined in 100 µL of OptiMEM media. 10 µL of Trans-IT LT1 transfection reagent (Mirus) was added to the plasmid mixture and incubated at room temperature for 20 minutes. Plasmid complexes were then added to 20,000 cells seeded in a 24 well plate and incubated for 72 hours. 1 µg/mL puromycin was then added and surviving cells were then plated into 96 well plates at 1 cell per well and single colony isolation was performed before protein extraction, and western blot analysis.

Chapter 3 – NER Deficient Cell Lines Are Sensitive To Nucleoside Analogues

Summary

Using defined and well characterised NER-deficient cell lines, the importance of several NER factors in resistance to nucleoside analogues is shown here, in a phenotypic manner that differs from classical NER sensitivities in relation to UV and 4-nitroquinoline-1-oxide (4NQO). We show that cell lines deficient in NER factors are sensitive to Gemcitabine and Cytarabine and that in the absence or mutation of these factors, the classical apoptosis pathway is activated. Most notably, XPC mutant cell line XP4PA, which is relatively resistant to 4NQO, is highly sensitive to the nucleoside analogues gemcitabine and cytarabine, and conversely, XPF deficient XP2YO which is sensitive to 4NQO, shows low sensitivity to nucleoside analogues.

Introduction

It is well established that NER-deficient cell lines of all complementation groups show sensitivity to UV radiation, due to the failure in repairing 6-4 photo products, and cyclo-pyrimidine dimers (CPDs) (de Lima-Bessa et al., 2008). Here we use the compound 4NQO which has been shown to produce lesions that are repaired via NER (Dollery et al., 1983; Waters et al., 1992). 4NQO is metabolised into 4-acetoxyaminoquinoline-1-oxide (Ac-4HAQO) which can covalently bind C^8 or N^2 of deoxyguaninine in dsDNA (Miao et al., 2006). 4NQO is used here as a classical NER substrate control because of its similar dose range to gemcitabine, and it allows for continuous exposure whereas UV exposure does not.

Previous work from our lab showed that in the model organism *S. pombe*, cells lacking the XPA homolog (Rhp14) or the ERCC1 homolog (Swi10) are sensitive to the nucleoside analogue gemcitabine (unpublished). This points towards the hypothesis that nucleoside analogues may be substrates for NER, and that incorporated gemcitabine is removed from genomic DNA via NER. We therefore wanted to test these findings in higher eukaryotic cells using the NER-deficient model of human fibroblasts from patients who suffered from Xeroderma pigmentosum.

We have selected several well characterised XP and CS cell lines for work in this project. Using cell lines that are deficient in GG-NER or TC-NER, or both, as well as 5' and 3' nuclease deficient, the significant steps of the classical NER process are represented in this selection. The cell lines are detailed below and summarised in Table 3.1.

XP12RO (XP-A)

The XP-A deficient cell line XP12RO was isolated from a Palestinian male XP patient who had severe neurological and dermatological symptoms (Muotri et al., 2002). The XP12RO cell line was generated from this patient in 1974 but not SV40 transformed until 1984 (Cleaver, 1975; Royer-Pokora et al., 1984). The XP12RO patient was found to be homozygous for a nonsense mutation (c.619 C>T) resulting in a stop codon at Arg207, and the resulting truncated XPA protein is 75% of the full length 273 amino acid sequence (Fig.3.1) (Roginskaya and Wood, 2005). This relatively small truncation has been shown to prevent XPA interacting with TFIIH, therefore preventing successful NER due to failed positioning of the XPF-ERCC1 complex. As a result, repair of UV damaged DNA as measured by unscheduled DNA synthesis (UDS), has been shown to be less than 5% of non-NER controls in this patient cell line (Muotri et al., 2002).

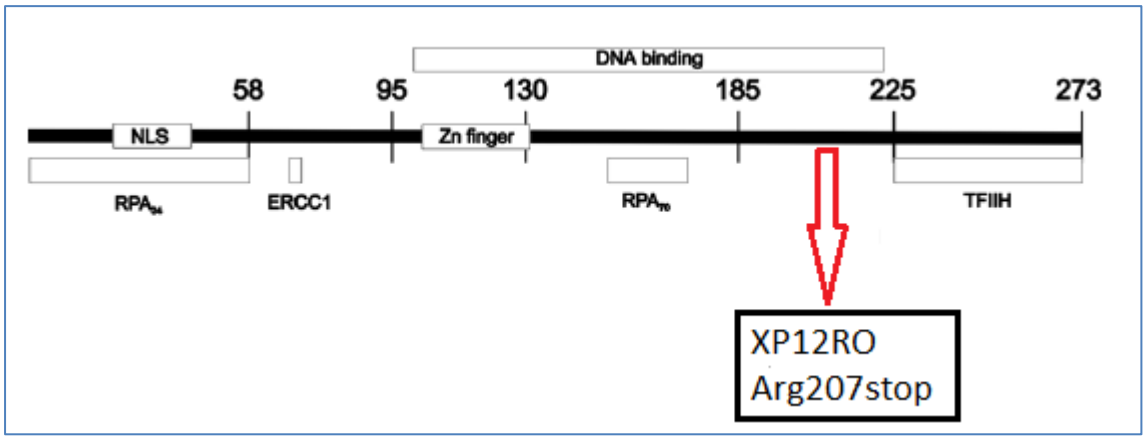

Figure 3.1: XPA Protein Domain Structure. Domain map of XPA protein showing XP12RO truncation mutation location in the DNA binding domain (Cleaver, 2000 modified).

XP4PA (XP-C)

The XP4PA cell line was isolated from an amniocentesis sample taken from a 40 year old pregnant woman who had previously had 2 children with XP in 1979 (Daya-Grosjean et al., 1987). The resulting cell line was then SV40 transformed in 1987 and found to be homozygous for a 2 base pair deletion (1643_1644delTG) resulting in the introduction of a frameshift truncating stop codon at amino acid position 573 (Fig.3.2) (Daya-Grosjean et al., 1987). This 50% truncation in XPC prevents GG-NER from recognising UV damaged DNA, resulting in a reported UDS after UV irradiation of 30%. This relatively high UDS percentage is due to the TC-NER recognition system being intact (Daya-Grosjean et al., 1987).

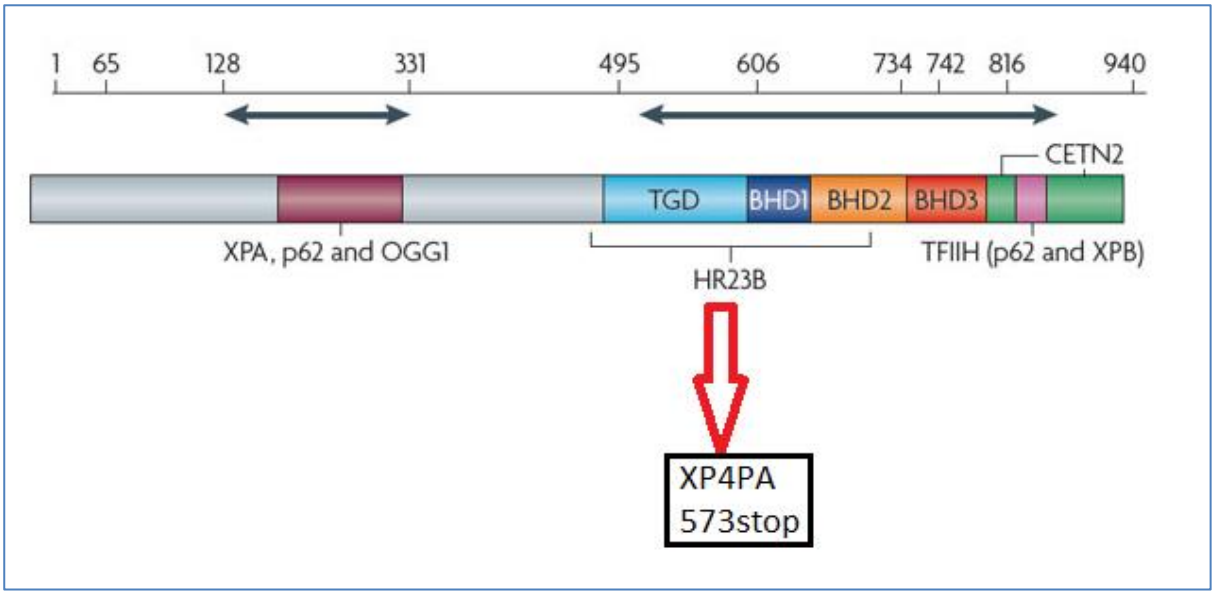

Figure 3.2: XPC Protein Domain Structure. Domain map of XPC protein showing XP4PA truncation mutation within the HR23B binding domain. (Cleaver et al., 2009 modified)

XP6BE (XP-D)

The patient XP6BE and her two sisters (XP5BE and XP7BE) suffered from a pure XP phenotype, with severe neurological symptoms. These siblings were all compound heterozygous for a large section deletion (codons 31-61) and a point mutation resulting in the codon change Arg683Trp (Fan et al., 2007). The XPD deletion mutation has been shown not to be the cause of the XP6BE patients phenotype, however the Arg683Trp mutation renders the XPD allele helicase deficient as this hydrophobicity change prevents the ATPase activity of the helicase (Takayama et al., 1995; Zhu et al., 2012). Other patients in the XPD complementation group have shown a variety of different pathology phenotypes including XP/CS, TTD and CS (Fig.3.3). The mapping of mutations which lead to various DNA repair

pathology phenotypes has led to greater understanding of the XPD protein structure. As shown in Figure 3.3, Pure XP phenotype mutations are mostly found in the DNA binding domains, HD1 and HD2 which prevent efficient helicase activity (Lehmann, 2008). However XP/CS mutations can also occur in the DNA binding domains but have been predicted to prevent ATPase activity which is required for the helicase function of DNA (Fan et al., 2008). TTD phenotypes occur in residues that are essential for the overall 3-dimensional structure of the XPD protein (Lehmann, 2008; Morice-Picard et al., 2009) It has been reported that the Arg683Trp mutation has no impact on the transcriptional function of the TFIIH complex, but prevents efficient NER from occurring, resulting in a reduced repair capacity measured by UDS of 15% (Winkler, 2000).

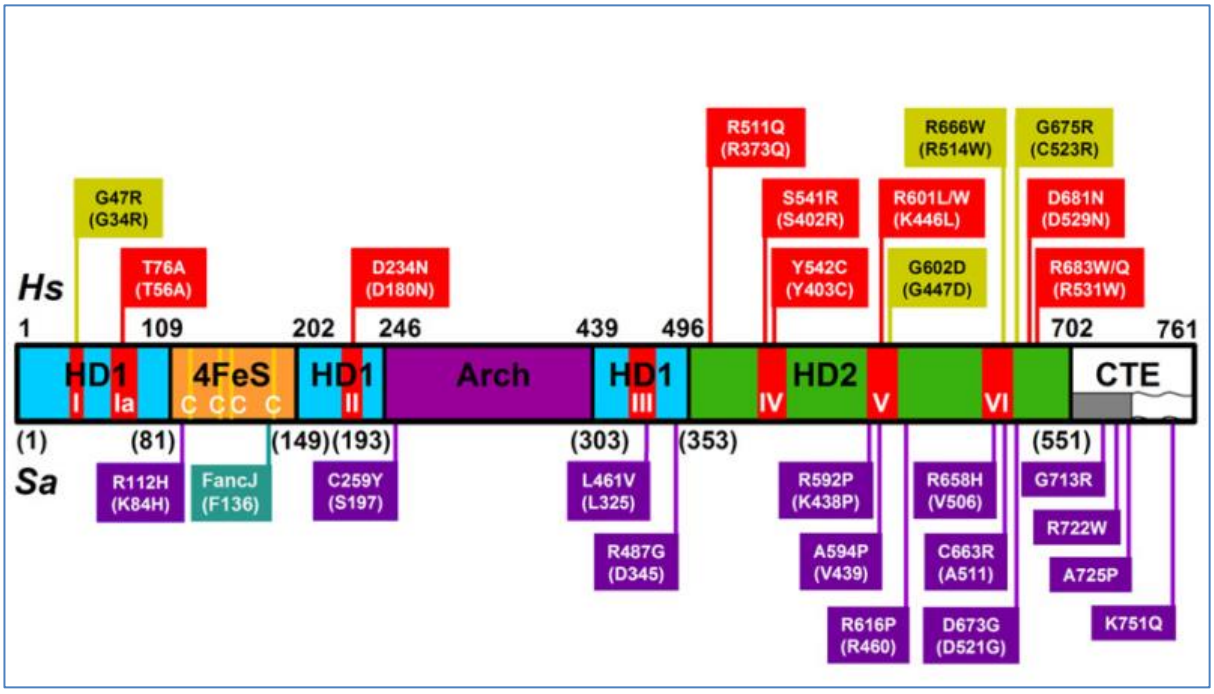

Figure 3.3: Structure of XPD Protein showing Phenotype Specific Mutations. Pure XP phenotype mutations (red) are localised to DNA binding domains HD1 and HD2. Whereas XP/CS associated mutations (yellow) cluster around residues which form the ATPase activity which may prevent conformational changes. TTD mutations (purple) occur throughout HD1 and HD2 DNA binding domains but are predicted to disrupt the overall structure of the XPD protein (Fan et al., 2008; Lehmann, 2008).

XP2YO (XP-F)

The XP2YO cell line is derived from a 64 year old female Japanese patient who exhibited a relatively mild pure XP phenotype (Mogi and Oh, 2006). Patient XP2YO was found to be compound heterozygous with one frameshift truncation mutation resulting from a single base deletion mutation (646fs) and a Thr556Ala codon changing point mutation (Fig.3.4). This amino acid substitution mutation has been shown to fail to bind with ERCC1 and form their hetero-dimer which prevents its nuclear localisation (Ahmad et al., 2010). The XP2YO cell line has been shown to exhibit 17% UDS after UV irradiation (Yagi et al., 1998).

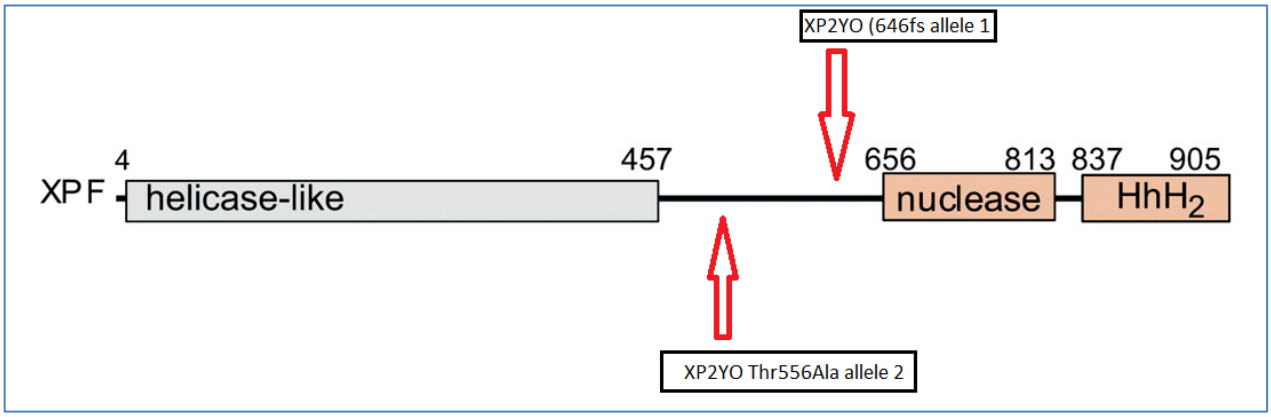

Figure 3.4: XPF Protein Domain Structure. XP2YO compound heterozygous mutations shown in the region between the helicase-like domain and the nuclease domain (Tsodikov et al., 2005 modified)

XPCS1RO (XP-G)

The XPCS1RO patient is one of three people first diagnosed into the XP-G complementation group. XPCS1RO exhibited a severe XP/CS phenotype with extreme neurological symptoms and dwarfism, and died before 6 years (Schärer, 2008b). This patient was found to be homozygous for a frameshift mutation resulting from a single base deletion (926fs) resulting in a stop codon at position 980. This truncation was later shown to prevent the translated XPG protein from interacting with the TFIIH complex which resulted in the extreme XP/CS phenotype (Clarkson, 2003). Other XP-G complementation group patients only display a pure XP phenotype, such as XP125LO (Ala792Val). This patient expressed full length XPG however the point mutation rendered the XPG protein nuclease deficient (Klungland et al., 1999; Schärer, 2008b). The structural integrity of the TFIIH complex dictates the severity of the phenotype shown in XP-G patients secondary to the endonuclease function (Staresincic et al., 2009). The XPCS1RO cell line exhibits low levels of UDS (7%) after UV irradiation

whereas nuclease deficient, XP only XP-G patients often have a UDS percentage greater than 30% (Clarkson, 2003; Staresincic et al., 2009).

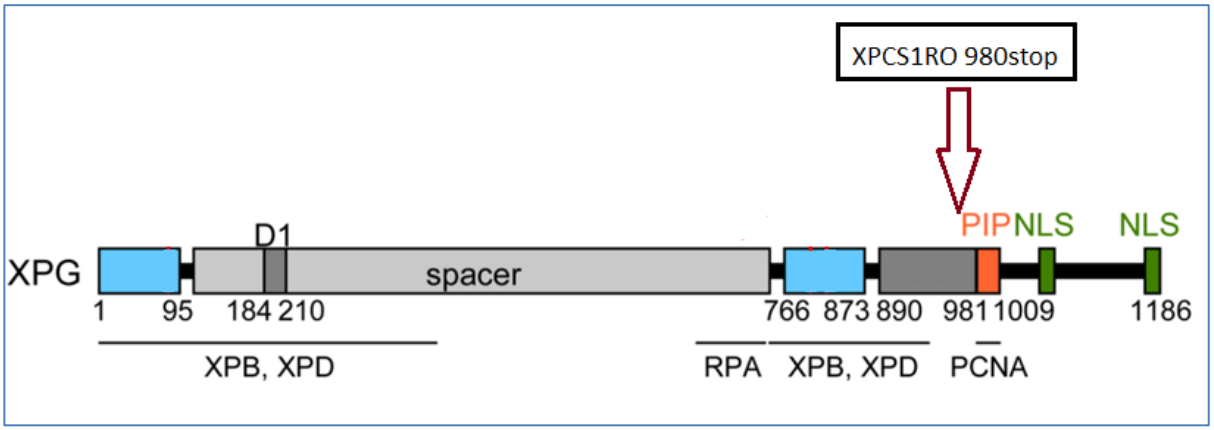

Figure 3.5: XPG Protein Domain Structure. XPCS1RO mutation position shown within highly conserved region (grey). Nuclease domains shown in light blue (Schärer, 2008 modified)

CS3BE (CS-A)

The CS3BE cell line was isolated from a patient with extreme Cockayne syndrome. This 13 year old male suffered from microcephaly and dwarfism, but had no dermatological symptoms. The patient was found to be compound heterozygous with one nonsense mutation resulting in a Glu13stop truncation and a point mutation (Ala160Val) (Nardo et al., 2009). This point mutated CSA protein was found to be unable to bind to RNA Pol II and initiate TC-NER resulting in a transcription related phenotype. The CS3BE cell line was found to have a high UDS percentage of 80% compared to XP and XP/CS phenotypes (Fousteri and Mullenders, 2008; Ridley et al., 2005).

Work in this chapter utilises the widely reported colony forming assay technique (Muotri et al., 2002; Nishiwaki et al., 2008; Pomp et al., 1996; Sidwell et al., 2006) in order to compare nucleoside analogue sensitivity to sensitivity to the lesions produced by 4NQO which have been shown to be repaired by NER (Dollery et al., 1983; Waters et al., 1992). In addition, nucleoside analogues are shown to activate the classical apoptosis cascade in NER-deficient cell lines at low concentrations which do not affect MRC5 cell line controls.

Table 3.1: NER Deficient Cell Lines Used

Cell Line	Genotype	Phenotype	Mutation	Patient	Reported UDS*
MRC5	-	WT	-	Male neonate	
XP12RO	XP-A	XP	Homozygous nonsense Arg207stop	Palestinian boy Severe neurological phenotype Died age 6	<5% (Muotri et al., 2002)
XP4PA	XP-C	XP	Homozygous r.1643_1644delTG Val548Ala>fsX25**	Male – cell isolated from prenatal amniocentesis	30% (Daya-Grosjean et al., 1987)
XP6BE	XP-D	XP	Compound heterozygous. Arg683Trp/ deletion 36-61	Female – Caucasian Severe neurological phenotype Died age 20	15% (Winkler, 2000)
XP2YO	XP-F	XP	Compound heterozygous. Thr556Ala/646fs**	Female – Japanese Mild phenotype – no neurological symptoms Died age 64	17% (Matsumura et al., 1998)
XPCS1RO	XP-G	XP/CS	Homozygous 926fs > 980stop**	Female – Caucasian Severe XP/CS phenotype Died age 5	7% (Staresincic et al., 2009)
CS3BE	CS-A	CS	Compound heterozygous. Ala160Val/E13stop	Male – Caucasian Severe CS phenotype Died age 13	80% (Vermeulen et al., 1993)

*UDS or unscheduled DNA synthesis measured by H^3 thymidine incorporation or 5-ethnyl-2'deoxyuridine (EDU) incorporation. ** fs indicates frameshift mutation

Results

Before beginning cytotoxicity testing, validation of NER-deficient cell lines was performed by western blotting in order to detect various truncation mutations and compare protein levels of various NER factors to the control cell line MRC5.

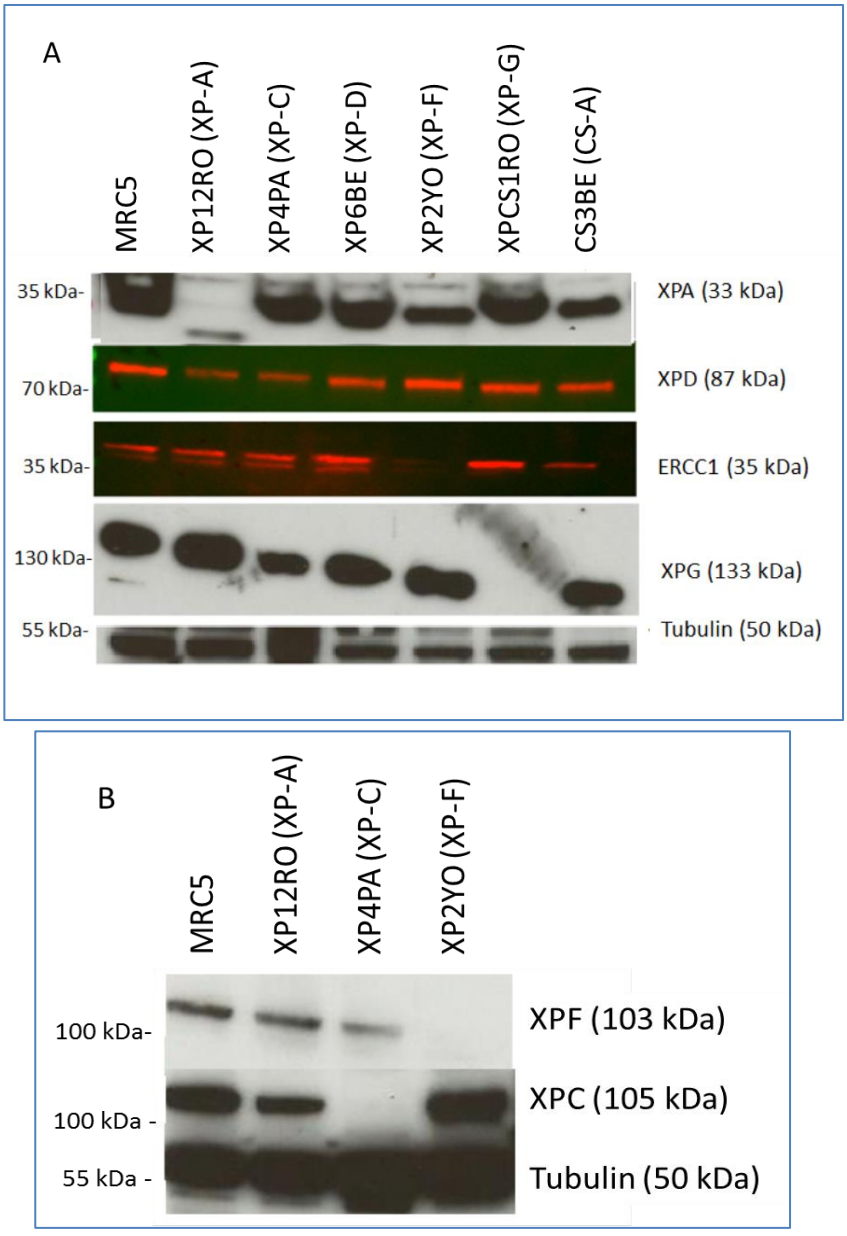

Figure 3.6: NER Protein Detection by Western Blotting. Whole cell lysates were prepared from confluent cells, 30 µg of total protein loaded onto 4-15% gradient gels and transferred to PVDF membranes. Resulting blots were probed with NER protein antibodies and developed via HRP ECL luminescence. (**A**) Detection of XPA, XPD, ERCC1 and XPG relative to tubulin controls. (**B**) Detection of XPF and XPC relative to tubulin controls.

All NER-deficient cell lines used here have been widely used and reported in the literature (Table 3.1). The XP12RO (XP-A) cell line has a truncation mutation resulting in a 206 amino acid protein instead of the full length 273 amino acid XPA protein (Fig.3.6A Lane 2). XP4PA (XP-C) also has a truncating nonsense mutation which ends translation at 573 amino acids of the full length XPC protein of 940. No XPC protein band can be detected in XP4PA cell (Fig.3.6B Lane 3). XPD is detectable in the XP6BE (XP-D) cell line as this patient is compound heterozygous for a short section deletion and point mutation which renders the expressed XPD protein present, but helicase deficient (Fig.3.6A Lane 4). The XPF deficient cell line XP2YO is also compound heterozygous containing a point mutation (Thr556Ala), however as this mutation has been shown to limit the XPF-ERCC1 hetero-dimerisation, both XPF (Fig.3.6 Lane 4) and ERCC1 protein levels (Fig.3.6A Lane 5), are significantly reduced. The XP-G cell line XPCS1RO has been shown to have a truncating frameshift mutation (926fs -980stop). This truncated form of the XPG protein is also undetectable via western blotting (Fig.3.6A Lane 6). CSA protein antibody was not tested.

Following NER factor detection via western blotting, each cell line was tested for sensitivity to 4NQO and to the nucleoside analogues gemcitabine and cytarabine by colony formation assays (Fig.3.7). Colony forming assays (CFAs) have been shown to accurately assay replicative ability following transient drug treatments or various radiation treatments (Pomp et al., 1996).

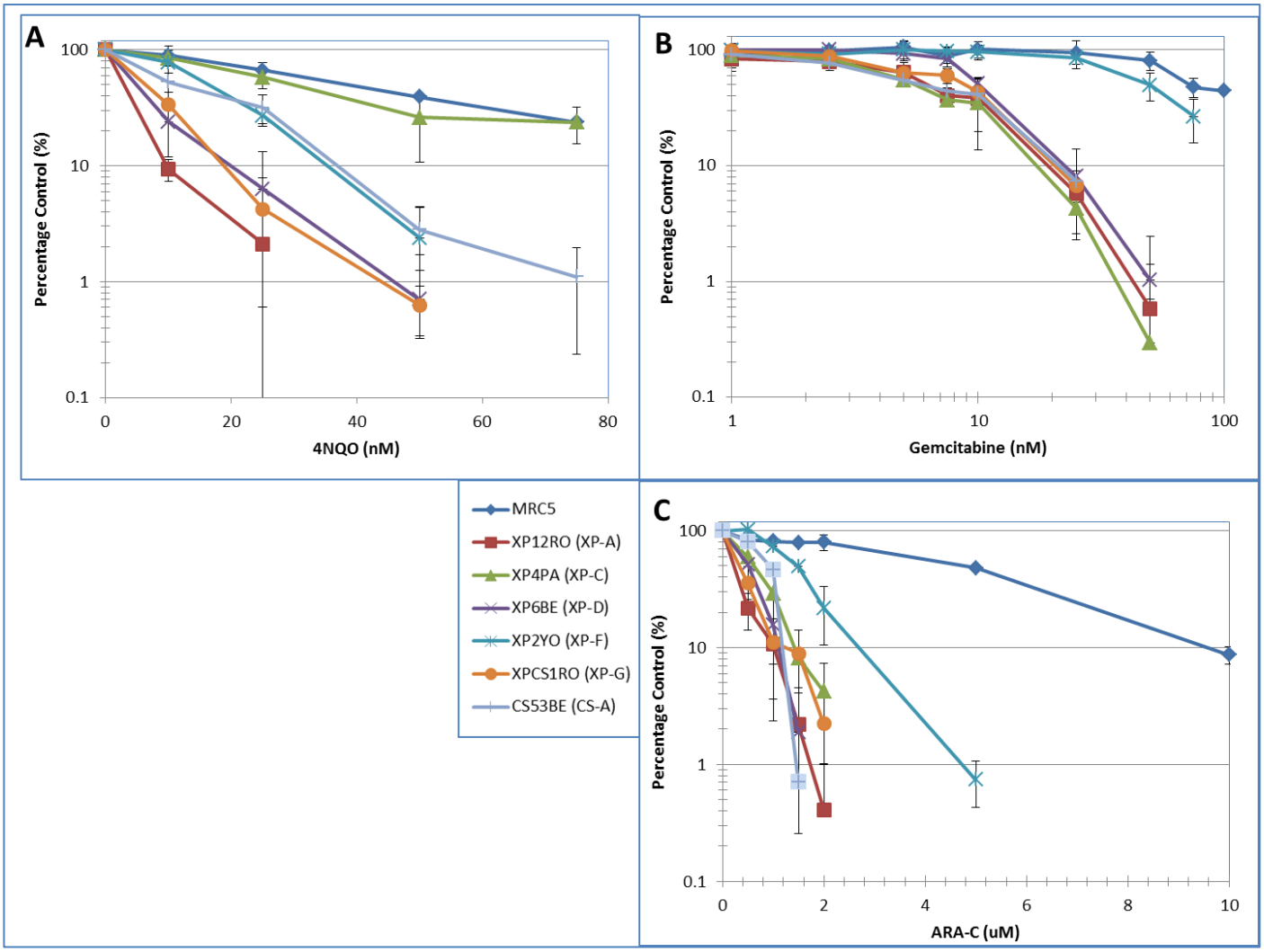

Figure 3.7: NER Deficient Cell Lines Are Sensitive to Nucleoside Analogues Gemcitabine and Cytarabine. Colony forming assay survival data after 24 hour drug exposure. (**A**) 4NQO, (**B**) gemcitabine (**C**) Cytarabine. Values expressed as mean percentage of each cell lines untreated controls. Error bars indicate standard deviations of 3 independent repeats. Gemcitabine X-axis scale is logarithmic due to multiple data points at low and high concentrations.

Table 3.2: LC_{50} dosages and fold increase sensitivity compared to MRC5 controls for Gemcitabine, Cytarabine and 4NQO extrapolated from survival data (Fig.3.7)

	Gemcitabine LC_{50} (nM) *	Fold Increase	Cytarabine (ARA-C) LC_{50} (μM)	Fold Increase	4NQO LC_{50} (nM)	Fold Increase
MRC5	72.3	1	5.0	1	45.0	1
XP12RO (XP-A)	9.25	7.8	0.6	7.8	5.8	7.8
XP4PA (XP-C)	8.45	8.6	0.8	6.1	39.4	1.1
XP6BE (XP-D)	13.7	5.3	0.7	7.0	8.0	5.6
XP2YO (XP-F)	52.7	1.3	2.0	2.5	17.0	2.6
XPCS1RO (XP-G)	10.6	6.8	0.7	7.3	8.6	5.2
CS3BE (CS-A)	9.4	7.7	0.9	5.4	16.0	2.8

*LC_{50} (lethal dose 50) defined as concentration required to produce 50% of the colonies seen in respective untreated controls.

The XPC cell line shows no significant sensitivity to 4NQO when compared to the MRC5 cell line control as shown in Figure 3.6A. The calculated LC_{50} value for the XP-C deficient cell line XP4PA is 1.1x that of wild type (Table 3.2). The CS-A and XP-F cell lines show intermediate sensitivity to 4NQO, which is consistent with their relatively mild patient phenotypes. Whereas cell lines deficient in the core NER factors XPA, XPG and XPD show highest sensitivity to 4NQO with extrapolated LC_{50} values of above 5-fold lower than the MRC5 cell line (Table 3.2). However, for both nucleoside analogues gemcitabine and cytarabine, all NER mutant cell lines are sensitive except XP-F deficient XP2YO cell line which shows the lowest sensitivity in comparison to MRC5 (LC_{50} 1.3x and 2.5x fold respectively, Table 3.2). For all NER-deficient cell lines except XP2YO (XP-F), the calculated LC_{50} values are greater than 5-fold higher than wild type. Whereas with 4NQO, XP-C shows weak sensitivity due to redundancy between GG-NER and TC-NER. However the CS53BE cell line shows a fold increase sensitivity to 4NQO of 2.8x, which is consistent with reports that 4NQO lesions are predominantly repaired by TC-NER (Dollery et al., 1983). Both XP4PA and CS53BE cell lines show high sensitivity to the nucleoside analogues gemcitabine and cytarabine suggesting that their role in initiating NER is different from their response to nucleoside analogues.

Based on colony survival data seen in Figure 3.7, we wanted to investigate the mechanism that kills NER-deficient cell lines in response to nucleoside analogues. Following the activation of the classical apoptosis pathway by the permeabilsation of the mitochondria membrane, pro-caspases are converted into active caspases which are responsible for cleaving many hundreds of substrates in preparation for cell death (Houtgraaf et al., 2006). One such substrate is the poly(ADP-ribose) polymerase (PARP-1) which is involved in BER and DSB repair (Soldani et al., 2001). Furthermore, the degradation of the cell membrane exposes phosphatidylserine to the external environment and renders the cell membrane leaky. The Annexin V antibody binds exposed phosphatidylserine phospholipids and propidium iodide is able to bind the DNA of cells with comprised cell membranes. The activation of classical apoptosis is indicative of these cellular markers being present.

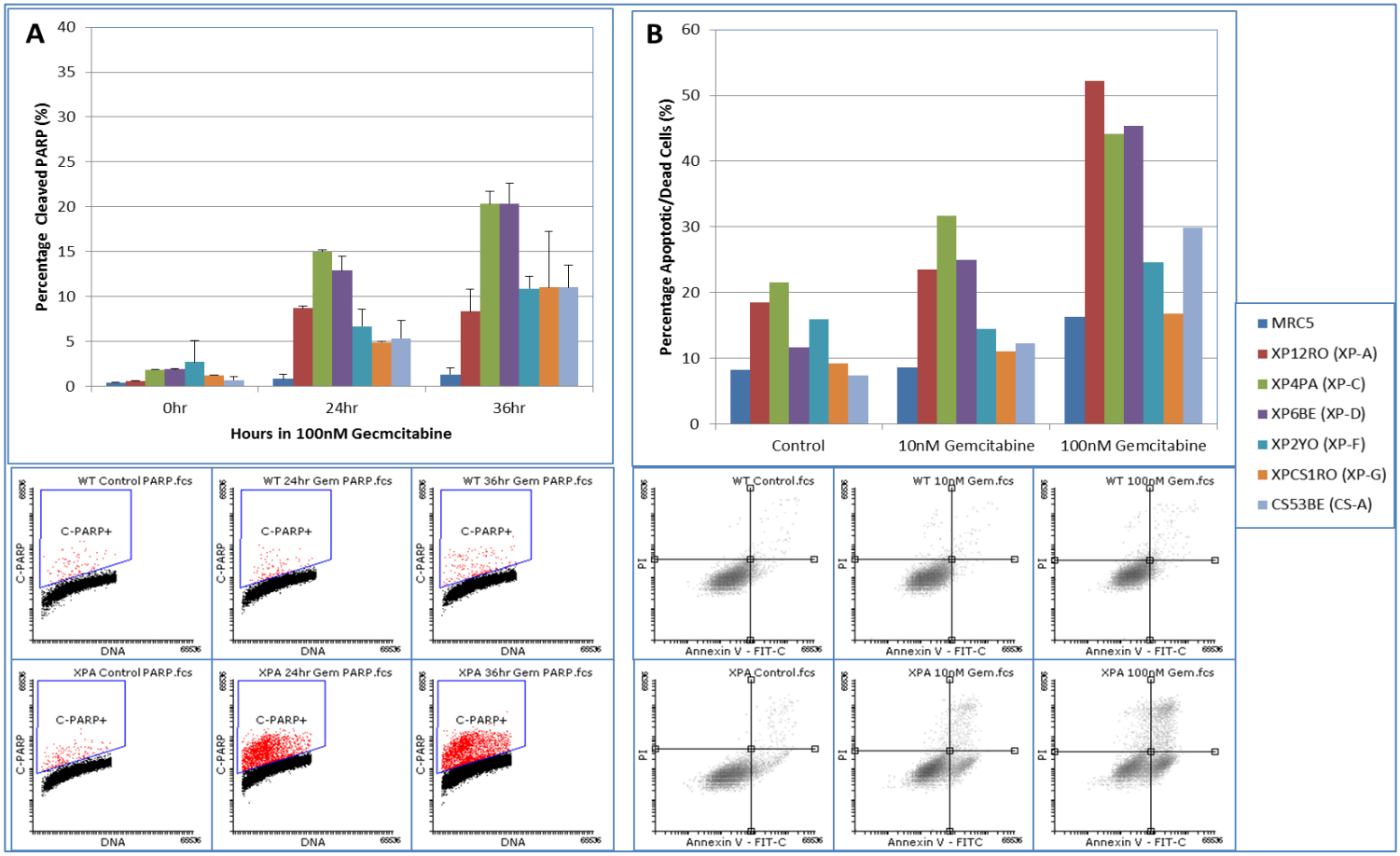

Figure 3.8: NER Cell Lines Undergo Classical Apoptosis in Response to Nucleoside Analogues. (**A**) Flow cytometry data using fluorescently labelled cleaved PARP antibody 24, and 36 hours after 100 nM gemcitabine treatment. (**B**) Annexin V/Propidium iodide apoptosis detection following 30 hour incubation with 10 nM or 100 nM gemcitabine, values show average of two independent repeats. Lower panels show increased cleaved PARP in XP-A cells (left) and increased Annexin V/PI staining (right) compared to MRC5 cells. Graphs above indicate percentage detectable apoptosis based on non-drug treated controls.

As shown in Figure 3.8, the increased cleaved PARP and increased annexin V signal shows NER deficient cell lines, particularly XP-A, XP-C and XP-D, enter classical apoptosis in response to gemcitabine, both time and dose dependently. This apoptosis data correlates with colony forming ability after gemcitabine treatment (Fig.3.8) showing that XP-A, XP-C and XP-D cells are the most sensitive to nucleoside analogue treatment. However although present, the apoptotic signal seen in XP-G deficient cell line (XPCS1RO) is weaker than expected, almost the same as MRC5, potentially suggesting that cell death in response to nucleoside analogues occurs necrotically, rather than via apoptosis. However, reports indicate that XP/CS cells enter apoptosis more readily after UV irradiation than pure XP phenotype cell lines due to increased Bax expression and low p53 nuclear localisation (Clément et al., 2007). It is therefore also possible that the XPCS1RO cell line enters a senescent state rather than entering apoptosis, which results in a low annexin V signal. inconsistently with colony forming data, XP-F deficient XP2YO cell line shows an apoptosis activation as measured by cleaved PARP and annexin V following gemcitabine incubation, possibly caused by differences between acute and transient drug exposure in colony forming and flow cytometry experiments.

Discussion

The classical mechanism by which nucleotide excision repair is carried out, from recognition, verification, incision and re-ligation has been well reported and characterised (Hanawalt, 2002; Kamileri et al., 2012; Vermeulen, 2011). This repair pathway, in conjunction with the translesion polymerase Polη, has been shown to be critical in humans in removing DNA damage resulting from UV irradiation, and also several chemotherapeutic crosslinking drugs such as cisplatin (Wang et al., 2004). Several reports linking nucleoside analogues to cisplatin adduct repair suggest that gemcitabine inhibits the repair synthesis stage of NER, thus preventing the closure of short ssDNA gaps (Crul et al., 2003; Moufarij et al., 2003). However, results here (Fig.3.7 and Fig.3.8) suggest that NER-deficient cell lines are sensitive to nucleoside analogues in the absence of any secondary DNA damaging agent such as cisplatin, therefore indicating that gemcitabine incorporation during replication is cytotoxic to cells that are unable to perform efficient NER.

The increased sensitivity seen in NER deficient cell lines to gemcitabine and cytarabine in this work seem to differ from data gathered from 4NQO controls (Fig.3.7). High sensitivity seen in both TC-NER and GG-NER deficient cell types and low sensitivity seen in XP-F deficient cell lines suggests that classical NER is unlikely to be the main mechanism in which gemcitabine is removed from DNA. Although all other NER-deficient cell lines tested here show high sensitivity, it may seem that several of these protein factors may be participating in other pathways for the removal of nucleoside analogues from DNA with non-NER functions that are yet uncharacterised. The results shown here are consistent with the previous results from the lab using the model organism *S. pombe* in that cells lacking XPA^{Rhp14} and $XPC^{Rhp41/42}$ are sensitive to nucleoside analogues, however XPG deficient human cells are sensitive to gemcitabine and cytarabine, whereas XPG^{Rad13} *S. pombe* mutants are not. The *S. pombe* XPF mutant homolog Rad16 showed no sensitivity to nucleoside analogues, however, results shown here indicate a weak sensitivity in human cells.

If it is the case that classical NER is not occurring at sites of gemcitabine incorporation, what can be the function of the NER proteins in protecting from DNA damage? Unique repair mechanisms have been suggested involving several NER factors in repair of novel nucleoside analogue induced lesions (Wang et al., 2007). A novel cytidine analogue, 2'-C-cyano-2'deoxy-1-β-D-arabino-pentofuranosylcytosine (CNDAC) which incorporates into newly synthesised DNA similarly to gemcitabine, however prevents 3' phosphodiester linkage to the next incorporated nucleotide, has been shown to cause sensitivity in some NER-deficient cell lines (Wang et al., 2008). This missing 3' phosphodiester bond causes a single-strand nick, which was shown to be recognised by CSB in TC-NER, followed by 3'-5' helicase unwinding by XPB, and the resulting flap removed by XPF-ERCC1. The 3' involvement of XPG and XPD was shown not to be required by this substrate (Wang et al., 2007, 2008, 2012). This 5' only model of NER does not fit with our observed sensitivities that XP-A, XP-C, XP-D, XP-G, CS-A and to a lesser extent XP-F cell lines are all sensitive to gemcitabine and cytarabine.

Several conflicting reports have linked gemcitabine survival to efficient HR in different cell lines. It has been shown that BRCA-1 deficient cells are sensitive to gemcitabine (Alli et al., 2011), however cells lacking the HR proteins BRCA-2 and RAD51 show reduced DSB

formation after nucleoside analogue treatment (Jones et al., 2014). It seems likely that efficient HR processes gemcitabine induced stalled replication forks and prevents replication forks collapsing and forming DSBs. However, no NER proteins have been reported to be involved in HR except the XPF-ERCC1 complex (Ahmad et al., 2008; Al-Minawi et al., 2009) which shows little sensitivity to gemcitabine (Fig.3.6). Therefore HR is unlikely to be the only mechanism that confers resistance to nucleoside analogues.

The role of checkpoint signalling is extremely important in overcoming DNA damage, either by enabling the successful recruitment of other repair factors, or halting the cell cycle to prevent further damage accumulation. XP-A deficient cells have been shown to lose their ability to successfully activate the damage signalling protein, ataxia telangiectasia mutated and rad3-related (ATR) following UV irradiation. This loss of DNA damage signalling was shown to be due to failed interaction between XPA and the ATR activating protein ATRIP (Bomgarden et al., 2006; Li et al., 2011; Wu et al., 2007). Although this work showed that XP-C, XP-G and XP-F cells were not deficient in ATR signalling following UV irradiation, it may be that the nucleoside analogue sensitivity seen in NER deficient cells is due to low or absent DNA damage signalling.

Chapter 4 – Nucleoside Analogues Prevent S-Phase Progression and Cause yH2AX Activation in NER Deficient Cell Lines

Summary

Gemcitabine is known to cause S-phase checkpoint activation via the ATR-CHK1-Cdc25A pathway which slows DNA replication (Parsels et al., 2010). Work in this chapter shows that in the absence of several key NER factors, S-phase checkpoint activation occurs at comparatively low concentrations of gemcitabine which causes yH2AX activation, S-phase accumulation and replication inhibition in these cell lines. Strikingly, NER-deficient XP4PA (XP-C) and XPCS1RO (XP-G) cell lines that are sensitive to gemcitabine continue to incorporate BrdU in the presence of gemcitabine indicating a defective S-phase checkpoint activation. In addition phospho-ATM activation is present in XP-C and XP-D deficient cell lines, but not other cell lines tested, potentially indicating the presence of DSBs.

Introduction

To successfully overcome DNA damage, efficient repair and appropriate signalling is required. DNA damage signalling is important to recruit the relevant repair machinery to the sites of damage, as well as to halt cell cycle progression, which serves to prevent further accumulation of mutations (Hanahan and Weinberg, 2011; Hanahan et al., 2000). Several key signalling cascades exist to quickly and effectively halt DNA replication and cell division at specific phases of the cell cycle. Damage to DNA from chemotherapeutic attack has been shown to cause checkpoint signalling activation, and cancer cells are able to overcome checkpoints if the relevant signalling kinases are mutated or upregulated.

S-Phase Checkpoint

Following the detection of DNA damage during cell division, it is important to halt further replication to reduce additional damage, prevent lesions collapsing into more serious DSBs,

and prevent mutations that may lead to cancer. In-between key phases of the cell cycle, checkpoints exist to ensure proper genomic integrity. For example, before the initiation of DNA replication, the pRb protein requires hyperphosphorylation by cyclin dependent kinase (CDK4/6) before releasing E2F transcription factors from its complex (Murphree and Benedict, 1984). This phosphorylation event forms the basis of the G1/S phase block which prevents entry into the cell cycle.

The S-phase checkpoint however does not control phase transition, rather the speed of replication during S-phase (Willis and Rhind, 2009). The PI3 kinase proteins ATR and ATM form the upstream regulation of the S-phase checkpoint. When activated, these two proteins phosphorylate checkpoint kinase proteins 1 and 2 (CHK1, CHK2) respectively (Fujinaka et al., 2012; Willis and Rhind, 2009). The activated CHK1 and CHK2 kinases are both able to phosphorylate Cdc25A phosphatase which prevents its proteolytic degradation (Fig.4.1). In the absence of Cdc25A, CDK2-CyclinE cannot be dephosphorylated which prevents the loading of Cdc45 onto DNA replication origins, which ultimately controls origin firing (Ewald et al., 2007; Jobson et al., 2009; Thompson et al., 2012). Without the initiation of new origin firing, S-phase progression becomes increasingly slower which allows time for relevant DNA repair to occur. The initiation of the S-phase checkpoint by ATR or ATM has been shown to be lesion specific. ATM is the main signalling molecule for DSBs, whereas ATR forms the signalling axis for ssDNA and stalled replication forks (Fujinaka et al., 2012; Lee et al., 2013; Uziel et al., 2003).

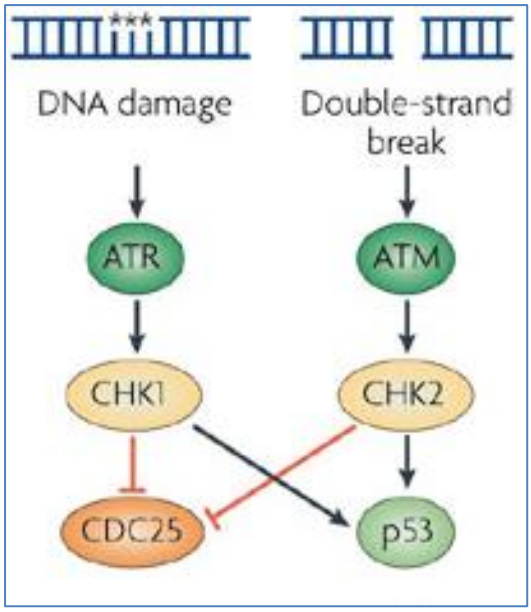

Figure 4.1: S-Phase Checkpoint Activation. Schematic showing DNA damage signalling kinases ATR and ATM activating CHK1 and CHK2 respectively following the detection of damage, resulting in the inhibition of Cdc25A and the activation of p53. (De Veylder et al., 2007 modified)

yH2AX

The most widely reported marker used for the detection of DNA damage is the phosphorylated variant of histone H2A termed yH2AX. Histone modifications have been shown to control many processes in chromatin dynamics, including gene silencing, and chromatin condensation (Bannister and Kouzarides, 2011). In response to DNA damage, the histone subunit H2A has been widely shown to be phosphorylated at serine 139 (Furuta et al., 2003; Kraemer, 2012; Turinetto and Giachino, 2015). This phosphorylation has been shown to be due to the activity of PI3 kinase family members ATM, ATR and DNA-PK (Dickey et al., 2009). At the sites of DNA damage, these signal kinases phosphorylate neighbouring H2AX histones at serine 139 which can vary from one megabase to tens of megabases (Pilch et al., 2011). Several DNA damage lesions are now thought to be marked by the activation of yH2AX, whereas previously thought of as purely a DSB marker, yH2AX has now been shown to be activated in response to stalled replication (Dickey et al., 2009; Elvers et al., 2012; Jones and Petermann, 2012). This stalled replication fork induced activation of yH2AX has been shown widely via co-localisation experiments using fork stabilising factors such as BRCA-1, RAD51, claspin, and RPA (Dickey et al., 2009). These experiments using agents that result in stalled replication forks such as interstrand crosslinking drugs (Mitomycin C), UV irradiation (CPD's and 6-4 photoproducts), hydroxyurea and gemcitabine have shown that yH2AX is activated in the absence of detectable DSBs (Bogliolo et al., 2007; Ewald et al., 2007; Maria Berra et al., 2013; Mogi and Oh, 2006). Where DSBs are present, yH2AX form clear foci across the nuclei, however, when cells are UV irradiated, or exposed to replication inhibitors, this foci pattern changes into a diffuse pan-cellular yH2AX activation (Marti et al., 2006).

Activated yH2AX seems to be a requirement for replication fork stabilisation via recruitment of FANCD2 in response to interstrand crosslinking agents (ICL) (Bogliolo et al., 2007). These experiments using H2AX point mutants (S136A and S139A) which are unable to be phosphorylated show dramatically reduced FANCD2 activation with a fanconi anemia like phenotype in mouse and immortalised cell line models. Similarly, homozygous S139A mice show increased chromosomal rearrangement and cancer predisposition indicating a role for yH2AX signalling in genomic stability (Bassing et al., 2003).

yH2AX is dephosphorylated by protein phosphatase 2A following completion of DNA repair (Ewald et al., 2007). However, if yH2AX activation persists, a second phosphorylation event occurs at tyrosine 142 (Y142) which switches yH2AX from a DNA repair recruiting role to a pro-apoptotic role (Furuta et al., 2003; Kaushik Tiwari and Rogers, 2013).

Gemcitabine and other nucleoside analogues have been shown to time and dose dependently activate yH2AX in S phase cells in the absence of DSBs (Ewald et al., 2007, 2008b). This yH2AX activation co-localises with activated ATM and the MRN complex, which form the main signalling axis for DSBs and DSB repair, as well as being associated with ATR and CHK1 activation which indicates the presence of stalled replication forks. This cross over between DSB signalling and replication fork signalling makes it difficult to precisely describe the effect of gemcitabine on the stalling of replication forks, and whether or not DSBs are formed from gemcitabine treatment alone.

The role of yH2AX in relation to nucleotide excision repair is poorly characterised. Following UV irradiation it has been shown that XPA and XPC deficient cells have far less yH2AX activation than G1 arrested wild type cells , indicating that initiation of NER is required to active yH2AX in the absence of S-phase replication (Marti et al., 2006). However, another study showed that XP-A cell lines exhibited a high level of yH2AX activation after treatment with interstrand crosslinking agents psoralen and MMC. Whereas XP-F deficient cells showed less yH2AX activation than even NER competent control cells using these two crosslinking agents (Mogi and Oh, 2006), suggesting that XPF is required to activate yH2AX in interstrand crosslink repair.

Work in this chapter relates yH2AX activation and cell cycle progression in gemcitabine treated NER deficient cell lines, where previously no data exists. All NER deficient cell lines tested exhibited greater yH2AX activation following gemcitabine treatment at concentrations that do not affect wild type. However the activation of ATM and the progression of S-phase as measured by flow cytometry and BrdU incorporation seem to be different for each NER deficient cell line tested indicating separate novel roles for each NER factor in resistance to nucleoside analogues.

Results

Before testing NER deficient cell lines for DNA damage signalling, γH2AX detection via flow cytometry in MRC5 control cells was optimised (Fig.4.2).

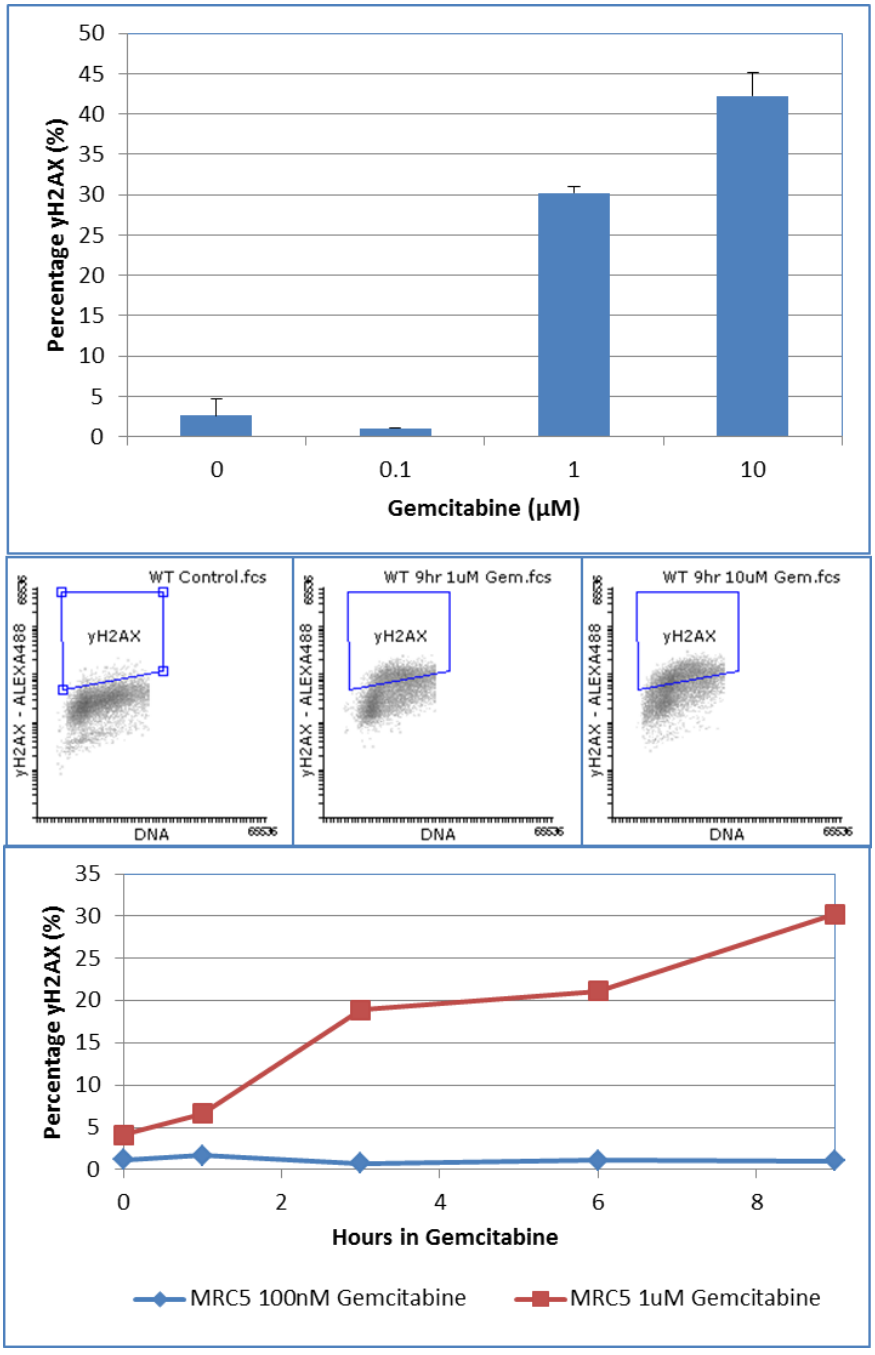

Figure 4.2: Gemcitabine Causes Time and Dose Dependent γH2AX Activation in MRC5 Cells. Flow cytometric γH2AX quantification of MRC5 cells treated with gemcitabine for 9 hours at indicated concentrations (Top), with related propidium iodide (PI)/γH2AX histograms (middle). Time dependent increase in γH2AX activation at 100 nM (blue) and 1 µM (red) (bottom). Data points show average of two independent repeats with γH2AX+ gates applied based on secondary antibody only treated samples and no gemcitabine controls.

As previously reported, gemcitabine induces time and dose dependent yH2AX activation (Ewald et al., 2007, 2008b). As seen in Figure 4.2, 1 µM gemcitabine is required to significantly increase yH2AX activation in MRC5 cells, which increases over 9 hours to 30% of the population, whereas 100 nM gemcitabine treatment fails to induce detectable yH2AX activation via flow cytometry.

Following yH2AX detection in MRC5 cells at high concentrations of gemcitabine, yH2AX activation was tested in NER-deficient cell lines at 100 nM concentration over several time points and further validated by western blotting (Fig.4.3).

As shown in Figure 4.3, NER-deficient cell lines exhibited increased yH2AX activation following comparatively low concentration of gemcitabine (100 nM), at which point yH2AX is not detectable in MRC5 cells. The yH2AX activation occurs in a different pattern in several of the tested cell lines. XP-D deficient XP6BE cells show a yH2AX increase of 20.8% after 1 hour of 100 nM gemcitabine exposure, indicating almost immediate yH2AX phosphorylation which continues to rise over 9 hours. Whereas XP-A, XP-C, XP-G and CS-A deficient cells show an increasing rise in yH2AX after 3 hours. Thirdly, XP-F deficient cell line XP2YO shows a much smoother and slower increase in yH2AX activation over 9 hours, potentially indicating a separate mechanism or signalling cascade in the absence of XPF compared to other NER-defective cell types. yH2AX activation via flow cytometry is validated here by western blotting (Fig.4.3B) showing increased yH2AX protein in extracts from XP-A and XP-C cells treated with 100 nM gemcitabine for 9 hours.

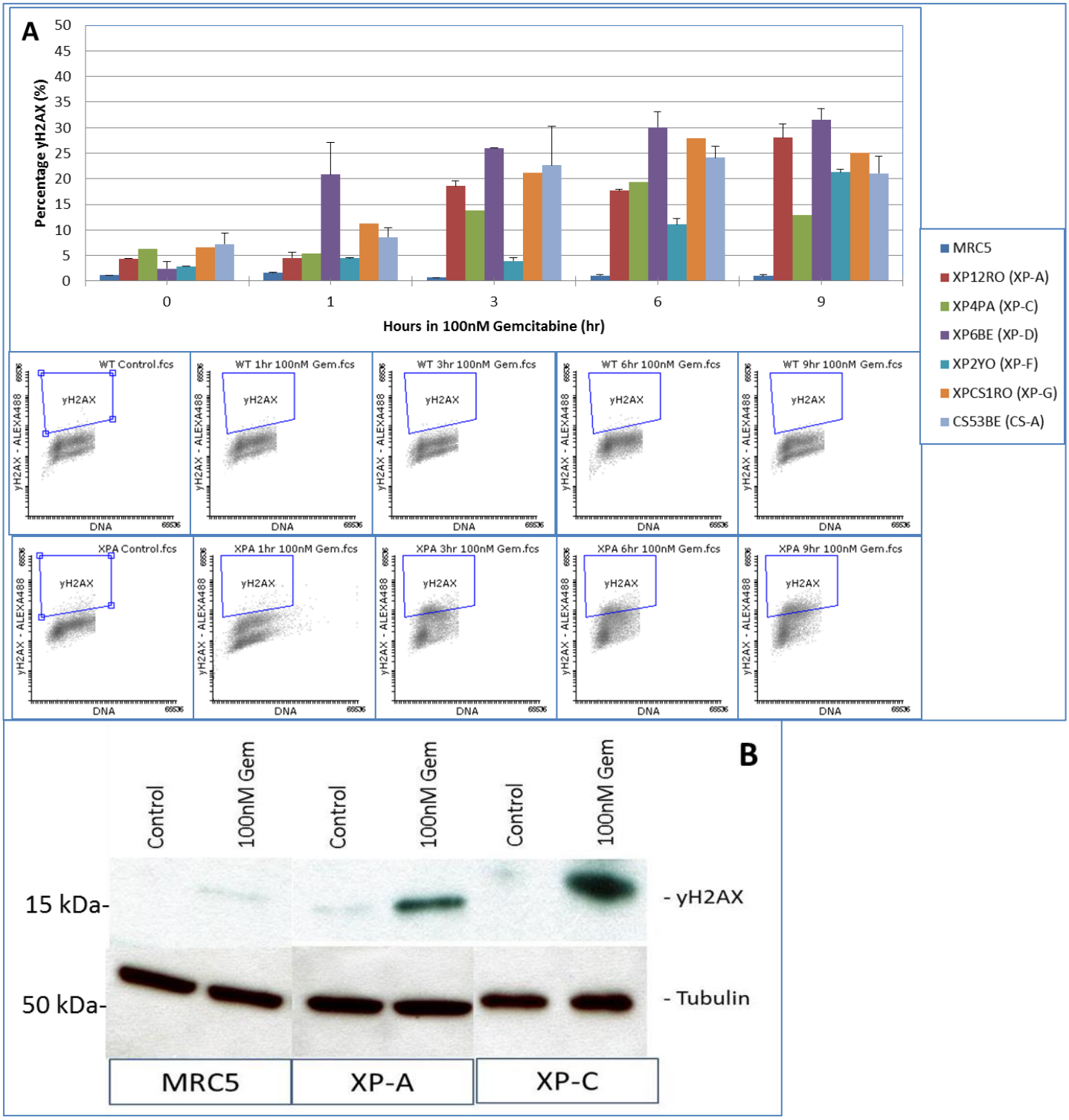

Figure 4.3: NER-Deficient Cells Lines Show Increased γH2AX Activation Following Gemcitabine Treatment. NER deficient cell lines show increased γH2AX activation over time following 100 nM gemcitabine treatment. (**A**) Flow cytometric average γH2AX quantification, error bars indicate standard deviation of 3 independent repeats (2 independent repeats without error bars), zero time point indicates untreated controls. Example histograms of MRC5 cells and XP-A deficient cells beneath. (**B**) γH2AX detection via western blotting showing protein extracts from MRC5, XP-A and XP-C cells treated with 100 nM gemcitabine for 9 hours.

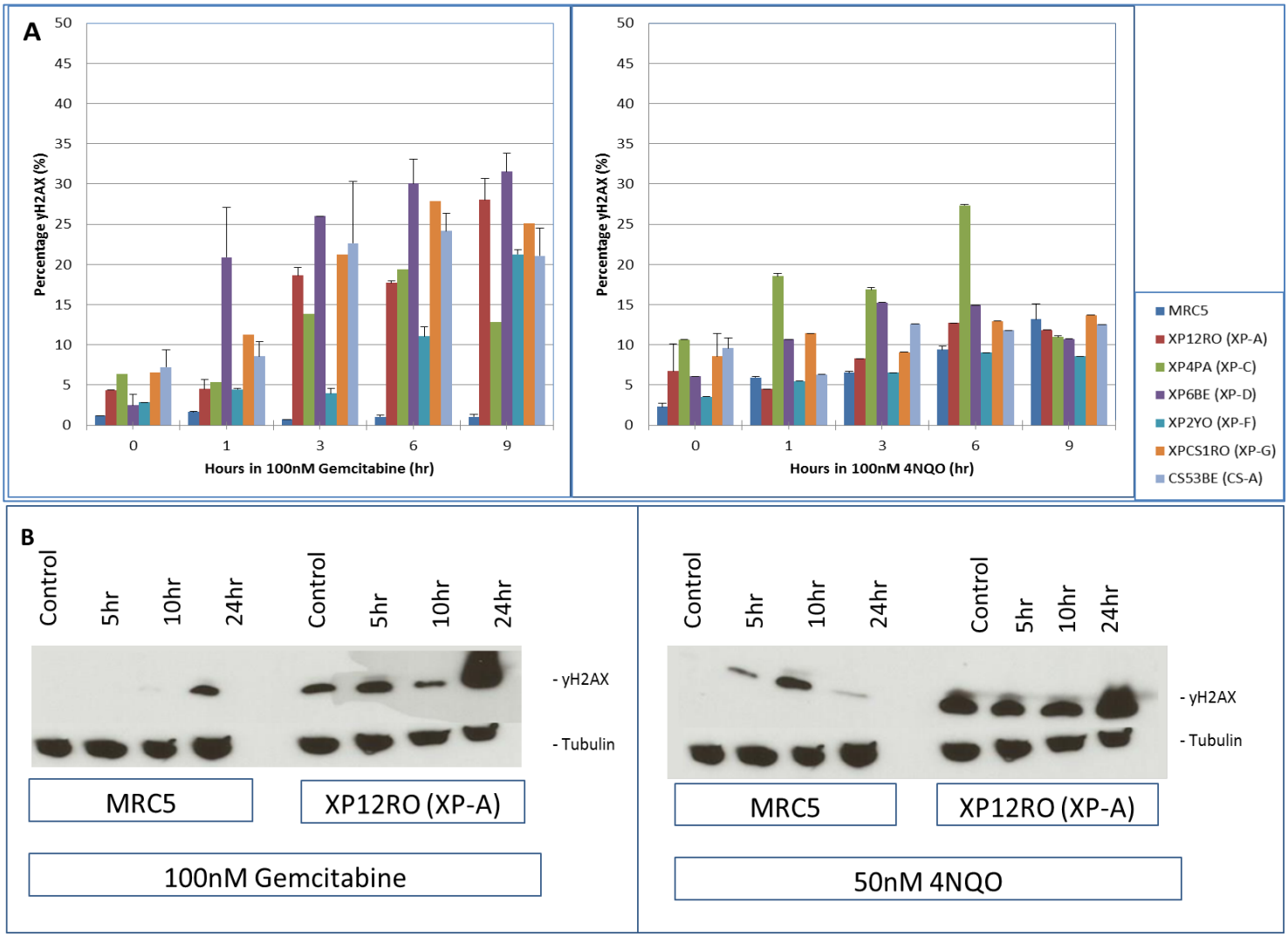

Figure 4.4: γH2AX Activation from Gemcitabine Treatment Occurs Phenotypically Differently Compared to 4NQO Treatment. Flow cytometric quantification of γH2AX (**A**) following 100 nM gemcitabine (Left), and 100 nM 4NQO (Right). Error bars indicate average of at least two independent repeats with standard deviation shown (**B**) Western blot detection of γH2AX following continuous gemcitabine treatment (Left) and 50 nM 4NQO treatment (right) over 24 hours showing tubulin loading controls.

As shown in Figure 4.2 and Figure 4.3A, γH2AX activation after gemcitabine treatment in NER-deficient cell lines occurs quickly, and rises over 9 hours. However as shown in Figure 4.4, following 4NQO treatment, no significant increases in γH2AX activation are seen compared to MRC5 cells, except for the XP-C deficient cell line XP4PA which conversely shows comparatively weak sensitivity to 4NQO (Fig.3.2). γH2AX activation seen in NER proficient cell line MRC5 treated with 4NQO peaks over 10 hours, and decreases again up to 24 hours, as seen in Figure 4.4B (right).

The presence of elevated yH2AX in NER deficient cell lines indicates DNA damage. Following the results in Figures 4.3 and 4.4, the signalling kinase which phosphorylates yH2AX was investigated. The activation of ATM as marked by phosphorylation at serine 1981 was targeted first via indirect flow cytometry.

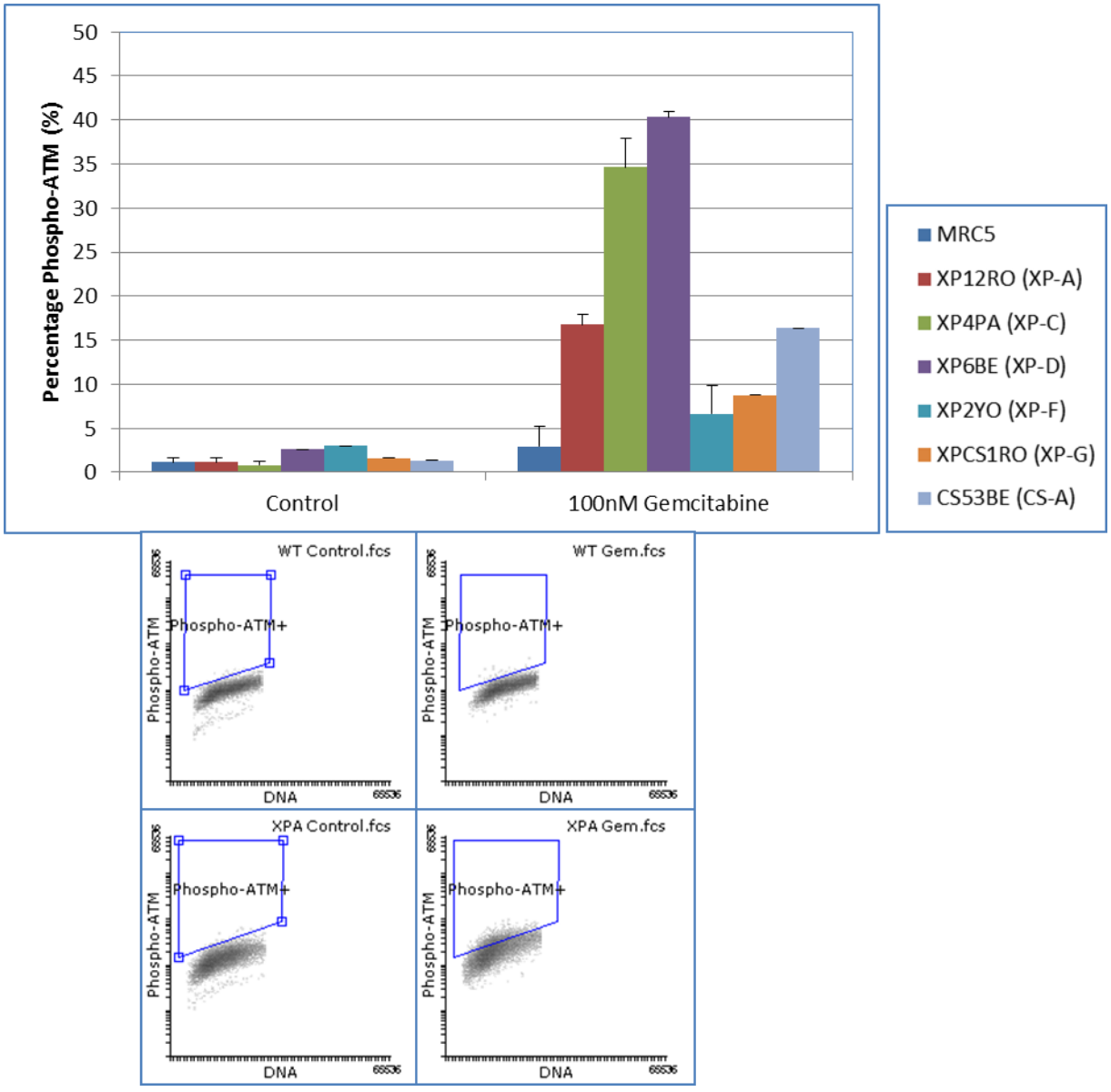

Figure 4.5: Gemcitabine Causes Strong ATM Activation in XP-C and XP-D Deficient Cell Lines. Activated ATM (phosho S1981) assayed via flow cytometry following 9 hour 100 nM gemcitabine treatment. Averages shown of 2 independent repeats with standard deviation. Example flow cytometry histograms shown below from MRC5 and XP-A XP12RO.

Activated ATM (phospho 1981) is the main signalling protein for DSBs which in turn phosphorylates CHK2 leading to S-phase checkpoint activation (Yan et al., 2014). Here, following 100 nM gemcitabine treatment, wild type cells exhibit only a small rise in

detectable ATM activation (2.9%) (Fig.4.5). However XPC and XPD deficient cell lines exhibited a high percentage increase in Phospho-ATM activation of over 35%, whereas XP-A and CS-A cell lines exhibit lower increases in signal. Smaller increases in phospho-ATM signal were detected in XP-F (6.6%) and XP-G (8.8%) cell lines.

Although present in XP-C and XP-D deficient cell lines, the phospho-ATM activation was shown to be relatively low for the remaining NER-deficient cell lines. We then wished to test the effect of ATM activation and the resulting yH2AX activation on the S-phase progression in these cell lines in order to find evidence of S-phase checkpoint activation. Cell cycle progression experiments were performed by single propidium iodide staining of asynchronous cultures and BrdU incorporation.

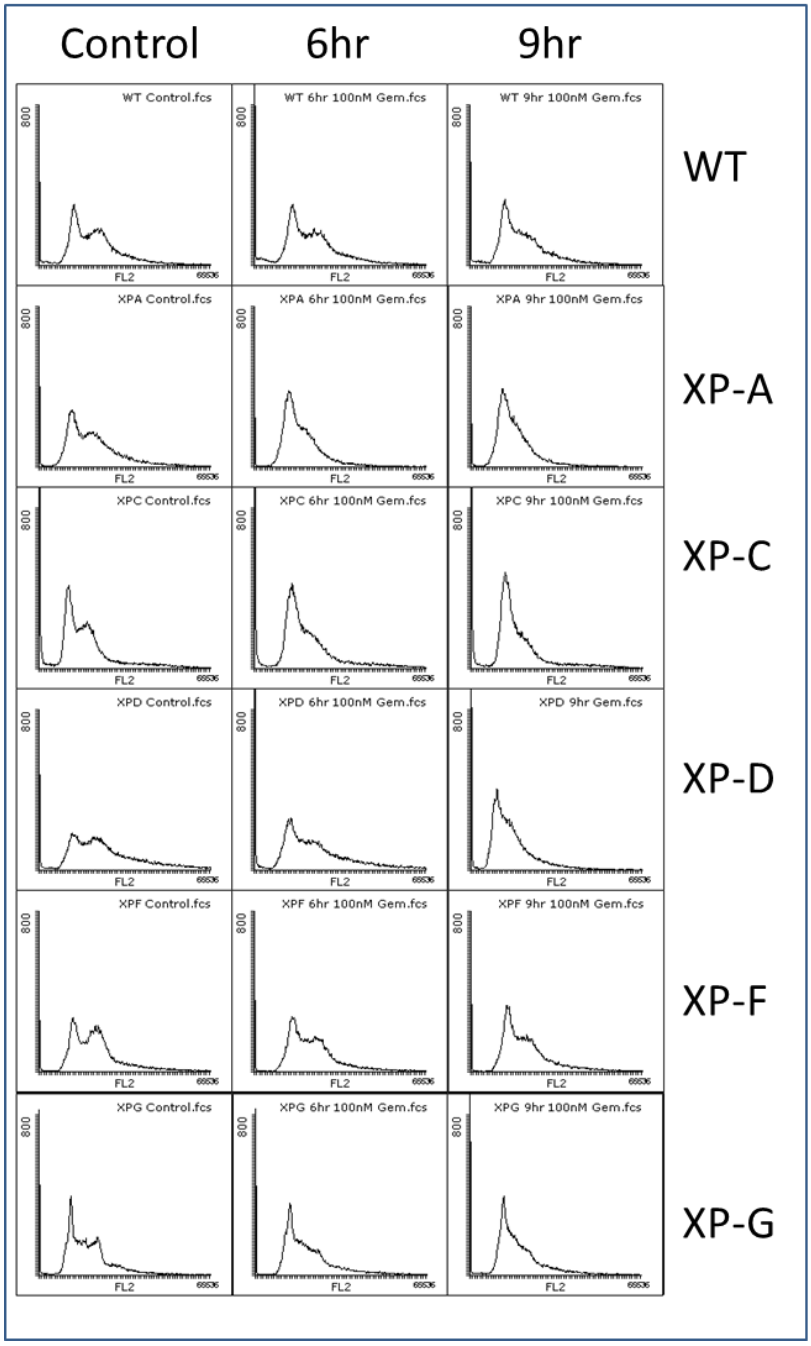

Figure 4.6: Gemcitabine Causes NER Deficient Cell Lines to Accumulate in G1/S. Cell cycle accumulation of NER deficient cell lines following incubation with 100 nM gemcitabine over 6 hours and 9 hours. Controls indicate untreated cell populations. (MRC5 cell line indicated as "WT")

As seen in Figure 4.6, XP-A, XP-C, XP-D, and XP-G deficient cell lines show significant G1/S accumulation following incubation with 100 nM gemcitabine as indicated by an increased G1 peak height and loss of the G2 peak, whereas WT and XP-F cells show a reduced G2/M peak indicating a slowing of S-phase and replication. This indicated that with the exception

of XP2YO (XP-F), NER deficient cell lines accumulate in early S-phase following exposure to gemcitabine. BrdU experiments were then performed to investigate whether this G1/S accumulation was due to a decrease in replication speed.

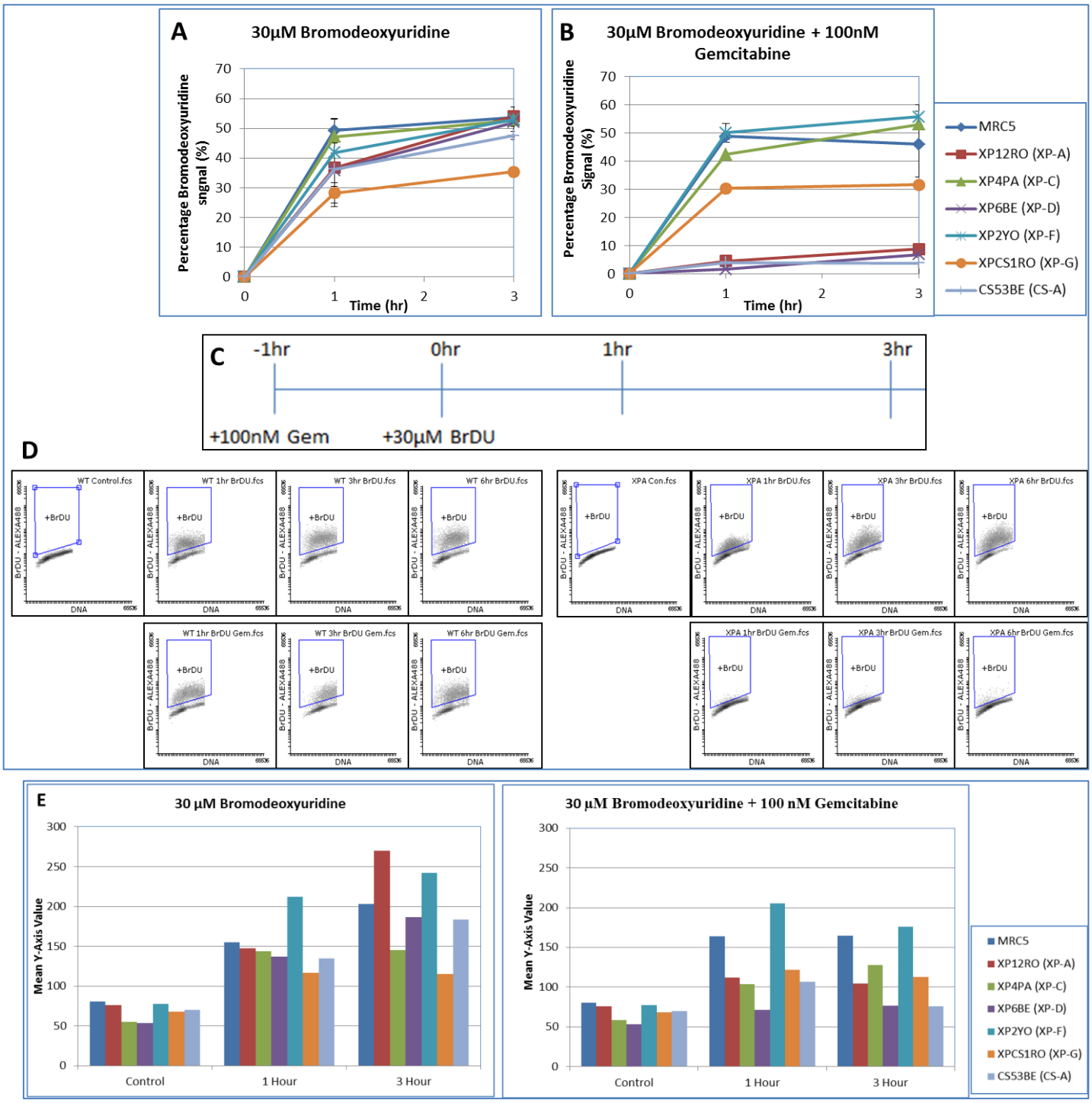

Figure 4.7: Gemcitabine Prevents S-Phase Progression in XP-A, XP-D and CS-A Deficient Cell Lines. Bromodeoxyuridine (BrdU) incorporation following 1 hour 100 nM gemcitabine incorporation assayed via flow cytometry. (**A**) Left, BrdU incorporation without gemcitabine exposure, right, incorporation of BrdU following 1 hour pre-treatment with 100 nM gemcitabine. Data points averaged over 3 independent experiments showing standard deviation. (**C**) Experiment schematic showing pre-incubation and 30 µM BrdU addition with respective assay time points. (**D**) MRC5 WT (left), XP12RO (XP-A) (right) histograms showing BrdU incorporation without gemcitabine (top) and with gemcitabine (bottom). (**E**) BrdU signal quantification expressed as mean Y-axis intensity for untreated controls, 1 hour and 3 hour time points following BrdU addition only (left), and pre-treatment with 100 nM gemcitabine (right).

As shown in Figure 4.7, MRC5 and NER deficient cell lines were pre incubated in 100 nM gemcitabine before the addition of 30 µM BrdU which is incorporated into newly synthesised DNA in the place of a thymidine base. BrdU incorporated molecules were then detected using a fluorescent conjugate antibody following DNA acid denaturing. Following the 1 hour pre-incubation with 100 nM gemcitabine, XP-A, XP-D and CS-A cell lines show dramatically reduced BrdU incorporation suggesting a powerful S-phase checkpoint activation, or a physical replication block. Whereas XP-C, XP-G and XP-F deficient cell lines incorporate BrdU in the presence of gemcitabine only slightly less efficiently than without drug exposure. XP-G and XP-C cells are highly sensitive to gemcitabine (Chapter 3) which potentially reveals separate roles for NER factors in activating the S-phase checkpoint in order to slow replication and encourage cell survival. The XPG deficient XPCS1RO was found to grow slowest of all tested NER cell lines in the absence of any damage which is reflected in slow BrdU incorporation in the absence of gemcitabine relative to the other cell lines (Fig.4.7A).

Discussion

Previous studies have shown that gemcitabine exposure causes yH2AX activation and S-phase checkpoint activation resulting in slower [^{3}H] thymidine incorporation (Ewald et al., 2007). Results presented here confirm these observations in MRC5 cells (Fig.4.2), where concentrations of 1 µM gemcitabine or more bring about time and dose dependent yH2AX activation. It is not known whether yH2AX has an active role in the stabilisation of replication forks, however co-localisation experiments have shown that following induction of DSBs by IR, or treatment with replication inhibitors such as gemcitabine, hydroxyurea, or MMC, yH2AX is rapidly activated by ATR or ATM and co-localises with MRN in DSB repair, and with Fanconi complexes in replication fork stabilisation (Bogliolo et al., 2007; Ewald et al., 2007; Mogi and Oh, 2006; Pilch et al., 2011).

NER-deficient cell lines tested here exhibit increased yH2AX activation compared to controls at concentrations (100 nM) that fail to cause activation in wild type (Fig.4.3). This increased sensitivity in yH2AX activation suggests a predisposition to replication fork stalling or reduced ability to resolve stalled forks resulting from gemcitabine exposure. Most NER mutant cell lines tested exhibit rapid yH2AX activation which persists. However, XP-F cell

line XP2YO shows a slow increase in γH2AX over time which suggests a different mechanism than the other NER factors, or that the XP2YO point mutation in the XPF protein may hold some residue interaction or activity with ERCC1. XPF has been shown to be required for γH2AX activation in response to inter-strand crosslinking agents, where its endonuclease activity is responsible for cleavage of splayed arm DNA substrates (Mogi and Oh, 2006), and in the absence of XPF, γH2AX activation is even less than MRC5 cells. However, in response to gemcitabine shown here, XP-F cell lines exhibit a slow increase in γH2AX indicating an accumulation of damage (Fig.4.3).

The percentage increase in γH2AX activation is far greater in cells treated with gemcitabine than with 4NQO, except for the MRC5 cell line, which shows very little increase in γH2AX signal following gemcitabine incubation. (Fig.4.4). Previous studies have shown that successful initiation of NER is required for γH2AX activation in response to UV via the ATR kinase, and in the absence of GG-NER recognition by XP-C and confirmation by XPA, γH2AX activation is reduced in cells outside of S-phase (Oh et al., 2011; Ray et al., 2013). However, results shown here (Fig.4.4A) indicate an increase in γH2AX activation in XP-C deficient cell line XP4PA following 4NQO exposure, even though the cell line exhibits no sensitivity to 4NQO based on colony forming assays (Chapter 3). As 4NQO lesions have been shown to predominantly be repaired via TC-NER (Waters et al., 1992), it raises the possibility that XPC is required for efficient and appropriate γH2AX signalling in response to transcription coupled NER lesions, as well as replication fork stalling brought about by gemcitabine.

ATM activation via phosphorylation of serine 1981 is also present in XP-A, XP-C, XP-D and CS-A deficient cell lines (Fig.4.5) indicating the presence of DSBs (Uziel et al., 2003). The minor ATM activation in XP-F and XP-G deficient cells suggests that the 5' and 3' endonucleases prevent the formation of DSBs from stalled replication forks. Alternatively, the lack of XP-A, XP-C, XP-D and CS-A may prevent the stabilisation of gemcitabine induced stalled replication fork, which leads to their collapse forming DSBs. The γH2AX activation seen in all NER-deficient cell lines tested does not correlate with the ATM activation seen in Figure 4.5 suggesting that the γH2AX activation seen in XP-F and XP-G cell lines is a result of ATR activation, not ATM, which further indicates that gemcitabine sensitivity seen in NER deficiency is a result of separate failed signalling and replication fork stabilising processes, different from the classical nucleotide excision repair pathway.

Further to the increased yH2AX and ATM activation seen in NER mutants, gemcitabine causes G1/S phase accumulation in most of the mutant cell lines tested (Fig.4.6). Accumulation at the early G1/S border in all cell lines, except XP-F indicates that S-phase is dramatically slowed in the presence of gemcitabine. XP-F cell line XP2YO cell cycle profile looks similar to MRC5 controls after 9 hours in 100 nM gemcitabine with slight S-phase delay. These observations are substantiated in Figure 4.7 where cells deficient in XP-A, XP-D and CS-A fail to incorporate BrdU in the presence of gemcitabine indicating a powerful S-phase checkpoint activation. For these three complementation groups this result correlates with their ATM activation which likely brings about S-phase arrest via the ATM-CHK2 axis. However, for XP-C deficient cell line XP4PA which shows G1/S phase accumulation, and ATM activation, BrdU is still incorporated in the presence of gemcitabine, (although over shorter gemcitabine incubation).

In the case of XP-C deficiency, it has been shown here that gemcitabine induces yH2AX activation, G1/S phase cell cycle accumulation, and phospho-ATM activation indicating S-phase checkpoint activation. However the XP-C deficient cell line XP4PA continues to incorporate BrdU in the presence of gemcitabine (Fig.4.7). The presence of markers of S-phase checkpoint activation, whilst continuing to incorporate BrdU potentially means that XPC may be involved in the downstream steps of the S-phase checkpoint which prevents new origin firing. Therefore the absence of XPC may result in the continued initiation of replication which is ultimately stalled by gemcitabine leading to the G1/S phase accumulation seen here.

The results presented here show increased yH2AX and ATM activation in NER-deficient cell lines in response to gemcitabine at concentrations that do not affect MRC5 controls. From these data the hypothesis that NER factors are involved in the stabilisation of gemcitabine induced replication forks develops, and in the absence of these factors, DNA damage signalling via either ATM or ATR is hyper activated. It is therefore prudent to test whether the inhibition of DNA damage signalling further sensitises NER deficient cell lines to gemcitabine (Chapter 5).

Chapter 5: CHK1 and ATR Inhibition Hyper Sensitises NER Deficient Cell Lines to Gemcitabine and Stalled Replication Forks

Summary

Previous results indicate that NER factors are required for the resistance to nucleoside analogues. Results from the literature indicate that inhibition of the ATR-CHK1-Cdc25A signalling cascade increases nucleoside analogue sensitivity. Therefore it is possible that NER factors are required for the stable and complete activation of the S-phase checkpoint or that the S-phase checkpoint recruits NER factors to stalled replication forks which helps in their stabilisation. Results presented here show that NER-deficient cells are intrinsically more sensitive to ATR and CHK1 inhibition than MRC5 cells, and that in the absence of NER factors the combination of ATR or CHK1 inhibition with gemcitabine treatment induces super-sensitisation, as well as increased yH2AX and ATM activation and increased apoptosis as detected via annexin V antibody binding.

Introduction

As previously discussed, the S-Phase checkpoint plays an essential role in slowing replication and preventing further origin firing when DNA damage is detected in order to allow efficient and accurate repair to occur. The results from Chapter 4 suggest that gemcitabine causes yH2AX activation and S-phase arrest in cell lines that are NER-deficient. It is therefore necessary to elucidate the mechanisms and signalling kinases that are responsible for causing the cell cycle delay seen in these cell lines in order to understand potential non-NER functions of these genes in overcoming nucleoside analogue stress. Identifying the DNA damage signalling axis responsible for the sensitivity observed will help to understand the specific lesions that NER deficient cell lines are struggling to repair and overcome.

The signalling cascades responsible for cell cycle arrest and yH2AX activation as seen in Chapter 4 and with various other types of DNA damage have extensive cross-talk; however there are 3 distinct upstream kinases responsible for sensing and initiating responses that

are lesion specific. Briefly mentioned in Chapter 4, these three PI3 kinase family members, ATM, ATR and DNA-PK are able to activate by phosphorylation a vast list of proteins which bring about cell cycle arrest, DNA repair factor recruitment, apoptosis and even changes in cellular metabolism (Fig.5.1) (Bernstein et al., 2002; Nutley et al., 2005; Prevo et al., 2012; Yang et al., 2004). In recent years these three DNA damage sensing/signalling kinases, as well as several of their downstream proteins, have been identified as potential drug-able targets which if inhibited, can lead to powerful sensitisation of cancer cells to existing and commonly used DNA damaging chemotherapeutics (Facchinetti et al., 2004; Madhusudan et al., 2005; Montano et al., 2013; Prevo et al., 2012).

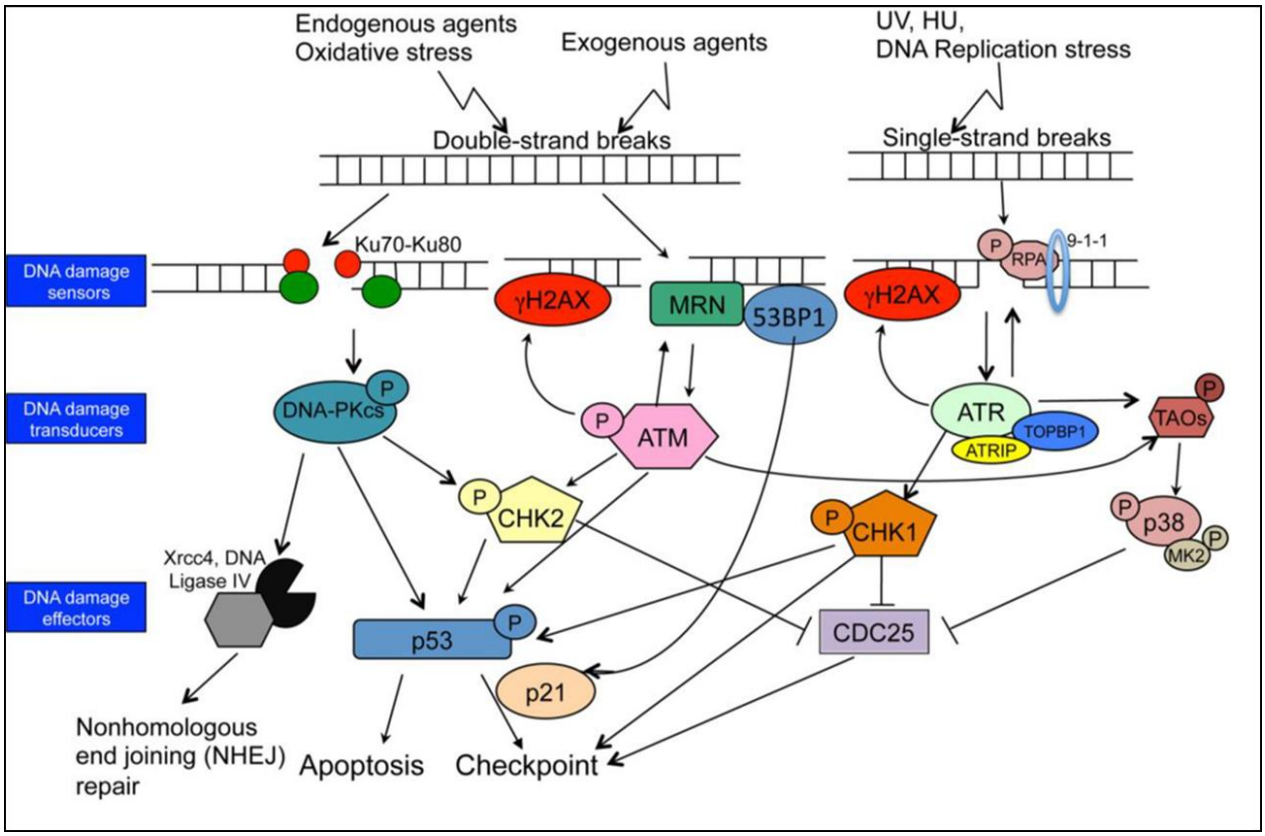

Figure 5.1: Schematic Diagram of S-Phase Checkpoint Signalling via DNA-PK, ATM and ATR Which Brings About Cell Cycle Arrest and/or Apoptosis. (Xiaofei and Kowalik, 2014 (modified) DNA damage kinases DNA-PK, ATM and ATR shown here and the cross talk between CHK1 and CHK2 to bring about the degradation of Cdc25A to halt S-phase progression as well as signalling p53 to mediate the induction of apoptosis. DNA-PK and ATM respond to DSBs, whereas ATR is activated by single-strand breaks and stalled replication forks.

DNA-PK

The proteins Ku70/80 initiate NHEJ repair by binding to the ends of DSBs. When Ku70/80 is loaded onto each end of a DSB, the 470 kDa kinase subunit DNA-PKcs protein is recruited.

This trimer complex is known as DNA-PK. The addition and activation of this kinase subunit causes the phosphorylation of NHEJ repair machinery which begins work on end processing before re-ligation can occur, as well as causing the phosphorylation of checkpoint kinase 2 (CHK2) which further phosphorylates Cdc25A preventing its degradation, leading to S-phase checkpoint activation (Furgason and Bahassi, 2013). Because of its role in DSB repair, cells highly expressing DNA-PK have been shown to be resistant to etoposide and ionising radiation, and inhibition of DNA-PK using the compound NU7026 brings about sensitisation to these DNA damaging agents in MCF-7 cells (Furgason and Bahassi, 2013; Munck et al., 2013). NU7026 is highly selective for DNA-PK over other PI3 kinases and is now in pre-clinical trials in combination with etoposide (Nutley et al., 2005; Palmitelli et al., 2015).

Ataxia Telangiectasia Mutated (ATM)

ATM kinase forms a subunit of the BRCA-1 associated surveillance complex (BASC), and is also responsible for DSB repair signalling (Christmann et al., 2003). ATM in its inactive state forms a homodimer which dissociates in response to phosphorylation or via auto-phosphorylation at serine 1981 (Goodarzi and Lees-Miller, 2004). Phospho-ATM in turn is then able to phosphorylate CHK2 at the tyrosine 68 residue similarly to DNA-PK, which brings about S-phase checkpoint activation (Jobson et al., 2009). As well as CHK2, phospho-ATM is able to activate yH2AX in response to double strand breaks, and conversely phosphorylate NBS1 leading to the co-localisation of the MRN complex with DSBs (Uziel et al., 2003). The activation of ATM leads to the promotion of double strand break repair via phosphorylation of RAD51 and BRCA-1 allowing their loading onto damaged DNA, as well as removing the CtiP mediated inhibition of BRCA-1 loading (Bernstein et al., 2002). In addition to S-phase checkpoint activation, phospho-ATM causes G1/S checkpoint activation via the phosphorylation of p53 at serine 15 which leads to its stabilisation, prevention of its nuclear export, releases it from its inhibitor MDM2 and leads to the transcription of the p21 gene which prevents entry into G1 (Christmann et al., 2003; Goodarzi and Lees-Miller, 2004). Inhibition of ATM activation using the molecule KU-55933 has been shown to sensitise cells to ionising radiation (Golding et al., 2010).

ATM and Rad3-related (ATR)

ATR is the main recognition and signalling kinase for single-stranded DNA and stalled replication forks. ATR has been shown to bind long patches of single-stranded DNA coated in RPA, which in conjunction with the activating protein ATRIP causes ATR activation. Following stalling of replicative polymerases, the helicase containing replication complex does not stop. This helicase uncoupling leads to long patches of ssDNA ahead of the replication fork which require stabilisation by RPA (Schwab et al., 2013). Long patches of ssDNA such as these recruit ATR as well as the 9-1-1 complex and TOPBP1 which causes ATR phosphorylation at serine 428 (Thompson, 2012). Activated ATR is also able to phosphorylate p53 leading to G1/S arrest as well as activate BRCA-1 loading. However, unlike ATM, the main target for ATR is the activation of CHK1 instead of CHK2. ATR phosphorylates CHK1 at serine 345 which in turn phosphorylates Cdc25A in the same way as DNA-PK and ATM (Dai et al., 2008; Myers et al., 2009). ATR activation has been shown to be essential in resisting a wide range of chemotherapeutic agents. ATR RNAi knockdown as well as inhibition using the compound VE-821 have been shown to increase sensitivity to cisplatin, camptothecin and gemcitabine (Dai et al., 2008; Karnitz, 2014; Prevo et al., 2012).

Checkpoint Kinase 1 (CHK1)

Following detection of stalled replication forks or ssDNA, ATR phosphorylates CHK1 at serine 345 and 317, in turn auto-phosphorylating itself at serine 296. As mentioned above, activated CHK1 phosphorylates Cdc25A protecting it from proteosomal degradation which prevents further origin firing (Ewald et al., 2007). However, in addition, CHK1 phosphorylates several other target proteins which results in their recruitment to the site of stalled replication forks leading to their stabilisation (Fig.5.2). CHK1 has been shown to recruit the stabilising proteins Claspin and Tipin to stalled replication forks as well as mediating the exchange of RPA subunits with RAD51 subunits which controls homologous recombination at sites of damage (Elvers et al., 2012). The inhibition of CHK1 in the absence of any other DNA damage insult has been shown to kill several cell types due to the increase in origin firing brought about by increased Cdc25A protein levels. This overactive origin firing causes an increased frequency in which replication forks collide with transcription machinery which ultimately increases the number of endogenous stalled replication forks (Thompson et al., 2012).

Unlike ATR inhibition which broadly sensitises cells to various DNA damaging agents, CHK1 inhibition by UCN-01, AZD7762 or the new and more specific drug MK-8776 specifically increases sensitivity to antimetabolites such as hydroxyurea and nucleoside analogues cytarabine and gemcitabine (Elvers et al., 2012; Karnitz, 2014; McNeely et al., 2010; Myers et al., 2009; Prevo et al., 2012). This increase in sensitivity using these inhibitors is associated with increased yH2AX activation (McNeely et al., 2010), and the conversion of stalled replication forks to DSBs (Karnitz et al., 2005). CHK1 inhibition confusingly increases its serine 345 phosphorylation due to feedback by increased ATR activation, whereas the auto-phosphorylation at serine 296 is blocked. Similarly ATR inhibition blocks CHK1 serine 345 phosphorylation (Karnitz, 2014).

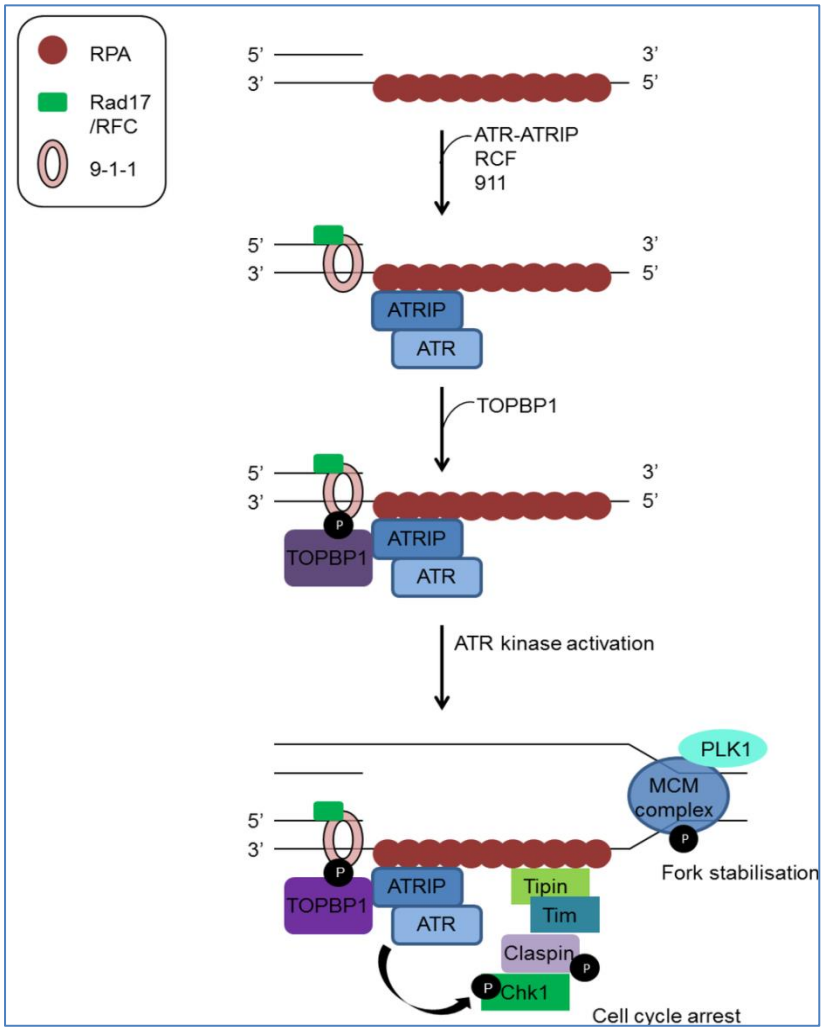

Figure 5.2: Model of ATR-CHK1 Activation At Sites of Stalled Replication (Hollingworth and Grand, 2015 modified). Once activated, ATR phosphorylates CHK1 leading to the recruitment of Tipin, Claspin and several other replication fork stabilising proteins which protect against nuclease attack.

Based on these reported findings, work presented here focuses on the role of ATR and CHK1 in resistance to gemcitabine in relation to the observed finding in NER deficient cell lines,

potentially showing a requirement for NER factors in replication fork stabilisation allowing for the removal of gemcitabine.

Results

To test the requirements of each of the core S-phase DNA damage kinases in resistance to nucleoside analogues, inhibitors of ATR, ATM and DNA-PK were used in combination with gemcitabine in MRC5 cells, before further testing in NER deficient cell lines.

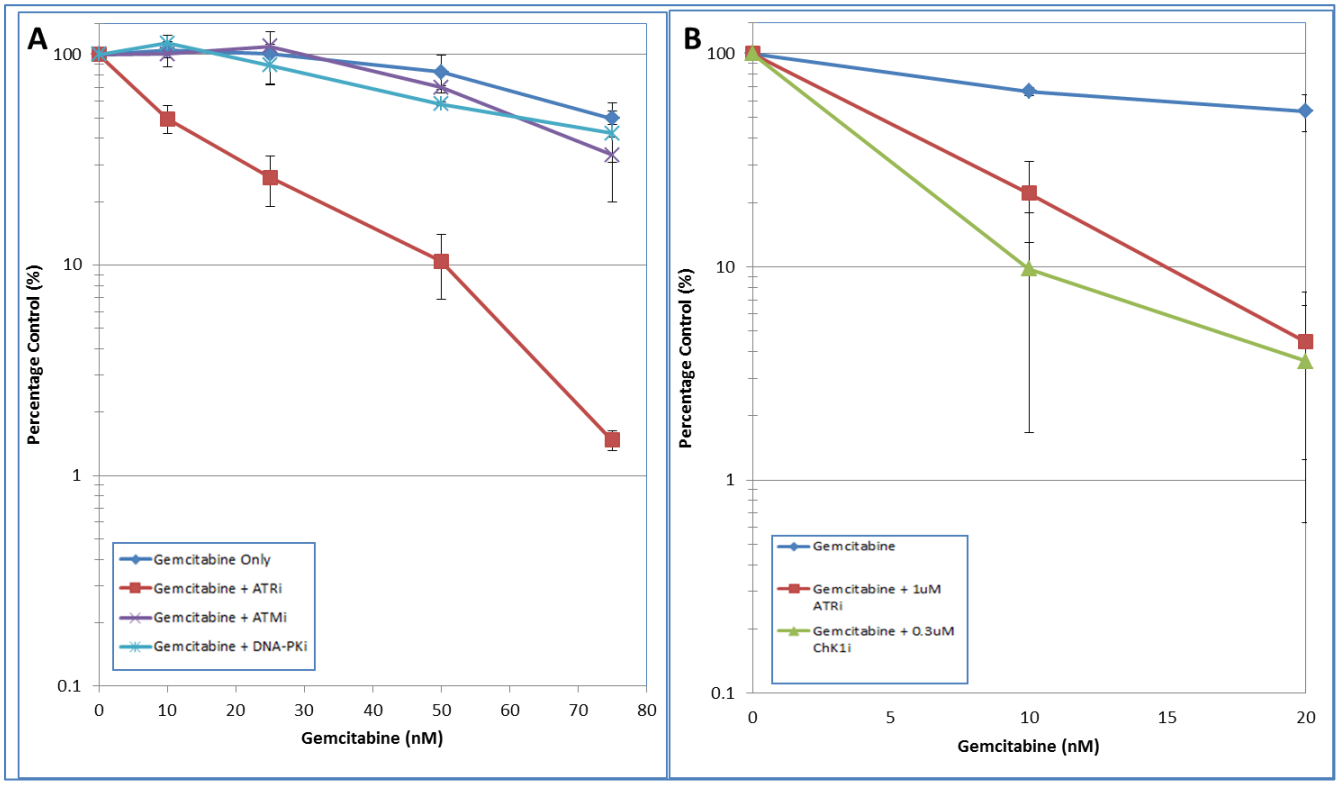

Figure 5.3: CHK1 and ATR Inhibition Sensitises MRC5 Cells to Gemcitabine. (**A**) colony forming assay data from MRC5 cells exposed to concentrations of gemcitabine as indicated for 24 hours +/- 1 µM ATR inhibitor VE-821, 1 µM ATM inhibitor KU-55933 and 1 µM DNA-PK inhibitor NU7026. (**B**) MTS assay data of MRC5 cells exposed to concentrations of gemcitabine for 72 hours +/- 1 µM ATR inhibitor VE-821 or 0.3 µM CHK1 inhibitor MK-8776. Values expressed as percentage untreated controls for gemcitabine only treated cells, or percentage inhibitor only treated controls where appropriate. Error bars show standard deviation of 3 independent repeats.

MRC5 cells treated with gemcitabine in the presence of DNA-PK inhibitor NU7026 or ATM inhibitor KU-55933 show no increased sensitivity compared to gemcitabine only treated cells (Fig.5.3). However, in the presence of the ATR inhibitor VE-821 sensitivity is dramatically increased. ATR inhibition increased the gemcitabine LC_{50} from 75 nM to 17 nM, corresponding to a 4.4-fold increase in sensitivity. Similarly ATR inhibition, as well as MK-8776 inhibition of CHK1 increased sensitivity of MRC5 to gemcitabine as assayed with MTS

cytotoxicity assay. These results indicate an essential role for the ATR-CHK1 S-phase checkpoint cascade in resistance to gemcitabine, whereas the signalling kinases DNA-PK and ATM are not required in MRC5.

The effectiveness of ATM, ATR and CHK1 inhibition was further measured via flow cytometry in the detection of both γH2AX and phospho-ATM flowing gemcitabine incubation.

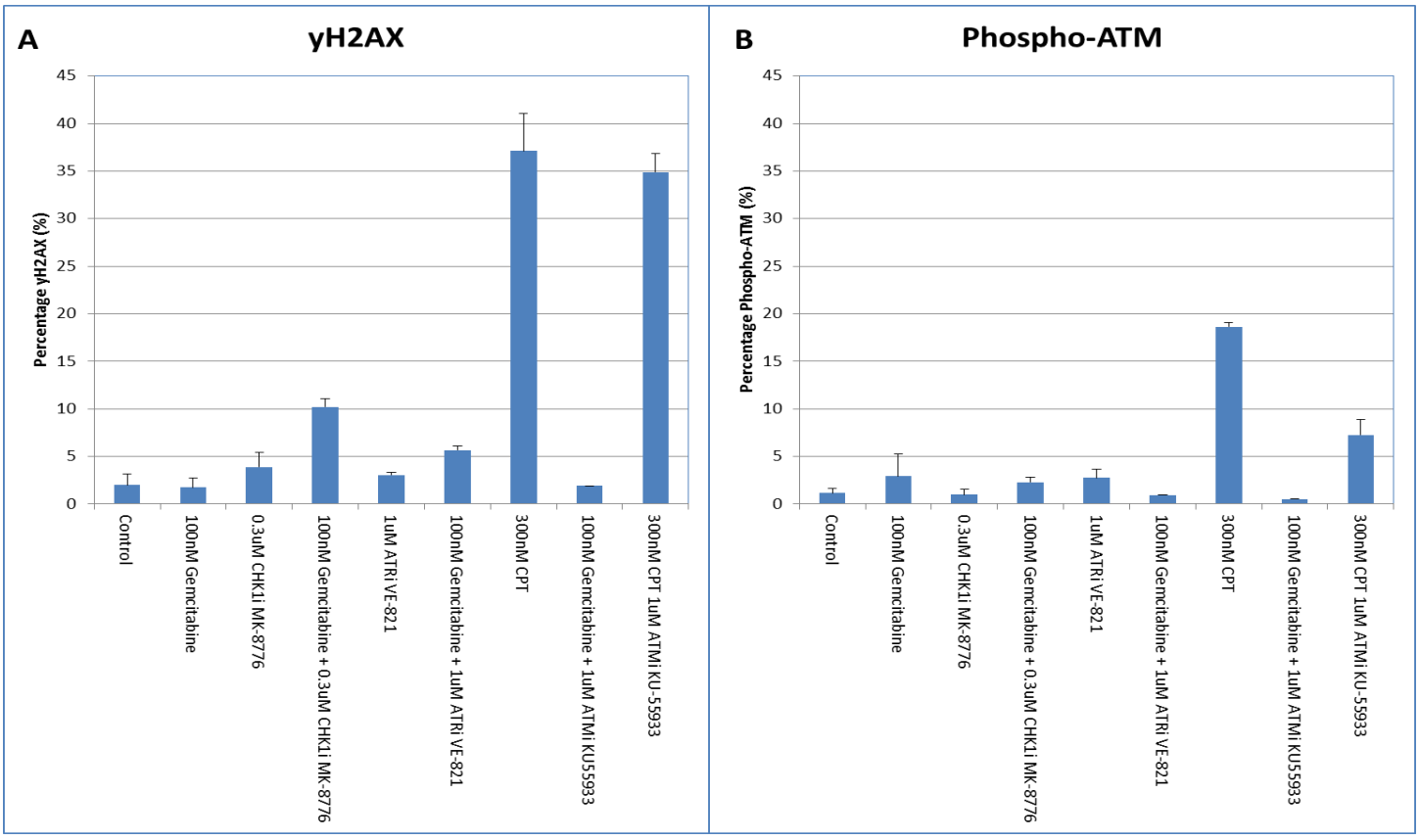

Figure 5.4: CHK1 inhibition in Combination with Gemcitabine Increases γH2AX Activation in MRC5 Cells. Flow cytometric detection of (**A**) γH2AX and (**B**) phospho-ATM following 9 hour incubation with gemcitabine alone, and in combination with CHK1, ATR and ATM inhibitors. Error bars indicate the standard deviation of 3 independent repeats.

As shown in Figure 5.4, minor increases in γH2AX were detected following co-treatment with ATR and CHK1 inhibitors with gemcitabine in MRC5 cells, whereas co-treatment with the ATM inhibitor KU-55933 failed to increase the γH2AX signal. 300 nM camptothecin (CPT) was used as a positive control for both γH2AX and phospho-ATM activation. Camptothecin induced an γH2AX signal increase to 37%, far greater than the nucleoside analogue gemcitabine. This CPT induced γH2AX activation was not significantly reduced by ATM inhibition; however the phospho-ATM activation caused by 300 nM CPT incubation was

reduced from 19% to 7% in the presence of KU-55933. This experiment confirms the activity of the ATM inhibitor at a concentration of 1 µM, which further validates the results seen in Figure 5.3.

The CHK1 inhibitor MK-8776 has previously been shown to induce cell death at high concentrations due to increased S-phase stress (Montano et al., 2013). Based on this observation the sensitivity of NER deficient cell lines to both ATR and CHK1 inhibition was tested.

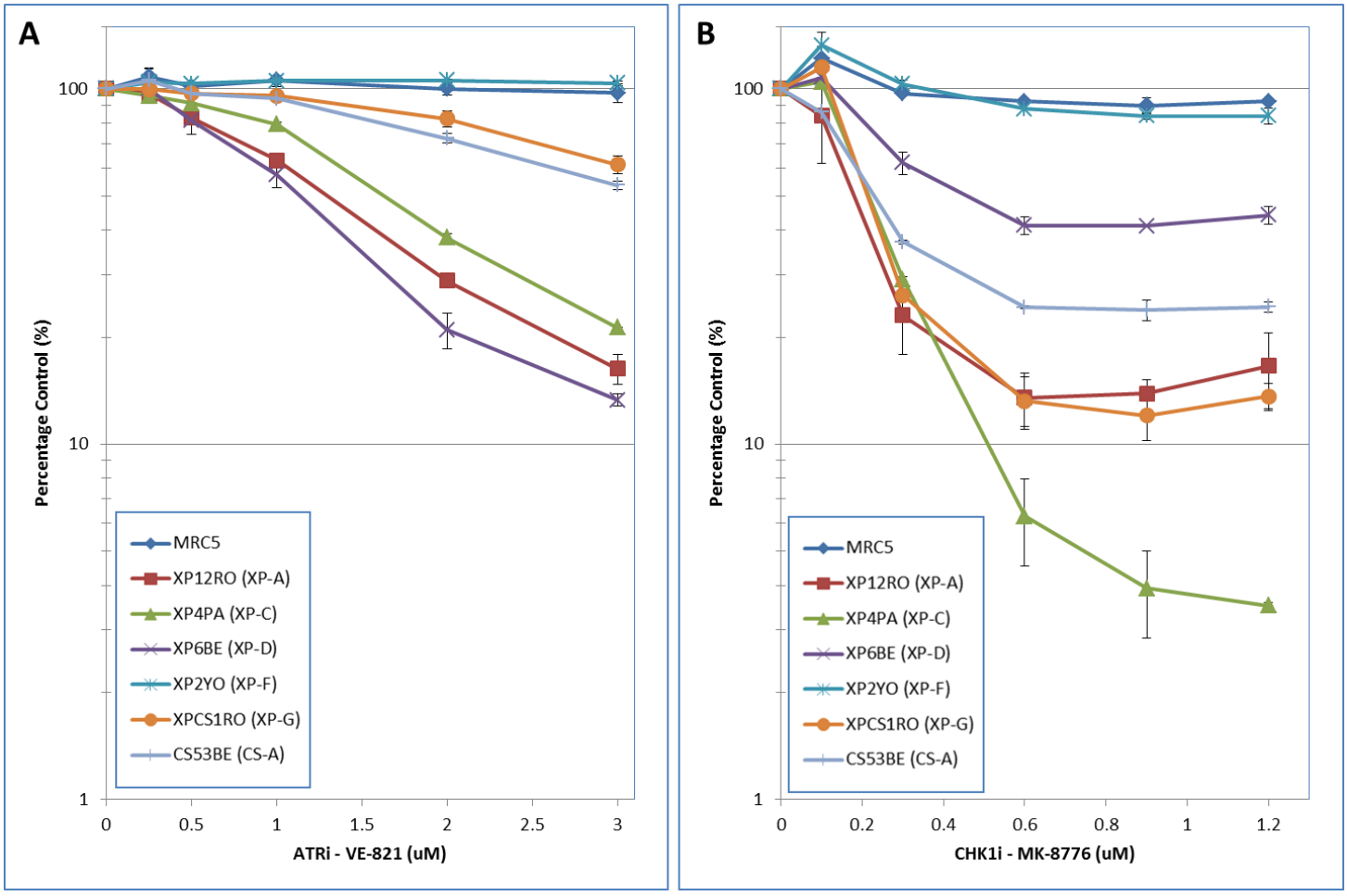

Figure 5.5: NER Deficient Fibroblasts Are Sensitive To ATR and CHK1 Inhibition. MTS 72 hour cytotoxicity of ATR inhibitor VE-821 (**A**), and CHK1 inhibitor MK8776 (**B**). Values expressed as percentage of untreated controls, showing standard deviations of two independent repeat experiments.

Results here demonstrate that most NER-deficient fibroblasts were intrinsically sensitive to inhibition of the ATR-CHK1 S-phase checkpoint signalling cascade (Fig.5.5). However the XP-F deficient XP2YO cell line showed no sensitivity to either ATR or CHK1 inhibition compared to MRC5. This observation combined with lower sensitivity to nucleoside analogues suggests

that XPF is not required for the stabilisation of gemcitabine induced stalled replication forks, or the signalling of endogenous replication stress. However, for some cell lines tested here, sensitivity to ATR inhibition does not necessarily correlate to CHK1 inhibition sensitivity, and vice versa. CS-A and XP-G deficient cell lines CS3BE and XPCS1RO show some intermediate sensitivity to ATR inhibition and high sensitivity to CHK1 inhibition in relation to other XP mutated cell lines tested. Whereas, XP-D deficient cell line XP6BE shows little sensitivity to CHK1 inhibition but tested the most sensitive to ATR inhibition. This observation points to separate roles for each NER factor in the regulation of the ATR-CHK1 pathway, or that in the absence of any of the NER factors, different delicate replication intermediates are formed which results in activation of ATR or CHK1 separately. Slight increases in cell viability at low dosages of CHK1 inhibitor MK-8776 correspond to reported increased new origin of replication firing, which ultimately leads to increased replication speed (Montano et al., 2013).

Based on the sensitivity of XP cell lines to nucleoside analogues and to both ATR and CHK1 inhibition shown here, the effects of combinations of drug treatments were measured next by colony formation assays as well as the MTS cell viability assay in order to test whether their sensitivity can be enhanced (Fig.5.6). XP cell lines were also assayed for induction of apoptosis via annexin V/PI staining (Fig.5.7).

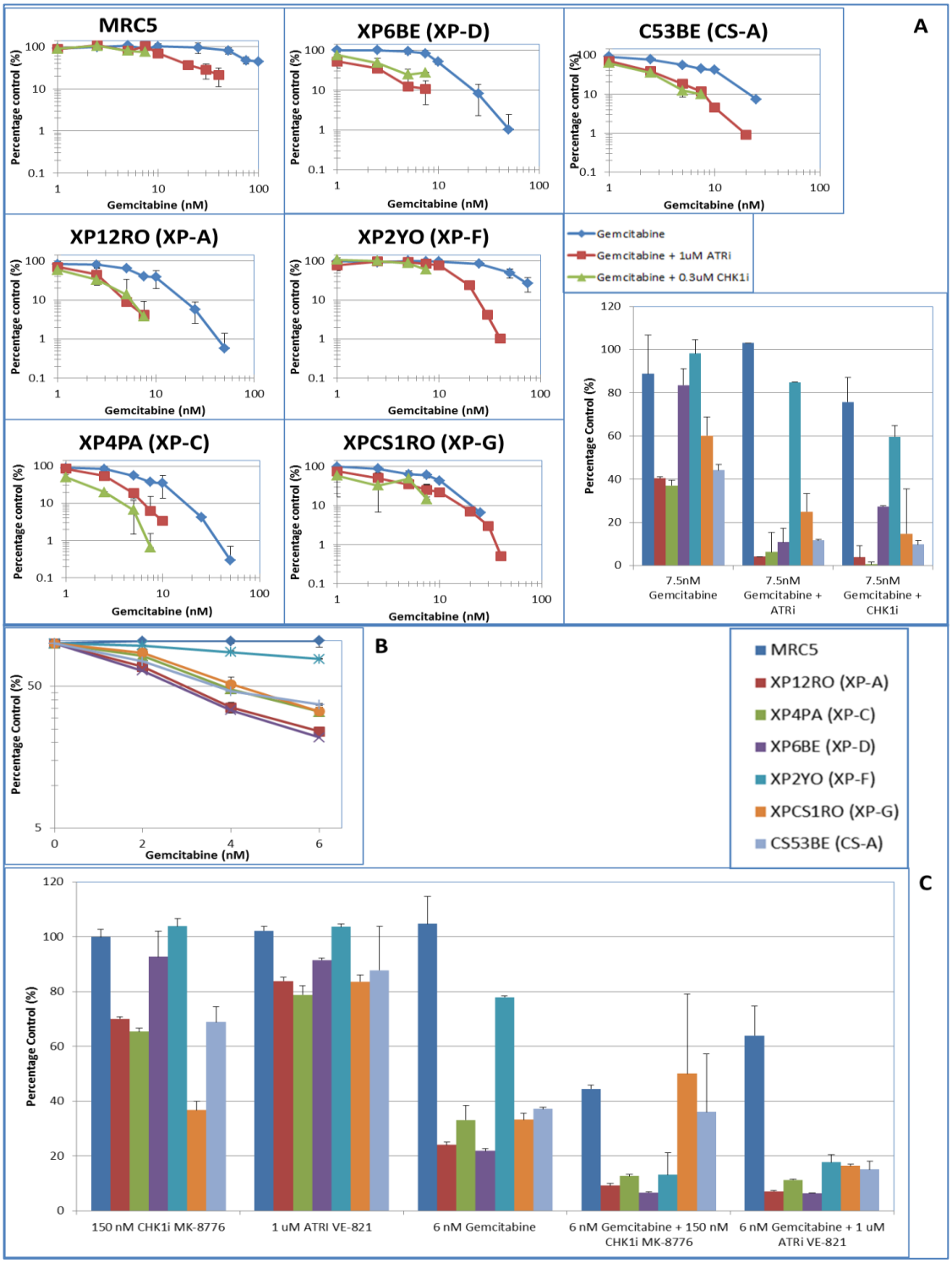

Figure 5.6: ATR and CHK1 inhibition Hyper Sensitise NER Deficient Cell Lines to Gemcitabine. ATR and CHK1 inhibition sensitises NER-deficient cell lines to gemcitabine. (**A**) Colony forming ability of NER-deficient cell lines after 24 hour gemcitabine incubation + 1 µM VE-821 (Red), +0.3 µM MK-8776 (Green), and Gemcitabine only (Blue). With 7.5 nM gemcitabine fixed dose sensitivity bar chart. All values shown indicated averages of three independent repeats, showing standard deviation. (**B**) Gemcitabine sensitivity assayed via MTS following 72 hour incubation. (**C**) Gemcitabine fixed dose (6 nM) sensitivity assayed via MTS following 72 hour incubation with 150 nM MK-8776 or 1 µM VE-821 (expressed as percentage inhibitor only control). MTS survival values shown indicate average of two independent repeats.

Table 5.1: Gemcitabine LC_{50} Values Extrapolated From Figure 5.6 Showing Fold Increase in Sensitivity with CHK1 and ATR Inhibitors

Cell Line	Gemcitabine LC_{50} (nM)	Gemcitabine + CHK1 inhibitor LC_{50} (nM)	Gemcitabine + ATR inhibitor LC_{50} (nM)
MRC5	91	19 (x4.8)	18 (x5.1)
XP12RO (XP-A)	6	2 (x3.0)	2 (x3.5)
XP4PA (XP-C)	6	1 (x6.0)	2 (x3.0)
XP6BE (XP-D)	9	3 (x3.1)	1 (x9.0)
XP2YO (XP-F)	45	11 (x4.0)	7 (x6.0)
XPCS1RO (XP-G)	8	2 (x4.0)	4 (x2.0)
CS3BE (CS-A)	7	2 (x3.5)	2 (x3.5)

Results shown in Figure 5.6 and Table 5.1 show that CHK1 and ATR inhibition increases the cytotoxicity of gemcitabine in NER deficient cell lines as well as in MRC5. Data from both colony forming assays (Fig.5.6A), and MTS survival (Fig.5.6C) experiments show hypersensitisation of cells to inhibition of the ATR-CHK1 signalling axis to varying degrees, whilst maintaining their relative sensitivity to each other. When combined with the CHK1 inhibitor MK-8776, gemcitabine sensitivity was enhanced on average by 4-fold, where XP-C deficient cells showed highest increase in sensitivity (x5.1) and XP-G deficient cells the lowest sensitisation of x3.2. However, when combined with the ATR inhibitor VE-821, gemcitabine sensitivity in NER-deficient cell lines varies depending on their complementation group. ATR inhibition in combination with gemcitabine in the XP-D deficient cell line XP6BE resulted in an 8.7-fold increase in sensitivity, whereas a much smaller increase in sensitivity of x2.1 is induced in XP-G deficient XPCS1RO cells. The results show that the inhibition of ATR or CHK1 increases sensitivity to gemcitabine in all cell lines tested here, however high increases in sensitivity from inhibition of one checkpoint kinase does not always correlate to increased sensitivity in the other, therefore indicating that NER deficient cell lines have different interactions with the ATR-CHK1 checkpoint cascade, and that ATR and/or CHK1 may be required in resistance to gemcitabine in combination with NER factors in separate stabilising mechanisms outside of the S-phase checkpoint cascade.

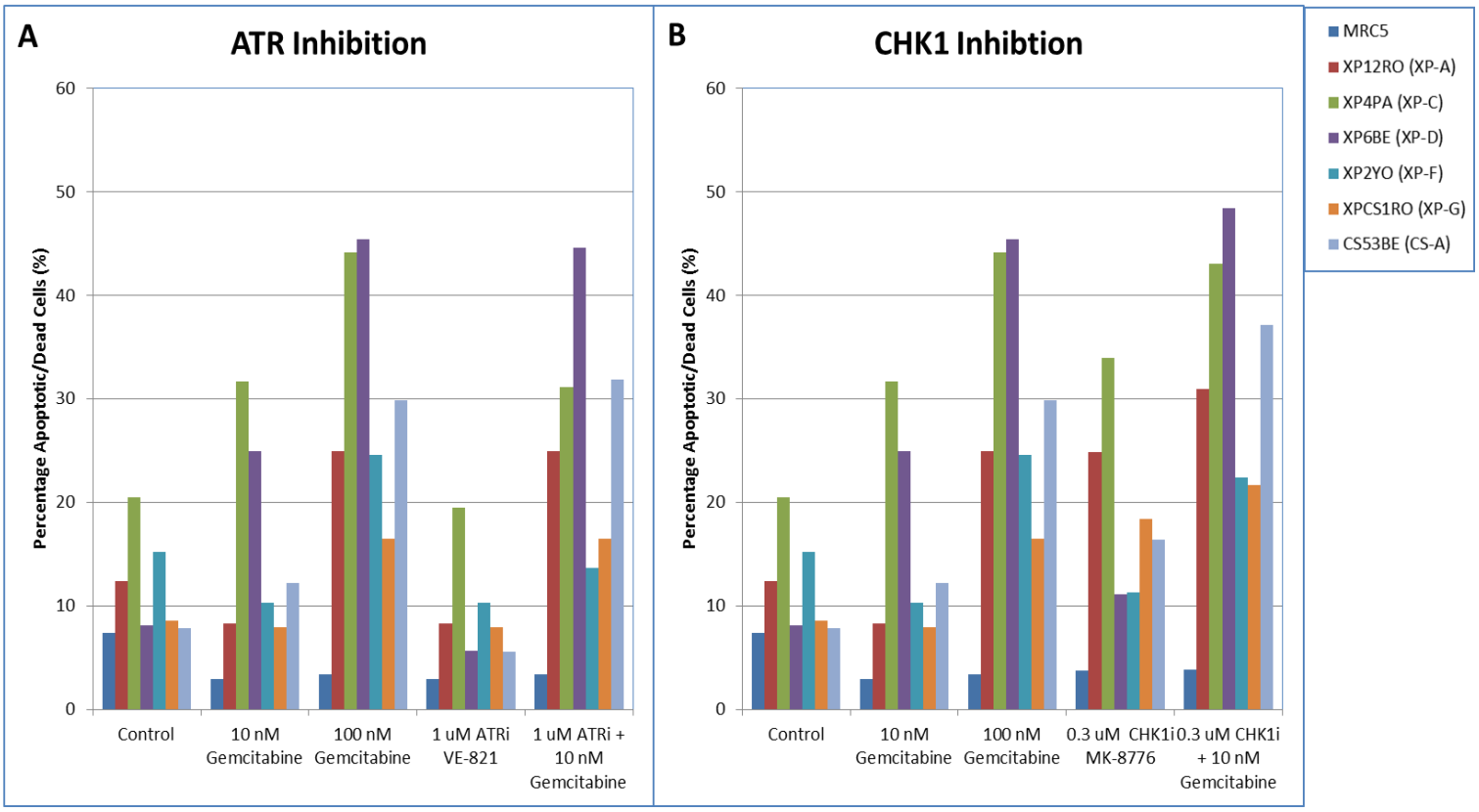

Figure 5.7: ATR and CHK1 Inhibition Increases Gemcitabine Induced Apoptosis in NER Deficient Cell Lines. Gemcitabine induced apoptosis following 10nM and 100 nM exposure over 30 hours as well inhibitor only and inhibitor with 10 nM gemcitabine. 1 µM VE-821 treatment (**A**) and 0.3 µM MK-8776 treatment (**B**). Values shown indicate average total apoptotic signal from 2 independent repeats.

Consistent with colony forming sensitivities tested in Figure 5.6, detectable apoptosis as measured by annexin V/PI staining is increased in most cell lines exposed to gemcitabine in combination with both ATR and CHK1 inhibition. For both ATR and CHK1 inhibition, percentage of apoptotic cells is increased to 100 nM gemcitabine equivalent levels when 10 nM gemcitabine is combined with the inhibitors. However, ATR inhibition combined with gemcitabine failed to increase detectable apoptosis in the XP-C cell line compared to 10nM gemcitabine only treated samples. CHK1 inhibitor MK-8776 mono-treatment increases detectable apoptosis more than ATR inhibition, particularly in XP-A and XP-C cell lines. ATR inhibition alone in several cell lines decreased detectable apoptosis to levels lower than seen in untreated controls, indicating that ATR activation is required for programmed cell death. After showing that gemcitabine sensitivity can be enhanced via inhibition of ATR and CHK1 in NER deficient cell lines, we wanted to test whether the increased DNA damage signalling seen in these cell lines in the form of γH2AX and ATM activation was also

increased or altered by ATR-CHK1 inhibition. Flow cytometric analysis of γH2AX and phospho-ATM was therefore repeated in the XP cell line library following gemcitabine exposure in combination with MK-8776 and VE-821.

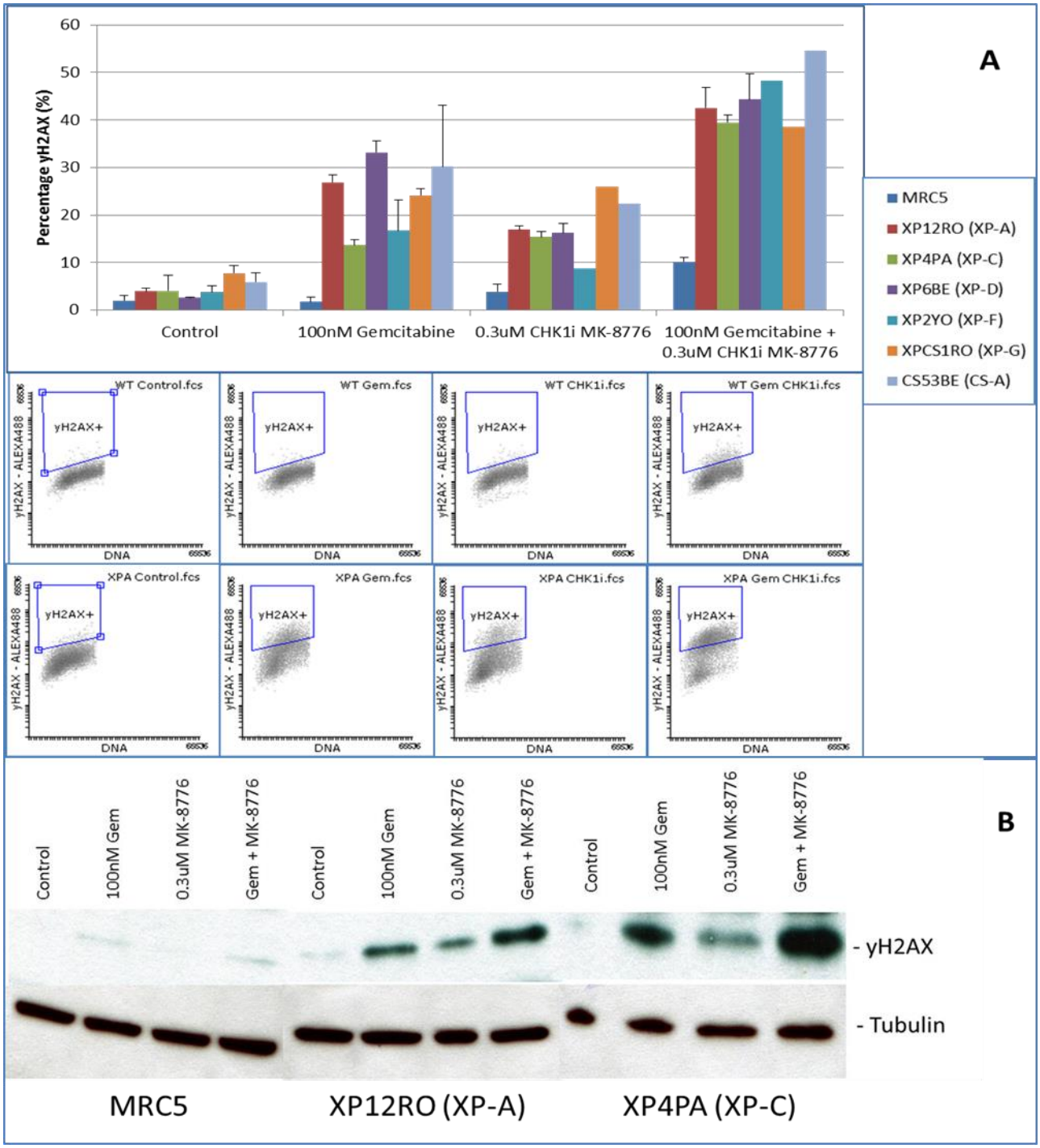

Figure 5.8: CHK1 Inhibition Increases Gemcitabine Induced γH2AX Activation. (**A**) Increased γH2AX activation from gemcitabine, MK-8776 and in combination seen in NER deficient cell lines assayed by indirect flow cytometry 9 hours following exposure, and example WT and XP-A deficient flow cytometry histograms beneath. Error bars indicate average of 3 independent repeats, (values without error bars are averages of two independent repeats) (**B**) Western blot γH2AX detection following 9 hour 100 nM gemcitabine exposure for WT, XP-A and XP-C deficient cell lines.

The activation of yH2AX seen in NER-deficient cell lines following gemcitabine treatment is enhanced when combined with CHK1 inhibition (Fig.5.8), and the treatment of NER-deficient cells with the CHK1 inhibitor MK-8876 alone induced an increase in yH2AX signal compared to controls. This observation is repeated via western blotting where yH2AX shows strong bands in XP-A and XP-C deficient cell lines following combination treatment with MK-8776 and gemcitabine (Fig.5.8B). XP-G deficient cell line XPCS1RO shows the smallest yH2AX activation of the NER deficient cell lines tested where gemcitabine treatment is combined with CHK1 inhibition which correlates to its low LC_{50} increase seen in Figure 5.6/Table 5.1.

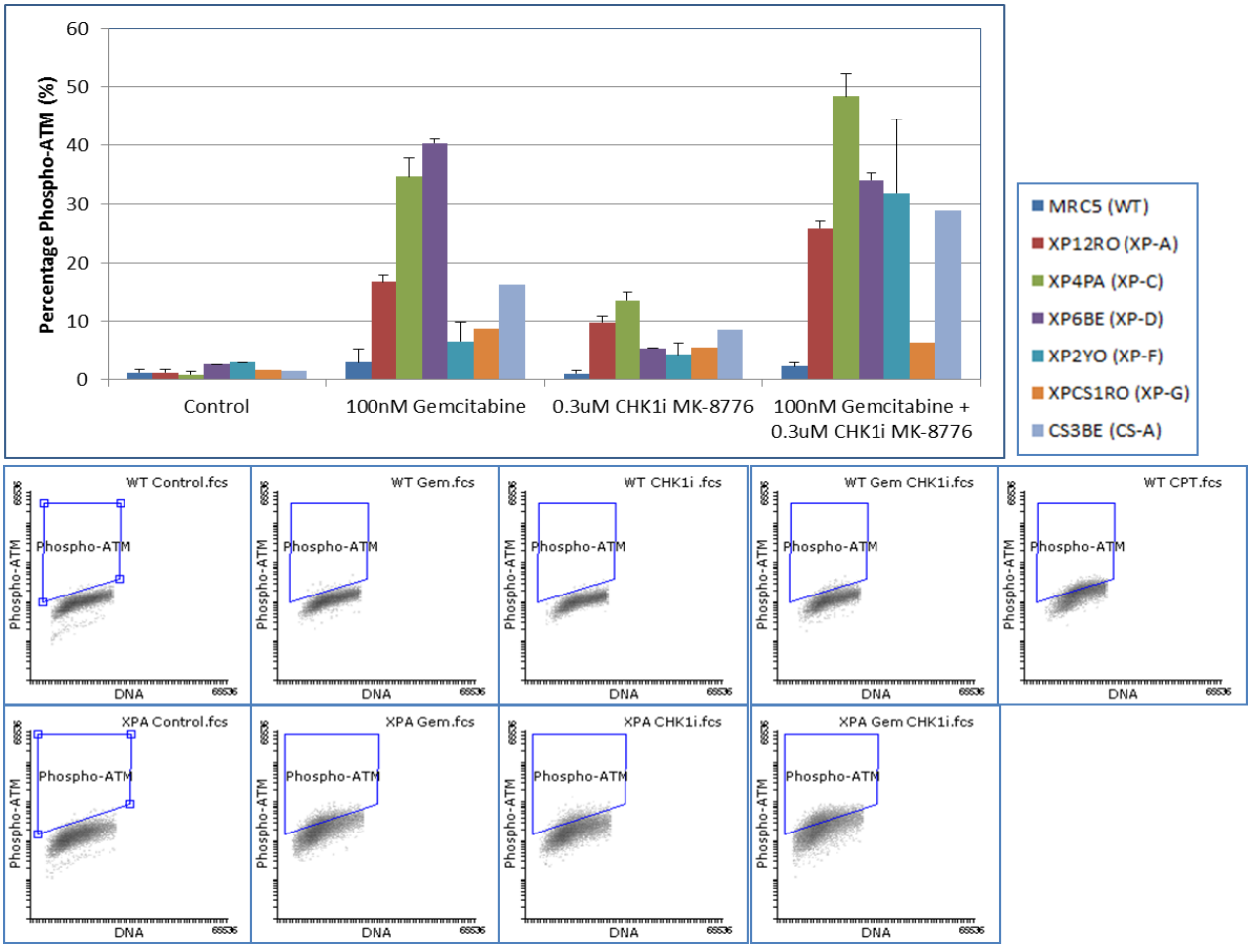

Figure 5.9: CHK1 inhibition Increases Phospho-ATM Activation in Several NER Deficient Cell Lines. Phospho-ATM activation assayed via indirect flow cytometry following 9 hour 100 nM gemcitabine incubation in combination with CHK1 inhibitor MK-8776. Error bars indicated standard deviation of three independent repeats, (values without error bars indicate average of two repeats). MRC5 and XP-A flow cytometry histograms below with wild type 300 nM CPT positive control (far right).

Phospho-ATM activation is seen in XP-A, XP-C, CS-A and XP-D deficient cell lines following 9 hour 100 nM gemcitabine incubation (Fig.5.9). However when CHK1 is inhibited in the

presence of gemcitabine, XP-F deficient cell line XP2YO, as well as CS-A deficient CS3BE exhibit a strong phospho-ATM signal. The ATM activation seen in XP-A and XP-C cell lines is increased following CHK1 inhibition whereas XP6BE cell line which is deficient in XP-D shows a slight decrease in phospho-ATM activation. The XP-G cell line XPCS1RO fails to significantly activate ATM following gemcitabine treatment, even in the presence of the CHK1 inhibitor MK-8776, which potentially reveals a requirement for XPG in ATM activation following gemcitabine treatment. 300 nM CPT treated wild type cells used as a positive control showed an 18.4% increase in phospho-ATM activation (data not shown).

Following the detection of increased yH2AX and ATM activation in XP cells when gemcitabine is combined with CHK1 inhibition (Fig.5.8 and 5.9), we wanted to test whether the S-phase checkpoint activation was still preventing several of the XP cell lines from incorporating BrdU in the presence of gemcitabine, even when CHK1 was inhibited. Therefore, the BrdU assay described in Chapter 4 (Fig.4.7) was repeated. As CHK1 inhibition has been shown to increase new origin firing, one may expect increased replication speed as measured by BrdU incorporation, or conversely, the increased DNA damage resulting from the gemcitabine and CHK1 inhibition results in a greater ATM dependent S-phase checkpoint response leading to decreased BrdU incorporation. The BrdU incorporation assay was therefore repeated in combination with ATR and CHK1 inhibition.

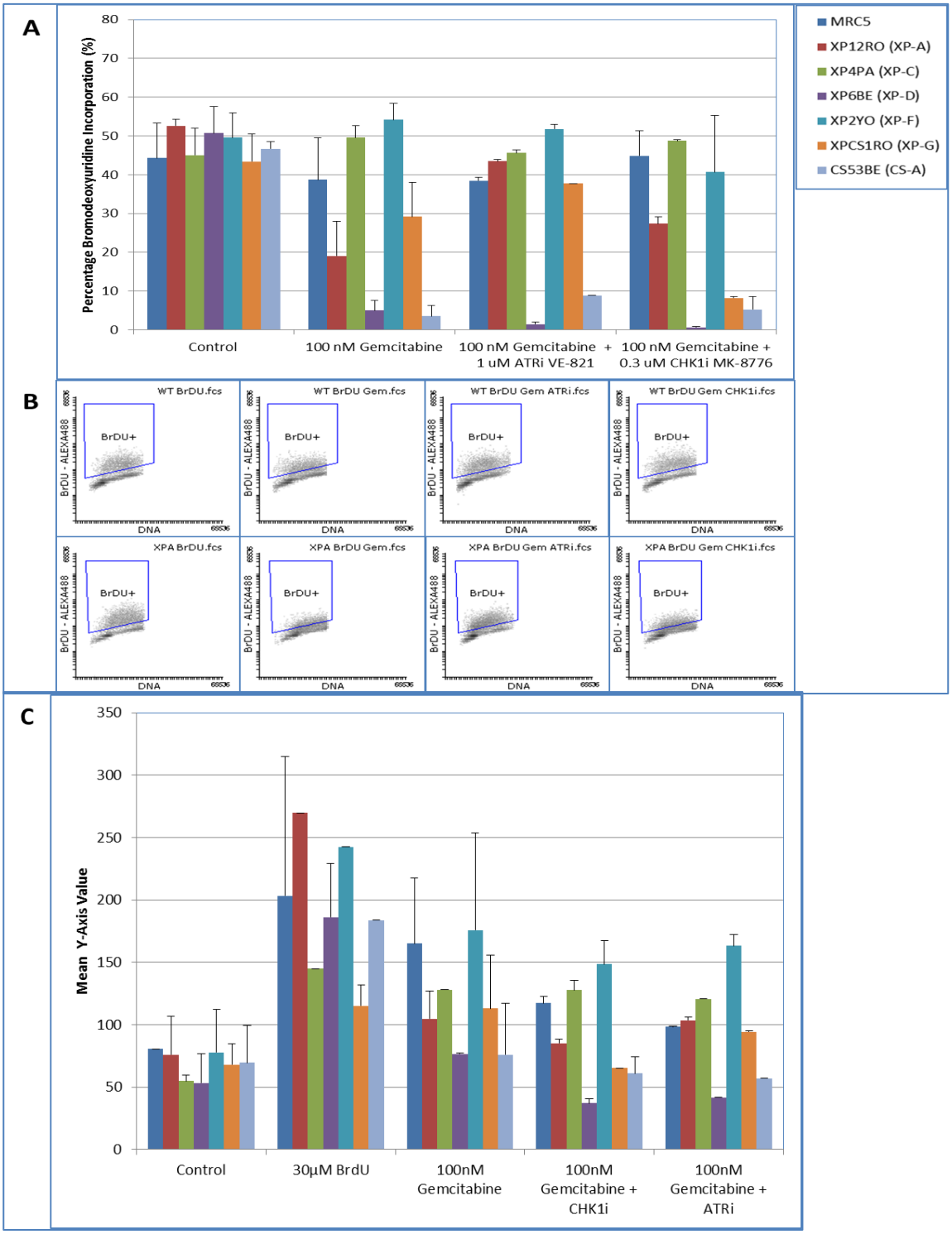

Figure 5.10: ATR and CHK1 Inhibition Alters Gemcitabine Induced S-Phase Arrest In Several NER Deficient Cell Lines. (**A**) BrdU incorporation following 1 hour 100nM gemcitabine incorporation assayed via flow cytometry. Data points averaged over three independent experiments showing standard deviation. (**B**) MRC5 (top), XP12RO (XP-A) (bottom) histograms showing BrdU incorporation +/- gemcitabine, and in combination with gemcitabine and ATR/CHK1 inhibition. (**C**) BrdU signal quantification expressed as mean Y-axis intensity for untreated controls, following BrdU addition only and pre-treatment with 100 nM gemcitabine +/- MK-8776 and VE-821.

As previously shown (Chapter 4), gemcitabine causes a reduction in S-phase progression, as measured by BrdU incorporation in the absence of XP-A, XP-D and CS-A, whereas S-phase speed is largely unaffected at 100 nM in XP-C, XP-F and XP-G cell lines. However, the inhibition of both ATR and to a lesser extent CHK1 causes XP-A deficient cell line XP12RO to incorporate more BrdU indicating a reduced S-phase checkpoint activation. Conversely, CHK1 inhibition, but not ATR inhibition, reduced BrdU incorporation in XP-G deficient cell line XPCS1RO following gemcitabine incubation. XP-D and CS-A cell lines remain strongly replication inhibited after gemcitabine treatment in combination with both ATR and CHK1 inhibition, potentially showing that the absence of XPD and CSA following gemcitabine treatment causes a physical replication block. MRC5 and XPF deficient cell line XP2YO remain largely unaffected by the combination of both ATR and CHK1 inhibition with gemcitabine, and continue to incorporate high levels of BrdU.

Following all previously presented results, we wanted to test whether ATR and CHK1 inhibition could also sensitise XP cells to the nucleoside analogue cytarabine, and whether the effect could be reproduced with stalled replication forks that result from hydroxurea mediated dNTP depletion as well as by nucleoside analogues. In addition, what effect the ATR and CHK1 inhibition has on 4NQO sensitivity, in order to further separate nucleoside analogue sensitivity seen in the absence of NER factors from the classical mechanism of NER. Figures 5.11, 5.12 and 5.13 relate to this question, showing colony forming assay (Fig.5.11) and MTS cell viability data (Fig.5.12, 5.13) where ATR and CHK1 inhibition is combined with cytarabine, hydroxyurea and 4NQO.

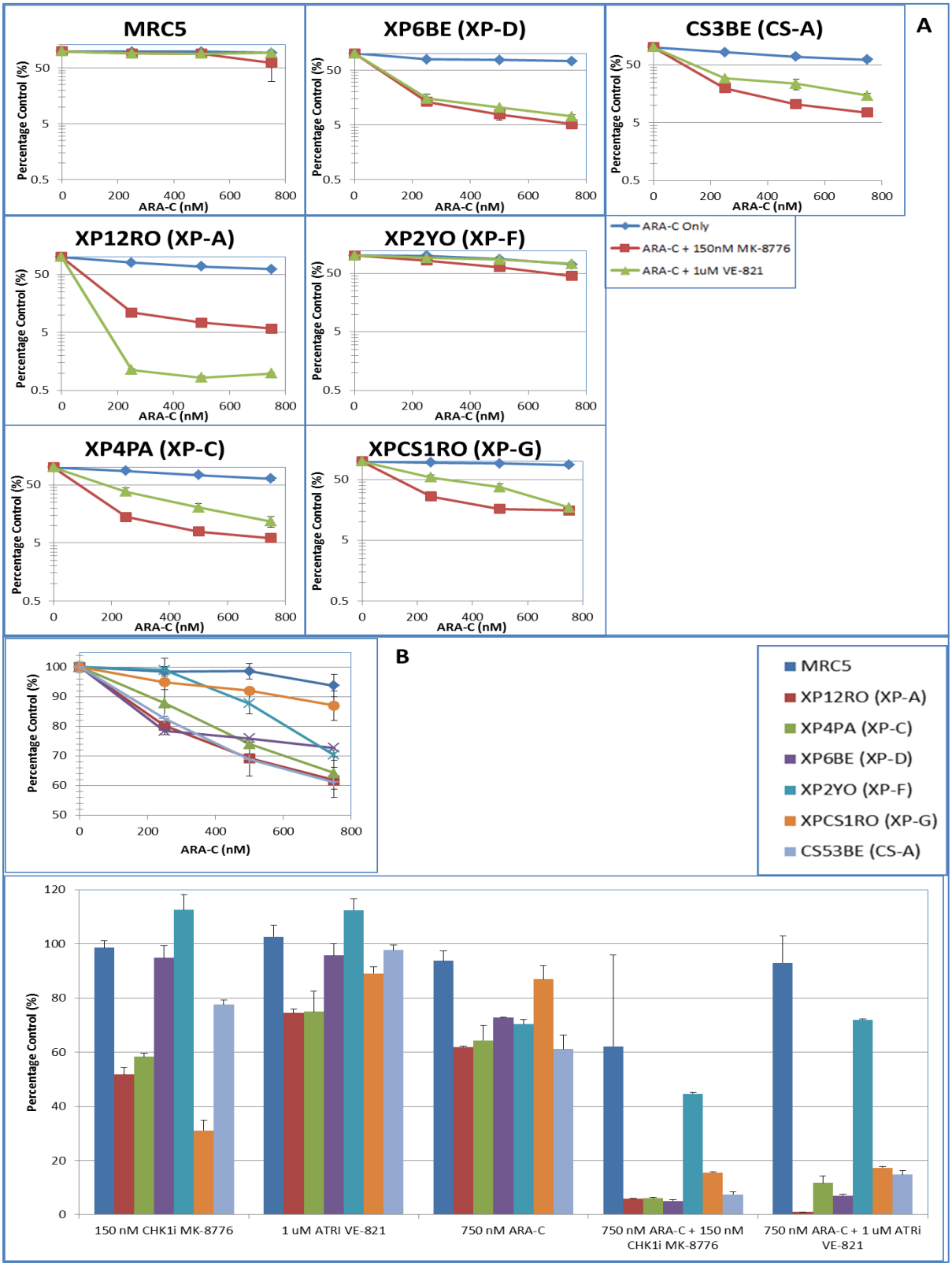

Figure 5.11: ATR and CHK1 Inhibition Hyper Sensitise NER Deficient Cell Lines To Cytarabine. (**A**) Cytotoxicity of cytarabine in NER deficient cell lines + 1 µM VE-821 (Red), 150 nM MK-8776 (Green), and cytarabine only (blue). (**B**) Cytarabine sensitivity assayed via MTS following 72 hour incubation (Top). Cytarabine fixed dose (750 nM) sensitivity assayed via MTS following 72 hour incubation with 150 nM MK-8776 or 1 µM VE-821 (expressed as percentage of inhibitor only control) (bottom). All values shown indicate averages of 2 independent repeats, showing standard deviation.

Table 5.2: Cytarabine LC_{50} Values Extrapolated From Figure 5.11 Showing Fold Increase in Sensitivity with CHK1 and ATR Inhibitors

Cell Line	Cytarabine LC_{50} (μM)	Cytarabine + CHK1 inhibitor LC_{50} (μM)	Cytarabine + ATR inhibitor LC_{50} (μM)
MRC5	8.70*	1.28 (x6.7)	7.05 (x1.2)
XP12RO (XP-A)	1.40	0.03 (x42.4)	0.01 (x116.7)
XP4PA (XP-C)	1.16	0.06 (x19.6)	0.20 (x5.8)
XP6BE (XP-D)	1.60	0.06 (x28.0)	0.07 (x22.9)
XP2YO (XP-F)	1.60	0.68 (x2.4)	1.67 (x0.95)
XPCS1RO (XP-G)	3.90	0.14 (x26.9)	0.31 (x12.3)
CS3BE (CS-A)	1.00	0.10 (x10.3)	0.18 (x5.5)

*MRC5 LC_{50} value not accurately extrapolated due to very low sensitivity.

The sensitisation to nucleoside analogues produced by the inhibition of CHK1 and ATR is considerably more powerful in treatment with cytarabine than gemcitabine. Results shown here in Figure 5.11 and Table 5.2 show up to a 42-fold increase in sensitivity to cytarabine when combined with CHK1 inhibition, and a 117-fold increase in combination with ATR inhibition in the case of XP-A deficient XP12RO. For XP-F deficient cell line XP2YO and MRC5 cells, ATR inhibition has no effect on cytarabine sensitivity, with only comparatively small increased sensitivity when combined with CHK1 inhibition. The results show that NER factors are required for resistance to cytarabine, and in the absence of effective ATR and CHK1 activation, NER factors are even more important.

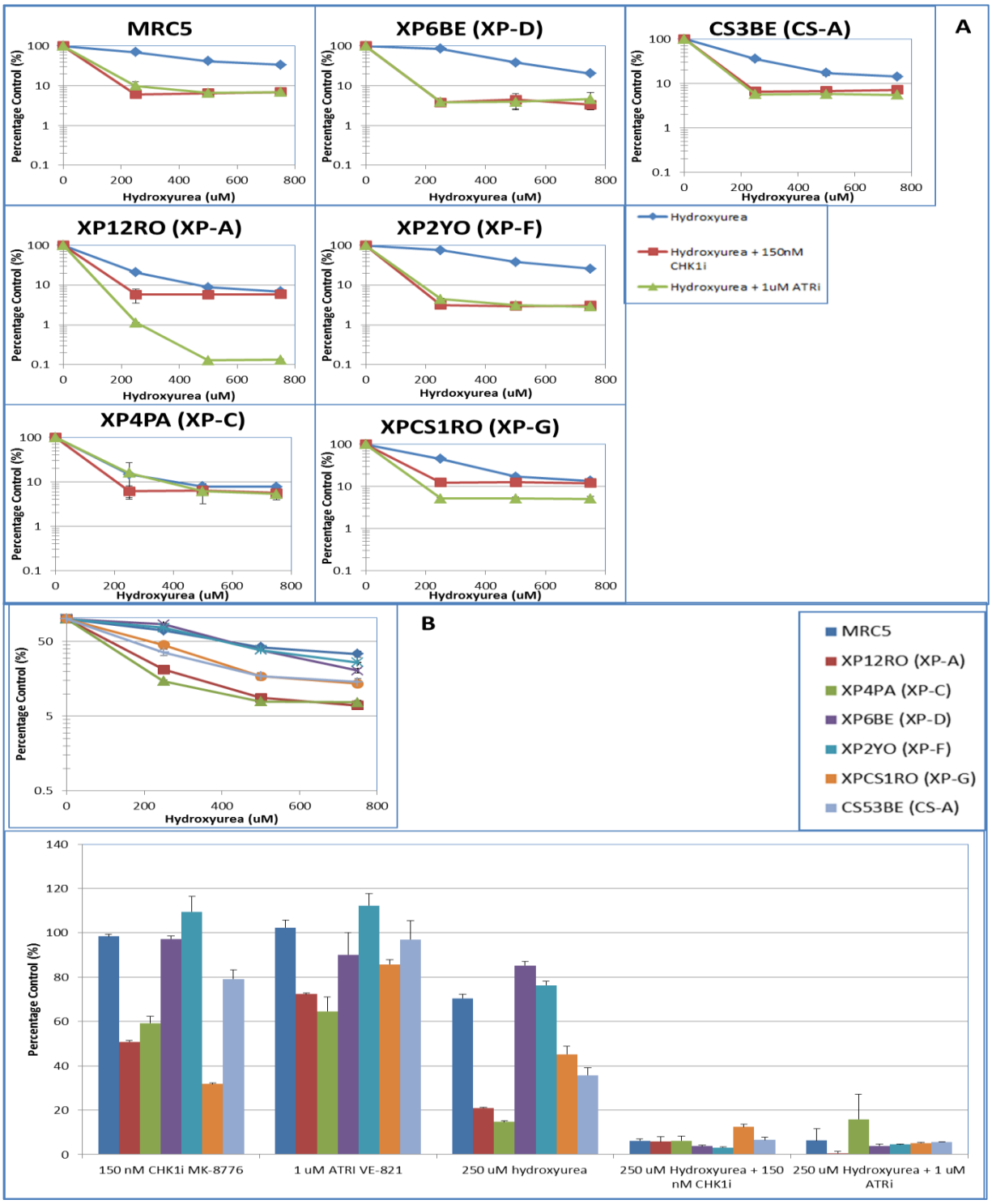

Figure 5.12: ATR and CHK1 Inhibition Hyper sensitises All Cell Lines to Hydroxyurea. (**A**) Cytotoxicity of hydroxyurea in NER deficient cell lines + 1 µM VE-821 (Red), + 150 nM MK-8776 (Green), and hydroxyurea only (Blue). (**B**) Hydroxyurea sensitivity assayed via MTS following 72 hour incubation (Top). Hydroxyurea fixed dose (250 nM) sensitivity assayed via MTS following 72 hour incubation with 150 nM MK-8776 or 1 µM VE-821 (expressed as percentage of inhibitor only control) (bottom). All values shown indicate averages of two independent repeats, with standard deviation.

The inhibition of ATR and CHK1 increases nucleoside analogue sensitivity in NER deficient cell lines. Therefore indicating that NER factors are involved in the stabilisation of nucleoside analogue induced stalled replication forks. From this observation the question of whether or not this can be repeated with stalled replication forks generated via hydroxyurea mediated dNTP depletion was asked. As shown in figure 5.12, hydroxyurea sensitivity phenotypes in NER deficient cell lines are similar to that of nucleoside analogues gemcitabine and cytarabine, in relative order of sensitivity compared to each other. However, the XP-D deficient XP6BE cell line which shows high sensitivity to nucleoside analogues is resistant to hydroxyurea, and the XP-G deficient cell line XPCS1RO which is highly sensitive to gemcitabine, is comparatively resistant to hydroxyurea. XP-A and XP-C deficient XP12RO and XP4PA cell lines show highest sensitivity to hydroyurea treatment. When combined with ATR and CHK1 inhibition, all cell lines tested showed a powerful sensitisation to hydroxyurea including wild type cell line MRC5, which indicates the effectiveness of both inhibitors on removing the S-phase checkpoint activation brought about by dNTP mediated replication fork stalling.

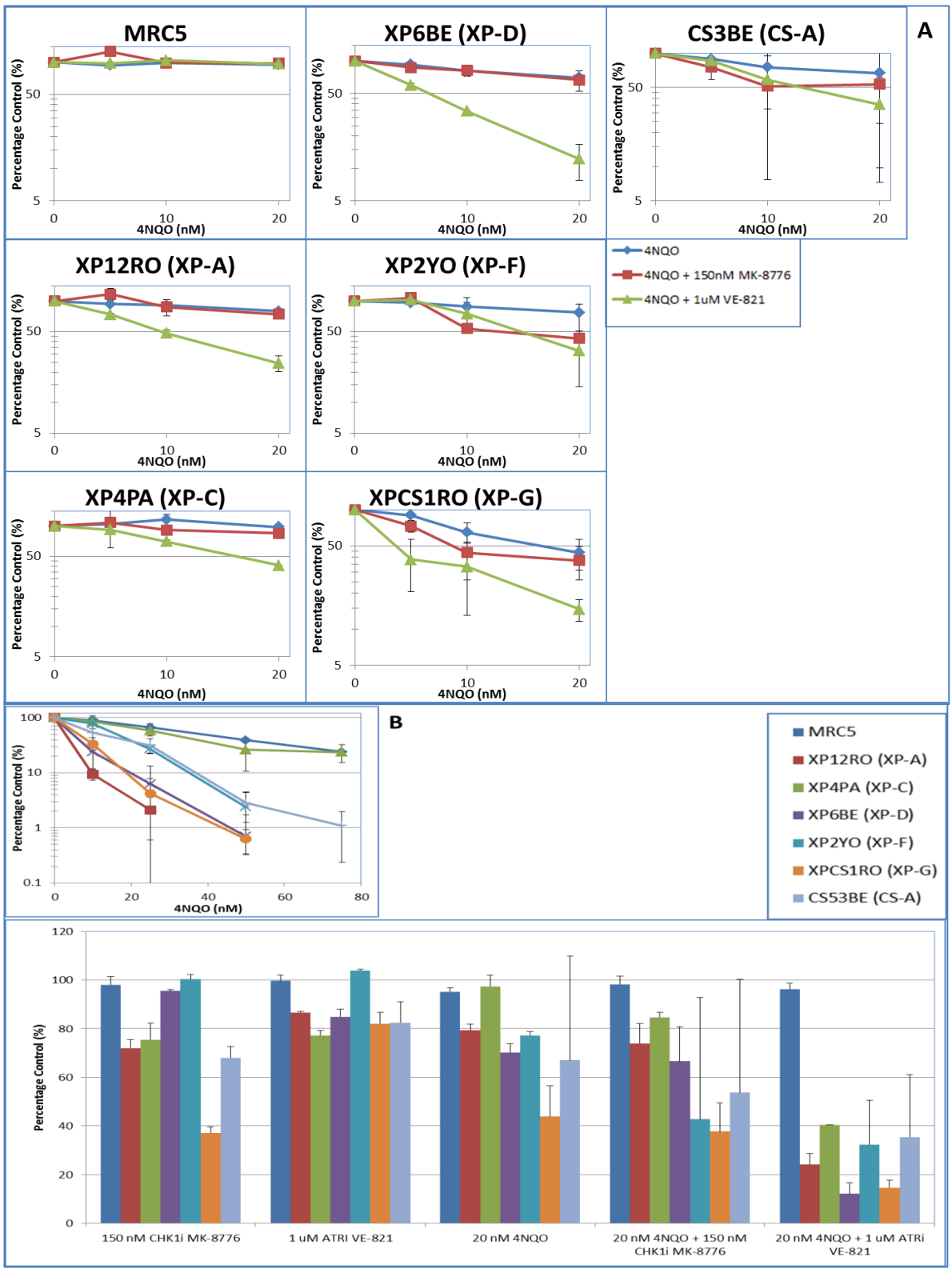

Figure 5.13: ATR Inhibition Increases 4NQO Sensitivity in NER Deficient Cell Lines. ATR inhibition sensitises NER deficient cell lines to 4NQO. (**A**) Cytotoxicity of 4NQO in NER deficient cell lines + 1 µM VE-821 (Red), 150 nM MK-8776 (Green), and 4NQO only (Blue). (**B**) 4NQO sensitivity assayed via MTS following 72 hour incubation (Top). 4NQO fixed dose (20 nM) sensitivity assayed via MTS following 72 hour incubation with 150 nM MK-8776 or 1 µM VE-821 (expressed as percentage of inhibitor only control) (bottom). All values shown indicate averages of two independent repeats, with standard deviation.

Following the results from ATR and CHK1 inhibition in sensitisation to nucleoside analogues and the replication inhibitor hydroxyurea, the effects of these inhibitors on a defined DNA damage NER substrate was tested. NER-deficient cell lines have previously been shown to be sensitive to 4NQO (Chapter 3). In combination with 4NQO, CHK1 inhibitor MK-8776 did not significantly increase sensitivity except for XP-F deficient cell line XP2YO, whereas the ATR inhibitor VE-821 increased 4NQO sensitivity in all cell lines tested except for wild type (Fig.5.13). This result indicates that ATR signalling during nucleotide excision repair is important in resistance to DNA damage, especially when NER is deficient, and that CHK1 signalling is not required.

Work presented here has shown that gemcitabine sensitivity can be enhanced by novel kinase inhibitors in cell lines deficient in NER. One further DNA repair pathway that NER factors have been shown to interact with is BER, specifically the glycosylases such as OGG1 (Melis et al., 2013). To examine whether interaction of NER factors with BER machinery in the results presented here, the novel APE1 inhibitor CRT0044876 was used in combination with gemcitabine. APE1 was identified as a good candidate for targeting as *in-vitro* data has suggested that this enzyme can remove the nucleoside analogue troxcitabine from various DNA substrates as a result of both its endo and exonuclease activity (Chou et al., 2000). APE1 was also identified as a protein potentially involved in gemcitabine removal from previous data from our lab using the LCMS/MS in *S. pombe* (Chapter 1).

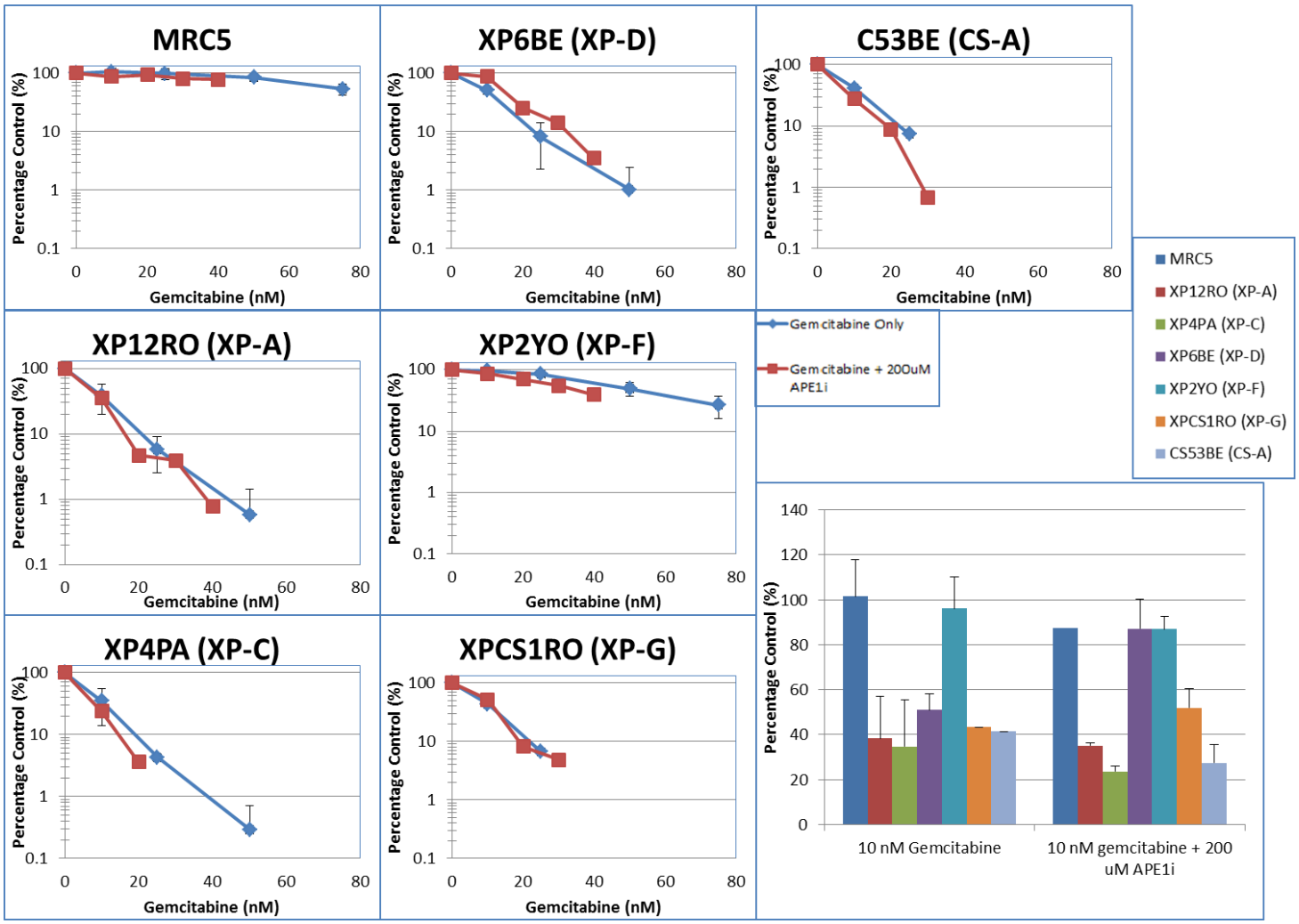

Figure 5.14: APE1 Inhibitor CRT0044876 Does Not Increase Gemcitabine Sensitivity In NER Deficient Cell Lines. Colony forming assay survival data of 24 hour gemcitabine exposure +/- 200 µM APE1 inhibitor CRT0044876. Values expressed as a percentage of untreated controls, and percentage inhibitor only treated controls for APE1 inhibition experiments. Error bars indicate standard deviation of two independent repeats.

The effect of APE1 inhibition on gemcitabine sensitivity showed no significant increases in cytotoxicity in either wild type MRC5 cells or NER deficient cell lines based on 24 hour drug and inhibitor exposure (Fig.5.14). Gemcitabine sensitivity was not increased in either XP-C or XP-G truncations which have been shown to interact with BER machinery *in vitro* (Melis et al., 2013; Menoni et al., 2012; Sugasawa, 2008a). The APE1 inhibitor CRT0044876 was further tested in MRC5 cell lines and was found to sensitise cells to hydrogen peroxide at the same 200µM concentration (data not shown).

Discussion

Previous reports have shown that gemcitabine resistance requires effective ATR-CHK1-Cdc25A signalling in a variety of cell types (Montano et al., 2013; Thompson et al., 2012). Consistent with this, results presented here (Fig.5.3) shows that DNA-PK and ATM inhibition did not increase gemcitabine sensitivity in MRC5 cells, whereas both ATR and CHK1 inhibition sensitised cells to the nucleoside analogue. However, the observation that several NER-deficient cell lines are intrinsically sensitive to ATR and CHK1 inhibition seems to be novel (Fig.5.5). In a phenotypic manner similar to the observed sensitivities to nucleoside analogues, XP-F deficient XP2YO is not sensitive to the effect of ATR inhibitor VE-821 or CHK1 inhibitor MK-8776, whereas other NER-deficient cell lines are either moderately or highly sensitive to these two inhibitors relative to NER proficient cells. Several reports have shown that inhibition of ATR and CHK1 with these two inhibitors causes increased origin firing due to the uncontrolled action of the Cdc25A phosphatase enzyme (Ewald et al., 2007). This overactive replicative action is reported to increase the frequency at which replication forks collide with transcriptional machinery which ultimately results in a high incidence of stalled replication forks which require stabilisation (Montano et al., 2013; Prevo et al., 2012; Thompson et al., 2012). It is therefore possible that several of the NER factors have a specific role in dealing with endogenous replication fork stresses that occur during S-phase, and in the absence of effective CHK1 or ATR signalling, this sensitivity is compounded.

The combination of gemcitabine or cytarabine with ATR or CHK1 inhibition dramatically increased sensitivity in NER-deficient cell lines (Fig.5.6, 5.7, 5.11). This increased sensitivity correlates with greater yH2AX activation in these cells, whilst only mildly increasing yH2AX in MRC5 (Fig.5.8). It has previously been shown that MK-8776 inhibition of CHK1 leads to increased yH2AX on its own and in combination with nucleoside analogues (Karnitz et al., 2005; Montano et al., 2013), however, the percentage increase in signal in NER deficient cell lines is far greater than in NER proficient cells. The mechanism by which CHK1 inhibition causes increased cell death in combination with gemcitabine has been suggested to be due to the lack of recruitment of replication fork stabilising proteins such as BRCA-1, claspin and RAD51 which protect against inappropriate degradation by Mus81 or MRE11 nucleases which collapse the fork into a DSB (Montano et al., 2013; Thompson et al., 2012). This

suggested mechanism correlates with the observation that NER deficient cell lines activated ATM in response to gemcitabine when CHK1 is inhibited (Fig.5.9), which is consistent with the presence of DSBs. However XP-G deficient XPCS1RO cell line failed to activate phospho-ATM when CHK1 inhibition is combined with gemcitabine, suggesting that XP-G might be responsible for cleaving replication forks into DSBs.

It is well known that hydroxyurea causes replication fork stalling due to depletion of dNTP pools required for DNA chain elongation (Fox, 2004; Lahkim Bennani-Belhaj et al., 2010). Hydroxyurea is used here as a replication fork stalling control (Fig.5.12). Results here largely show similar phenotypes to the sensitivity of NER-deficient cells to nucleoside analogues indicating that their sensitivity is due to issues at the replication fork rather than an involvement in the classical NER pathway. However, XP-D cell line XP6BE which is highly sensitivity to nucleoside analogues, is resistant to hydroxyurea. This indicates that the sensitivity seen in XP-D deficient cells maybe unique to nucleoside analogue induced replication fork stalling rather than the lesion generated by hydroxyurea. However consistent with replication fork models, hydroxyurea in combination with ATR and CHK1 inhibition results in complete sensitisation due to destabilisation (Elvers et al., 2012; Montano et al., 2013).

To further functionally separate nucleoside analogue sensitivity seen in NER-deficient cells from the pathway of classical nucleotide excision repair, CHK1 and ATR inhibitors where tested in combination with 4NQO (Fig.5.13). It has been reported that ATR is required for effective NER to occur (Li et al., 2011; Ray et al., 2013), consistent with this, ATR inhibition by VE-821 increased the sensitivity to 4NQO in all NER cell lines tested. However, the effect of CHK1 inhibition on 4NQO sensitivity was far less, indicating that the role of CHK1 in combination with NER factors is unique to nucleoside analogue and hydroxyurea induced replication fork stalling rather than classical NER.

As previously seen in Chapter 4, gemcitabine causes S-phase stalling in NER deficient cell lines from complementation groups XP-A, XP-D and CS-A, as measured by bromodeoxyuridine incorporation. The effect of inhibiting ATR and CHK1 signalling in the presence of gemcitabine on bromodeoxyuridine incorporation is varied for different NER factor deficiencies (Fig.4.7). The XP-D and CS-A cell lines tested fail to incorporate BrdU even

when ATR and CHK1 are inhibited after gemcitabine treatment. As these two cell lines show high phospho-ATM activation after nucleoside analogue treatment, it seem likely that the S-phase checkpoint activation is predominately controlled by the ATM-CHK2-Cdc25A pathway rather than ATR-CHK1 which prevents BrdU incorporation, or that in the absence of these two NER factors, replication is physically blocked by gemcitabine, rather and checkpoint activations. However, the XP-C deficient cell line XP4PA which also shows high Phospho-ATM activation is able to incorporate BrdU after gemcitabine treatment even when ATR and CHK1 are inhibited. In the absence of CHK1 signalling the phospho-ATM activation in XP-C deficient cells was even higher than with gemcitabine alone (Fig.5.9). It would therefore be expected that ATM-CHK2-Cdc25A activation would be very high and result in the S-phase checkpoint activation as seen in XP-D and CS-A cell lines. This result therefore suggests that XP-C deficient cell lines have a defect in halting replication in response to gemcitabine via the activation of ATM, leading to increased sensitivity.

However, in the case of XP-A absence, ATR and CHK1 inhibition caused increased BrdU incorporation following gemcitabine treatment (Fig.5.10). This result potentially means that cells lacking XPA rely on the ATR-CHK1 activation to halt S-phase progression in order to overcome stalled replication forks, and in the absence of ATR or CHK1 activation, further attempts to replicate result in BrdU incorporation, increased yH2AX activation and increased cell death.

Furthermore, in cells lacking XPG, which did not produce ATM activation following gemcitabine treatment, even when combined with CHK1 inhibition, BrdU incorporation is increased when ATR is inhibited yet decreased when CHK1 inhibitor MK-8776 is added. In the absence of CHK1 signalling, increased replication origin firing would be expected (Montano et al., 2013), which should increase BrdU incorporation, however in XP-G cell line XPCS1RO, CHK1 inhibition dramatically decreased BrdU incorporation. Without detectable ATM activation, and ATR inhibition having no effect on S-phase progression, it is hard to postulate the interaction between XP-G and CHK1 in gemcitabine survival.

Several NER factors have been shown to interact with BER machinery (Maria Berra et al., 2013; Melis et al., 2013; Soltys et al., 2012). To test the possibility that the BER pathway is involved in the removal of gemcitabine from stalled replication forks in NER deficient cell

lines, the APE1 inhibitor CRT0044876 was used (Fig.5.14). APE1 is a key enzyme in BER as it has both the ability to cleave the phosphodiester backbone of DNA at abasic sites, as well as 3'-5' exonuclease activity (McNeill et al., 2009). RNAi knockdown experiments have shown that cells lacking in APE1 are sensitive to oxidative damage due to hydrogen peroxide as well as several alkylating agents (McNeill et al., 2009; Xiong et al., 2010). In addition APE1 has been shown to be able to remove the nucleoside analogue troxacitabine from 3' ends of DNA substrates (Chou et al., 2000). Using the APE1 inhibitor CRT044876 which has been shown to strongly sensitise cells to oxidative damage (Madhusudan et al., 2005), gemcitabine sensitivity was tested in NER deficient cell lines which showed no significant increases in sensitivity (Fig.5.14). However, due to no increased sensitivity in the MRC5 cell line, and potential redundancies with additional BER glycosylases such as APE2, it can not be concluded that the absence of the reported interaction between both XPG and XPC with BER machinery is responsible for the sensitivity to nucleoside analogues seen in NER-deficient cell lines XP4PA (XP-C) and XPCS1RO (XP-G).

The results presented in this chapter raise the possibility that the replication instability brought about by the absence of core NER factors leads to difficulty in resolving nucleoside analogue induced stalled replication forks, and in the absence of ATR and CHK1 activation, this instability proves fatal for the cell. Furthermore, evidence shown here indicates that individual NER factors may contribute to replication fork stability in different ways based on each cell lines preference to either activate ATM or not, which signals the presence of DSBs, and the effect of inhibiting ATR and CHK1 which seems to alter replication arrest differently for each NER complementation group. It therefore indicates that in addition to the reported role of the ATR-CHK1 signalling pathway that brings about S-phase arrest, CHK1 and ATR maybe also interacting with NER factors outside of the signalling axis.

Chapter 6: Use of XPG E791A and XPF D676A Cell Lines and NER Factor Knockdown Attempts

Summary

Work in this chapter describes results from experiments using the XP2YO and XPCS1RO cell lines transfected with cDNA constructs expressing wild type or catalytically inactive XPF and XPG nucleases respectively. cDNA is readily detectable in these cell lines by PCR, however protein production is weak, particularly in XP2YO (XP-F). Low production of wild-type XPF in the XP2YO cell line rescues sensitivity to 4NQO but not gemcitabine. This chapter describes several attempts at NER factor knockdown by esiRNA and shRNA technology as well as attempting gene knockout using the relatively new technology CRISPR/Cas9.

Introduction

Catalytically Inactive XPG and XPF

In order to study the specific catalytic roles of both XPG and XPF in DNA repair, nuclease deficient cDNA constructs have been used which contain amino acid substitutions at highly conserved residues which renders the protein unable to cleave DNA. For the case of XPG and XPF, this technique allows for the separation of phenotypes observed from truncated endonucleases seen in XP patients which may be due to structural requirements of the proteins, and those of purely absent nuclease activity. This also allows for the complementation of the cell line with a wild-type cDNA endonuclease to determine if catalytically active nuclease action restores function.

Within the XPG complementation group, only 3 patients have been identified which produce full length XPG without nuclease function. Cell lines form two siblings XP125LO and XP124LO, were found to have an XPG mutation A792V and patient XP65BE showed an A874T mutation (Emmert et al., 2002; Schärer, 2008b). All three of these XPG patients showed a strikingly less extensive phenotype than other XPG complementation group patients who have XPG truncation mutations, which results in a XP/CS pathology. However,

one patient, XPCS4RO, was found to have full length XPG, but had a single amino acid substitution mutation within the N-terminal region (Pro72His), which was shown to result in miss-folding and degradation of the translated XPG protein (Clarkson, 2003; Schärer, 2008b). As mentioned in Chapter 3, this requirement for full length XPG is thought to be due to the structural integrity of the TFIIH transcription factor complex of which XPG is an important stabilising subunit (Thorel et al., 2004). Cell lines without full length XPG have also been shown to be sensitive to oxidative DNA damage whereas XP125LO fibroblasts with full length nuclease deficient XPG protein are not (Soltys et al., 2012). From this, it has been suggested that nuclease deficient XPG maintains its interaction with BER machinery, while truncated XPG protein does not (Klungland et al., 1999). It is therefore necessary to use nuclease deficient XPG and XPF models to determine separate roles for these NER factors.

To study the role of XPG nuclease function several studies have used the nuclease-deficient E791A mutation. This substitution of the highly conserved glutamate residue has been shown to be nucleolytically inactive in both higher eukaryotes and the *S. cerevisiae* XPG homolog Rad2 (Clarkson, 2003; Constantinou et al., 1999; Hohl et al., 2003). Using the XPG deficient XPCS1RO cell line, it has been shown that transfection with XPG E791A cDNA rescues UV sensitivity by 50% compared to the unmodified cell line, and that XPF incision precedes XPG in the classical model of cut and patch nucleotide excision repair (Staresincic et al., 2009). The XPF deficient cell line XP2YO transfected with a nuclease-deficient XPF cDNA construct (D676A) shows no UV sensitivity rescue, which suggests that XPF nuclease activity is absolutely required for effective nucleotide excision repair. In contrast, XPG nuclease activity is only partly required (Gaillard and Wood, 2001; Staresincic et al., 2009). Both XP2YO (XP-F) and XPCS1RO (XP-G) transfected with wild-type cDNA nuclease constructs have been shown to completely restore UV resistance (Ellison et al., 1998; Staresincic et al., 2009; Yagi et al., 1998). For both XP2YO and XPCS1RO the nuclease-deficient and wild-type transfected cell lines were supplied to our lab by Prof Alan Lehmann (University of Sussex).

siRNA, esiRNA, and shRNA

siRNA technology has been used in several cell types to reproduce UV sensitivities seen in XP patient cell lines (Kang et al., 2011). siRNA constructs which bind complementary mRNA

for a chosen gene, utilise the RISC complex (RNA-induced silencing complex) to degrade transcripts which ultimately reduces protein levels of a selected gene (Filipowicz et al., 2008). Several studies have shown that siRNA knockdown of NER factors reproduces UV sensitivity of XP cell lines as well as observed sensitivity of XP-C and XP-G cell lines to oxidative damage (Ito et al., 2007; Maria Berra et al., 2013; Soltys et al., 2012). Endoribonuclease prepared siRNA (esiRNA) technology is very similar to standard siRNA knockdown, however, instead of a mix of 2-3 oligo sequences, complementary RNA is generated by RNase III digestion of much larger RNA constructs (Yang et al., 2002). This technique offers greater numbers of target sequences whilst increasing gene target specificity (Kittler et al., 2004). XPC Mission esiRNA® were purchased from Sigma and used as per manufacturer instructions as described in Chapter 2.

Short hairpin RNA (shRNA) knockdown technology has also been used to target NER factors, as well as checkpoint kinases to reproduce results seen in XP cell line models. shRNA uses the same principle of mRNA degradation as siRNA, however, the complementary RNA oligo sequences in DNA form are integrated into the genome as part of a larger construct usually including antibiotic selection and fluorescent marker cassettes, before being transcribed into the active RNA form (Xiang et al., 2006). Once integrated, shRNA constructs stably express RNAi oligo sequences which decrease protein production of the targeted gene. This has the advantage over siRNA which induce transient gene knockdown, where shRNA integration can produce stable knockdown cell lines (Wang et al., 2011). XPG shRNA constructs encapsulated in lentiviral particles were purchased from Santa Cruz and transfected into the MRC5 control cell line as described in Chapter 2.

CRISPR/Cas9

CRISPR/Cas9 is a new generation gene editing system derived from immunological systems found in bacteria and archaea, which is now being used to generate gene knockout cell lines as well as mouse models (Riordan et al., 2015). CRISPR/Cas9 gene knockout requires transfection with a plasmid containing one or more guide RNA (gRNA) sequences of 20 nucleotides which are complementary to a specific gene locus, with an attached tracrRNA (trans-activating CRISPR RNA) (Fig.6.1). A second transfected plasmid contains the Cas9 nuclease which cleaves DNA at the specific locus where the gRNA sequence is bound. Cas9

wild-type nuclease cleaves both strands of genomic DNA, however modified Cas9 is available which creates a single-strand nick (Mou et al., 2015). The DSB which is created within a selected gene can be further exploited by targeted integration of a homologous construct containing antibiotic and fluorescent marker cassettes which allows for clonal selection and generation of stable cell lines which are deficient in expression of the chosen gene. CRISPR/Cas9 knockout kits are now commercially available to target many hundreds of genes. Efficient gene knockout/editing offers an attractive tool to study the role of various genes in DNA repair compared to use of immortalised cell lines derived from patients with pathologies such as XP because transcription of a targeted gene can be halted within the very start of open reading frames, whereas patient cell lines may still contain residual gene expression which may mask phenotypes.

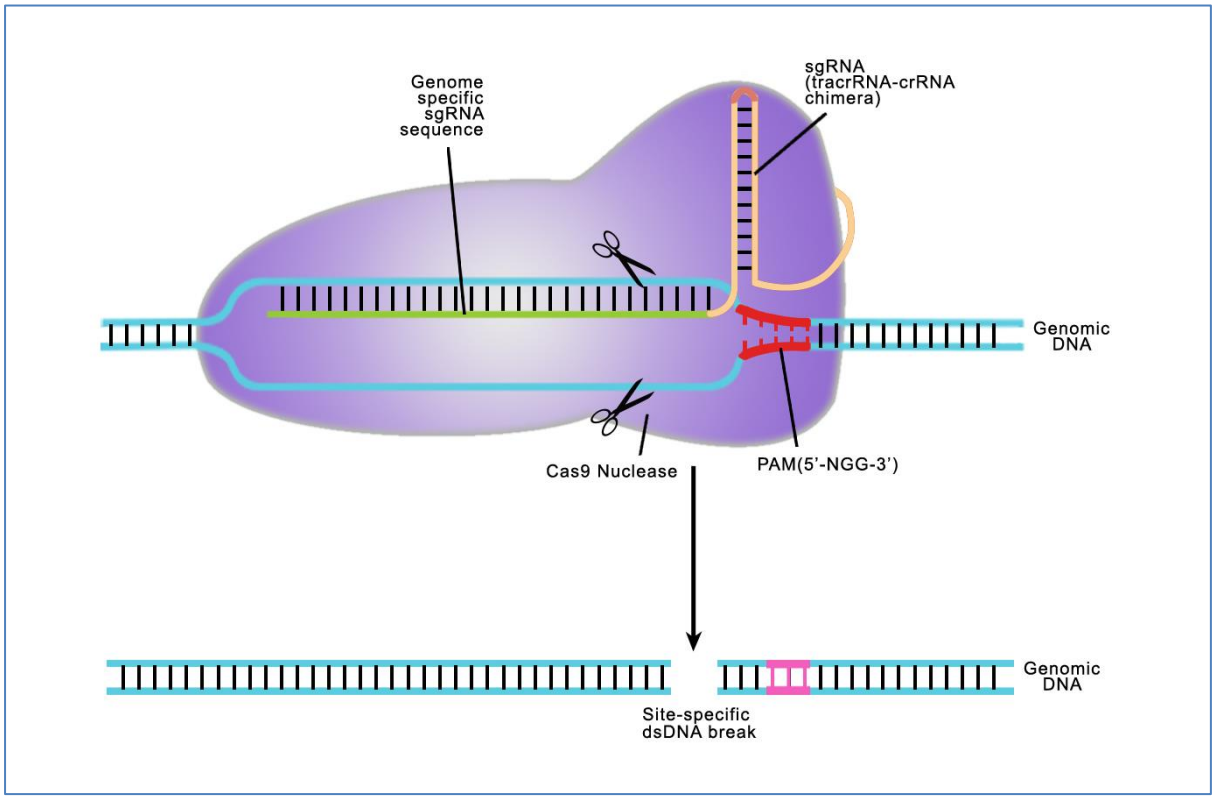

Figure 6.1: CRISPR/Cas9 Genomic DNA Cleavage. gRNA sequence (green) bound to tracerRNA (pink) binds to complementary bases at specific locus and recruits the Cas9 nuclease (purple) which cleaves the DNA at the site of the desired gene (Riordan et al., 2015).

Due to previous results from our lab which showed that in the yeast model *S. pombe* cells lacking the XPF homolog (Rad16) were not sensitive to gemcitabine, whereas cells lacking the XPF binding partner ERCC1 (Swi10) are (unpublished), a ERCC1 CRISPR/Cas9 gene knockout kit was purchased from Origene in order to generate a ERCC1 -/- cell line.

Results

In order to compare the nucleoside analogue resistance of XP-G and XP-F deficient cell lines to their cDNA transfected nuclease deficient and wild-type complemented counterparts, validation XPCS1RO and XP2YO cell lines was first performed by PCR and western blotting in order to confirm the presence of nuclease-deficient and wild-type constructs in each cell line, as well as analyse the relative levels of XPG, and XPF proteins relative to MRC5 controls.

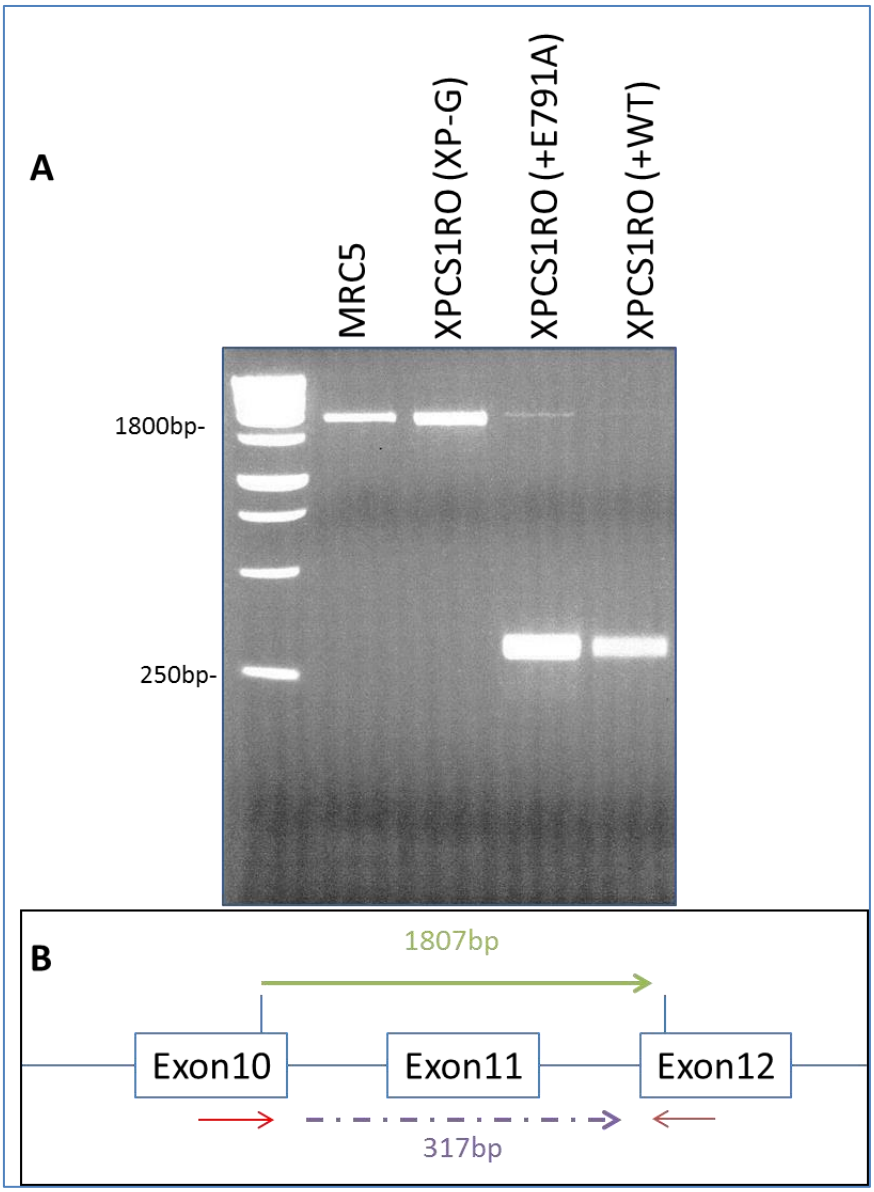

Figure 6.2: PCR Detection of XPG cDNA In XPCS1RO Cell Line. (**A**) PCR products run on 2% agarose gel with Bio-Rad 1kb ladder. (**B**) Diagram showing PCR primer positon and base pair size of expected product size. 1807 base pair band expected product from genomic XPG detection including introns (green), (purple) 317 base pair band expected in cDNA transfected cells.

Primers were designed to sequence the region between the end of exon 10 and beginning of exon 12 in the *XPG* gene. As shown in Figure 6.2, a 1.8 kb base pair DNA band was detected after PCR in both MRC5 and the unmodified XPCS1RO cell line. This fragment includes both the intron sections between exons 10 and 12. This band is very weakly detected in both cDNA transfected cell lines. However, in the case of XPCS1RO cells transfected with the XPG E791A and wild-type XPG constructs, a 317 bp band becomes detectable. This band corresponds to the predicted size of XPG cDNA between exon 10 and 12 which does not contain the intron sequences. Due to the random integration of the cDNA constructs, both bands are detected in the transfected cell lines. These two smaller bands were excised and purified by gel extraction before being sent for sequencing (Eurofins), and confirmed as *XPG* E791A and wild type *XPG*. The cDNA transfected cell lines were found to grow poorly under the conditions at which MRC5 and unmodified XPCS1RO cell line were grown, therefore survival experiments following 4NQO and gemcitabine exposure were not successful.

Previous experiments have shown that XPF nuclease function is required for efficient NER and that XPF truncation mutations result in lower ERCC1 protein levels due to proteosomal degradation (Ahmad et al., 2010; Staresincic et al., 2009). The XP2YO cell line and cDNA transfected clones were therefore tested for sensitivity to 4NQO and gemcitabine by colony formation assays, before being probed for XPF and ERCC1 protein levels by western blotting and cDNA integration by PCR.

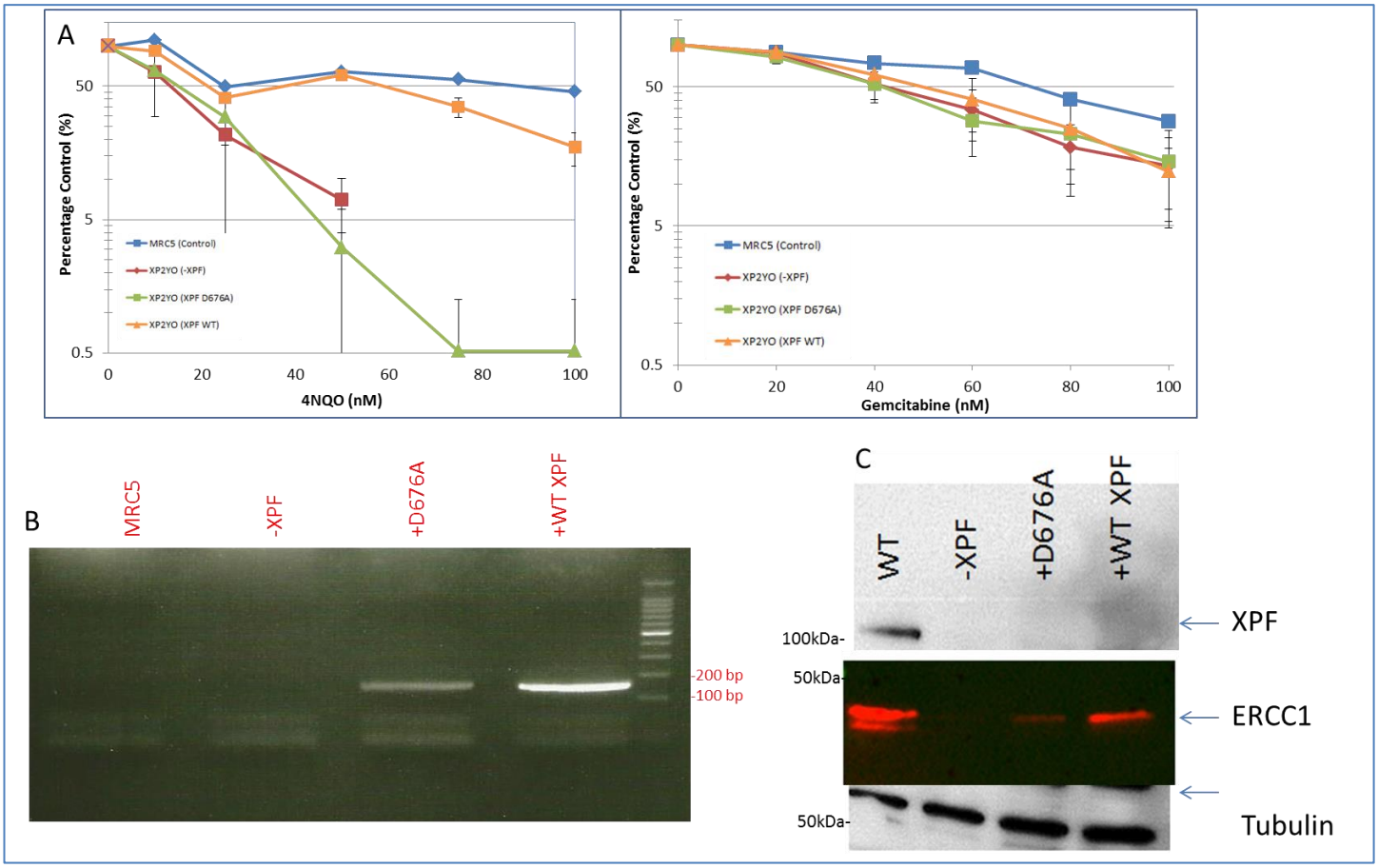

Figure 6.3: Nuclease Deficient XPF cDNA Construct XP2YO Cell Line Is Sensitive to 4NQO. (A) XP2YO Sensitivity to 4NQO (left) and gemcitabine (right) compared to MRC5 as well as nuclease deficient XPF (D676A) and wild-type XPF cDNA transfected XP2YO cell lines. (**B**) PCR detection of cDNA constructs in XP2YO clones. (**C**) Western blot detection of XPF and ERCC1 proteins in XP2YO clone cell extracts.

As shown in Figure 6.3, both XP2YO cell and its clone transfected with XPF D676A are sensitive to 4NQO whilst the XP2YO cell line transfected with wild-type *XPF* shows full rescue. This is consistent with results related to UV exposure using XPF D676A transfected cells (Staresincic et al., 2009). However, when treated with the nucleoside analogue gemcitabine, there is very little difference between the native XP2YO cell line and both the nuclease deficient and wild-type complemented clones.

The XP2YO clones were then screened for the presence of cDNA XPF constructs. As shown in Figure 6.3B, both XPF D676A and wild-type XPF transfected XP2YO cell lines show a fragment at the expected size of 157 bp. The expected 7 kb genomic DNA fragment including a large intron which spans between exon 9 and 10 is not detectable in MRC5 cells or any XP2YO clones under these conditions. Additionally, XPF protein levels were determined by western blotting were it can be seen that XPF and ERCC1 are not detectable

in the native XP2YO cell line (Fig.6.3C, lane 2). However, complementation wild-type XPF seems to restore the stability of the ERCC1 protein, but XPF protein levels are still very low. It therefore seems that very low levels of XPF-ERCC1 are able to restore resistance to UV as well as 4NQO, but does not reduce the mild sensitivity seen in the XP2YO cell line to gemcitabine.

To validate the sensitivity phenotypes seen in XP patient cell lines to nucleoside analogues, esiRNA and shRNA knockdown attempts were made with several NER factors. We also wished to knockdown expression of other NER genes in XP cell lines in order to create double NER deficient cells to further dissect the observed phenotypes.

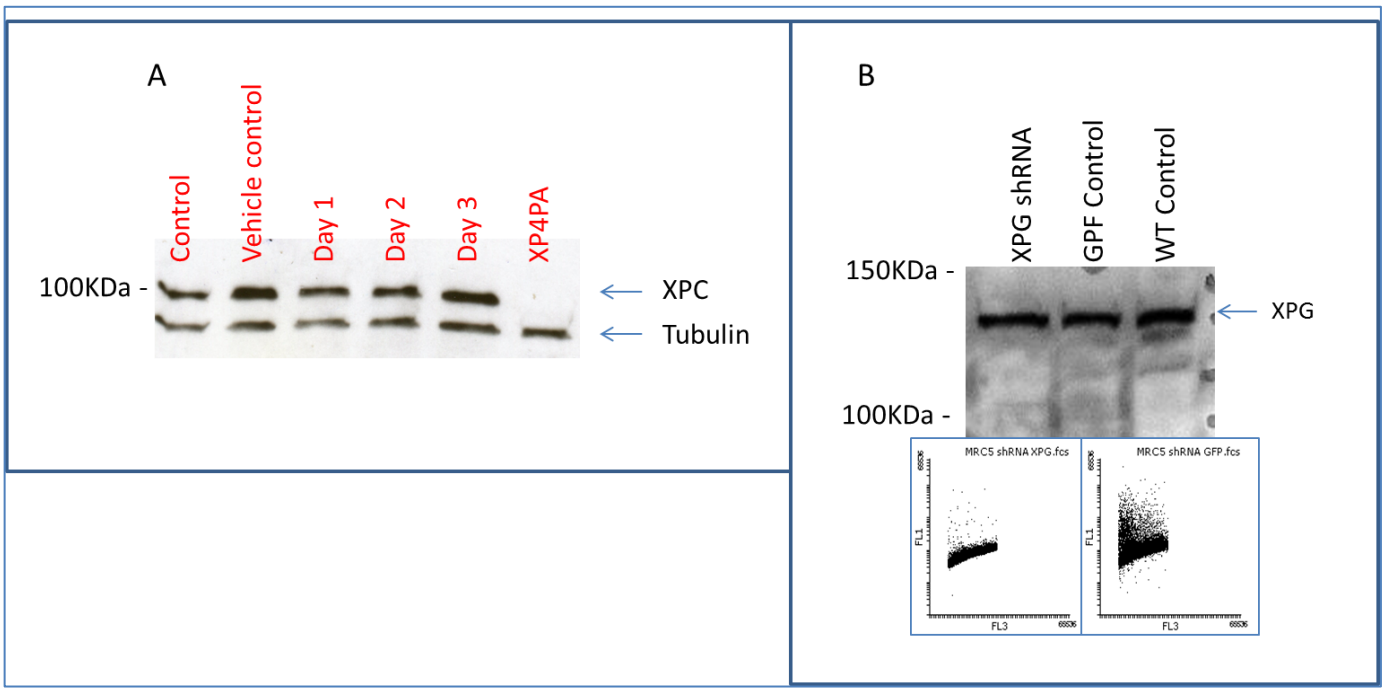

Figure 6.4: Unsuccessful esiRNA and shRNA Knockdown Attempts for XPC and XPG (**A**) Western blot detection of XPC protein over 72 hours from MRC5 cell line extracts transfected with XPC esiRNA showing absent XPC only in XP4PA cell line negative control. (**B**) Western blot detection of XPG protein from MRC5 cells transfected with XPG shRNA construct showing positive GFP expression as assayed by flow cytometry but no significant reduction in XPG protein levels.

esiRNA knockdown was attempted first in MRC5 fibroblasts. esiRNA was complexed in my optiMEM media with lipofectamine RNAiMAX transfection reagent and applied to media in 6 well plates. Protein extracts were then prepared every 24 hours and probed via western blotting for XPC expression in conjunction with untreated and lipofectamine only controls as well as an extract prepared from XPC deficient XP4PA cell line (Fig.6.4A lane 6). As shown in Figure 6.4A, XPC levels are not significantly reduced over 72 hours in MRC5 cells after

esiRNA transfection, whereas XPC protein is not detectable in XP4PA. After several attempts at XPC esiRNA knockdown, it was decided that this technique was unsuccessful.

Secondly, MRC5 cells were transfected with XPG shRNA lentiviral particles and grown for 1 week in complete media containing 1 μg/mL puromycin before protein extraction as well as analysis of GFP expression by flow cytometry. As shown in Figure 6.4B, GFP expression in vector only controls is readily detectable by flow cytometry, however shRNA transfection again fails to reduce XPG expression when probed by western blotting. Due to repeated failed attempts at both esiRNA and shRNA knockdown, we decided to attempt CRISPR/Cas9 gene knockout.

As previously mentioned, in *S. pombe* the lack of the ERCC1 homolog Sw10 has been shown to induce sensitivity to the nucleoside analogue gemcitabine whereas cells lacking Rad16 (XPF) are not sensitive (unpublished data). We therefore decided to attempt to reproduce this phenotype in human cell lines using the CRISPR/Cas9 gene knockout system and compare the sensitivity to that seen in the XP2YO (XP-F) cell line.

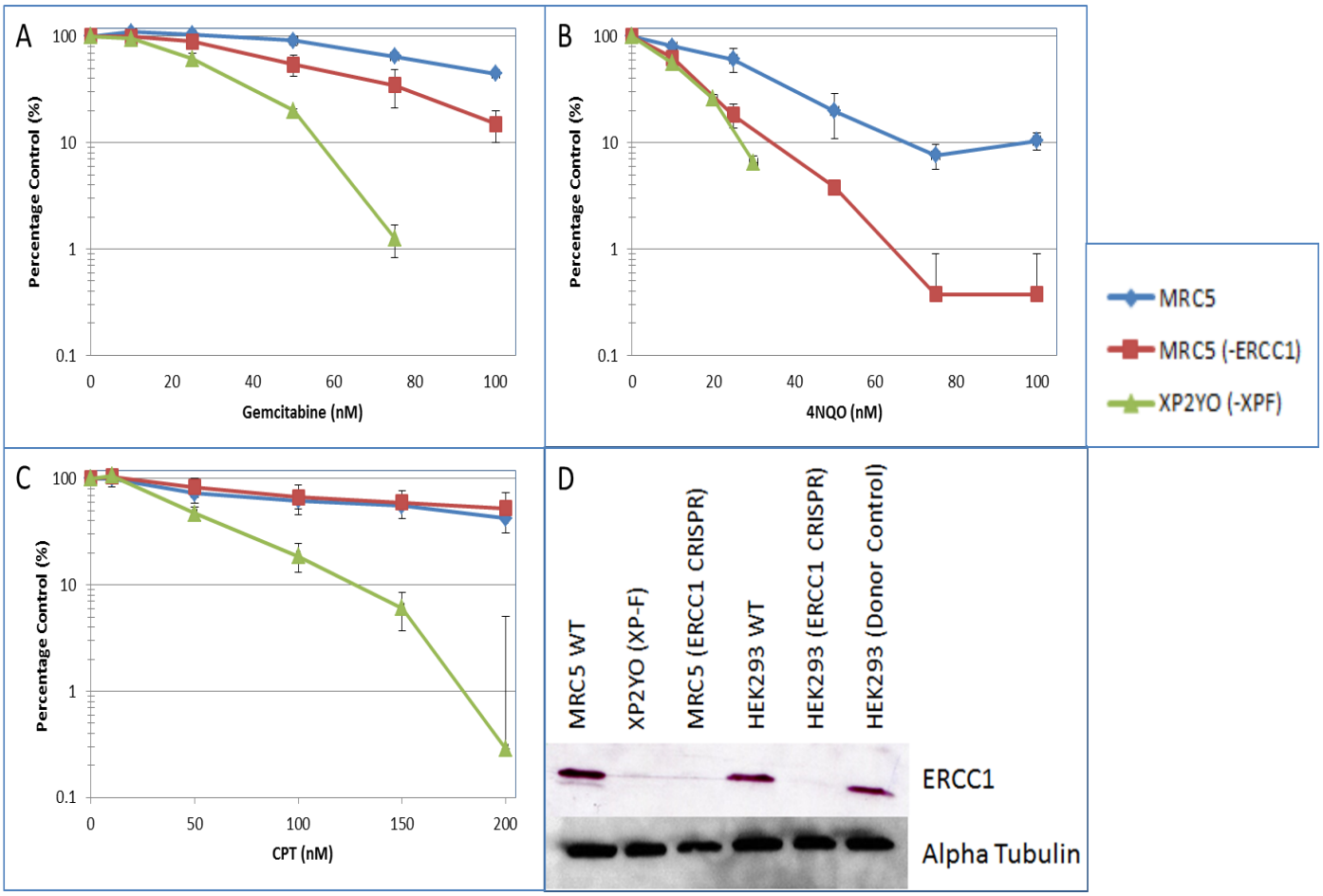

Figure 6.5: CRISPR/CAS9 Generated ERCC1 Knockout Sensitises Cells to Gemcitabine, CPT and 4NQO. Colony forming assay data from ERCC1 knockout cell line following 24 hour exposure to gemcitabine (**A**), 4NQO (**B**) and camptothecin (**C**). (**D**) Western blot detection of ERCC1 in MRC5 clones and HEK293 clones transfected with ERCC1 CRISPR/CAS9 knockout plasmids.

The MRC5 clone isolated following CRISPR/Cas9 plasmid transfection was probed for ERCC1 protein levels after protein extraction and found to have significantly reduced ERCC1 bands on western blots (Fig.6.5D). The MRC5 cell line lacking ERCC1 protein (MRC5 –ERCC1) was then maintained in complete media containing 1 µg/mL puromycin and tested for sensitivity to the nucleoside analogue gemcitabine as well as 4NQO and the topoisomerase I poison CPT by colony forming assays. XP2YO and MRC5 –ERCC1 cell lines were found to be equally sensitive to 4NQO confirming the lack of efficient nucleotide excision repair. However, the lack of ERCC1 does not seem to affect CPT sensitivity. Additionally, ERCC1 knockout seems to produce an intermediate sensitivity to gemcitabine compared to XP2YO (XP-F) and MRC5.

Discussion

Work in this chapter outlines several attempts at NER factor knockdown and the use of nuclease-deficient and wild-type complemented *XPG* and *XPF* cDNA transfected cell lines. The XPCS1RO and XP2YO cell lines which were kindly provided by Professor Alan Lehmann's lab (University of Sussex) containing cDNA constructs of their respective mutated nucleases were validated by PCR detection (Fig.6.2). These cell lines originally created by Dr Orlando Schärer (Stony Brook University, New York) have been used in several studies to investigate the mechanics of the classical NER pathway (Constantinou et al., 1999; Staresincic et al., 2009; Tsodikov et al., 2007). However, due to low protein levels seen in western blots (Fig.6.3C), it was decided that these cell lines provided an unreliable model for us to further investigate. Although XP2YO (XP-F) cell line transfected with wild-type *XPF* cDNA restored survival following 4NQO treatment, very little effect was seen following gemcitabine incubation. This observation, combined with the low XPF expression, as well as altered ERCC1 protein levels resulted in no further work being carried out with the XPCS1RO and XP2YO cDNA transfected cell lines.

esiRNA and shRNA knockdown of XPC and XPG respectively proved unsuccessful after several attempts at both. In the case of esiRNA, it maybe that low transfection efficiency in MRC5 fibroblasts prevented effective knockdown, or that the XPC protein has a long enough half-life to prevent siRNA interference from producing an effect. However as RT-PCR was not attempted we cannot be sure. In the case of shRNA knockdown targeting XPG, positive controls produced a high GFP signal and isolated clones were strongly puromycin resistant. XPG expression however was unaffected and no increase in sensitivity to gemcitabine was detected (not shown). Due to time constraints, we were unable to attempt siRNA or shRNA knockdown in more commonly used cell lines such as HEK293 or U2OS.

CRISPR/Cas9 knockout of *ERCC1* showed more success than other knockdown attempts. *ERCC1* was targeted here to try and investigate the role of the XPF-ERCC1 heterodimer in nucleoside analogue sensitivity, specifically aiming to separate the function of the two subunits. Unlike *S. pombe* data from our lab which showed that cells lacking Swi10 (ERCC1) were sensitive to gemcitabine, whereas deletion of Rad16 (XPF) had no effect, in higher eukaryotes this does not seem to be the case. XPF-deficient cell line XP2YO seems to be more sensitive to gemcitabine than MRC5 cells without ERCC1 (Fig.6.5A). This MRC5-ERCC1

cell line was further tested with 4NQO and shown to have very similar sensitivity to XP2YO cell line (XP-F) (Fig.6.5B). Further work with this CRISPR/Cas9 generated cell line is required to investigate the role of ERCC1 in nucleoside analogue survival, however, due to time constraints, this has not been possible here.

Chapter 7: The Role of Mre11 Nuclease in the Resistance of Nucleoside Analogues and Camptothecin

Summary

Results shown here indicate a role for the Mre11 nuclease in the resistance of trapped topoisomerase, as well as a role of this nuclease in resistance to the nucleoside analogues gemcitabine and cytarabine. Using Mre11 D60N and H129N point mutant chicken DT40 cell lines, the requirement of the nuclease function in resistance to CPT induced topoisomerase I lesions, as well as nucleoside analogue induced lesions discussed in earlier chapters is shown. In the absence of Mre11 nuclease activity, DT40 cells are sensitive to CPT which causes slow S-phase progression and G2/M accumulation. Similarly, nucleoside analogues gemcitabine and cytarabine cause increased intra-S-phase accumulation and cytotoxicity in the absence of Mre11 nuclease function.

Introduction

Work in this chapter uses the DT40 chicken cell line which is derived from a bursal B cell lymphoma which resulted from injection with avian leukosis virus (ALV) (Winding and Berchtold, 2001). The DT40 cell line which has been used widely in gene disruption experiments, was first generated in 1984, and was found to have a generation time of 8 hours when grown at 39°C (Adachi et al., 2004; Baba and Humphries, 1984; Winding and Berchtold, 2001). As a B lymphocyte precursor, DT40 cells have been shown to undergo constant recombination of the IgG gene, and due to the rapid generation time, as well as high levels of homologous recombination, DT40 cells have been an attractive model for gene knockout experiments (Wakasugi et al., 2007; Winding and Berchtold, 2001). 70% of DT40 cells are in S-phase under optimum conditions due to the extremely short G1 and G2 phases seen in this cell type, and they are grown in suspension (as described in material and methods) (Ji et al., 2009; Yamazoe et al., 2004).

Following the successful generation of Mre11 point mutated DT40 cells in our lab by Dr Ellen Vernon, work here shows the potential role of this nuclease in the resistance of trapped topoisomerase I enzymes from genomic DNA during replication, and also, following on from

work in earlier chapters, the potential role of Mre11 in resistance to the nucleoside analogues gemcitabine and cytarabine. The roles of topoisomerase enzymes and the MRN complex which contains the Mre11 nuclease are introduced below.

Topoisomerases

Topoisomerase enzymes are responsible for relieving topological stress within the DNA helix. During replication and transcription when the DNA is unwound, positive and negative supercoiling occurs ahead and behind the replication fork which unless resolved, can cause replication arrest and DNA damage. Topoisomerase enzymes cleave DNA and relieve topological stress which prevents physical damage to DNA during constant transcription and chromatin remodelling (Durand-Dubief et al., 2010). 7 topoisomerase enzymes have been identified in higher eukaryotes which can be separated into two classes. Type 1 topoisomerases which include TopI, TopIIIα and TopIIIβ as well as the mitochondrial specific top1mt cleave only one DNA strand and rotate this ssDNA section around itself before resealing the broken strand, thereby restoring the DNA double helix (Pommier et al., 2008). Type 1 topoisomerases are classed as tyrosine recombinases due to the tyrosine residue located within the active site. Other tyrosine recombinase enzymes include Flip and Cre recombinases (Pommier, 2003). Type 2 topoisomerases include TopIIα, and TopIIβ which cleave both strands of DNA and rotate around themselves to relieve super helical tension (Montecucco and Biamonti, 2007). Type 2 topoisomerases have been shown to resolve complex catenaned and knotted DNA structures that occur during replication (Martínez-García et al., 2014).

TOP1 is an essential gene in higher eukaryotes located on chromosome 20q11.2 in humans which encodes for the 100 kDa Top1 topoisomerase enzyme. *TOP1* deletion in higher eukaryotes causes embryonic lethality in mouse knockout models, as well as conditional knockout cell lines, however, in yeast models cells remain viable (Jacob et al., 2005). The active site of Top1 topoisomerase controls both its endonuclease activity as well as the stabilisation of a covalent bond between the central tyrosine residue and the 3'end of the induced DNA nick. This covalent bound Top1 to DNA bond is referred to as Top1-cc or the cleavable complex (Tomicic and Kaina, 2013). Once the Top1-cc is formed, controlled rotation of the DNA helix relaxes the superhelical tension and the 3'DNA end is then

released from the tyrosine residue by re-ligation of the 5'end. Due to the fact that the re-ligation step in the Top1-DNA interaction is considerably faster than the endonuclease and rotation action, the ratio of Top-cc to free Top1 is shifted away from multiple DNA single-strand gaps occurring at any one time which helps prevent DNA damage and checkpoint activation (Pommier et al., 2008; Tomicic and Kaina, 2013). However, the Top-cc half-life can be extended by cleavage at abasic sites, oxidised bases, single-strand nicks and collision with replication forks (Giovannetti et al., 2005). Extending Top-cc time leads to activation of DNA damage signalling and potential collapse of various substrates into DSBs which may trigger mutations or apoptosis.

Topoisomerase poisons are a class of chemotherapeutics which bind Top-cc substrates and prevents the re-ligation of induced DNA breaks, thus increasing the half-life of Top-DNA complexes. The Top1 specific poison camptothecin (CPT) was originally extracted from the *Camptotheca acuminata* tree and was found to inhibit the growth of leukemia cells (Wall et al., 1966). Semi-synthetic CPT derivatives such as topotecan and irinotecan have since been developed and are routinely used in the treatment of colorectal and lung cancers (Giovannetti et al., 2005; Tomicic and Kaina, 2013). In the presence of CPT, Top1 is bound to DNA considerably longer which shifts the ratio of free Top1 to Top-cc bonds towards the production of single strand nicks with covalently bound 3'-topoisomerase. The induced CPT-Top-cc complex is able to eventually reverse itself in the absence of replication, however when transcription or replication machinery collide with the Top-cc complex, the trapped topoisomerase enzyme becomes irreversibly attached because the 5'DNA end is no longer in a position to re-ligate due to the increased distance between the two DNA ends (Jacob et al., 2005; Tomicic and Kaina, 2013).

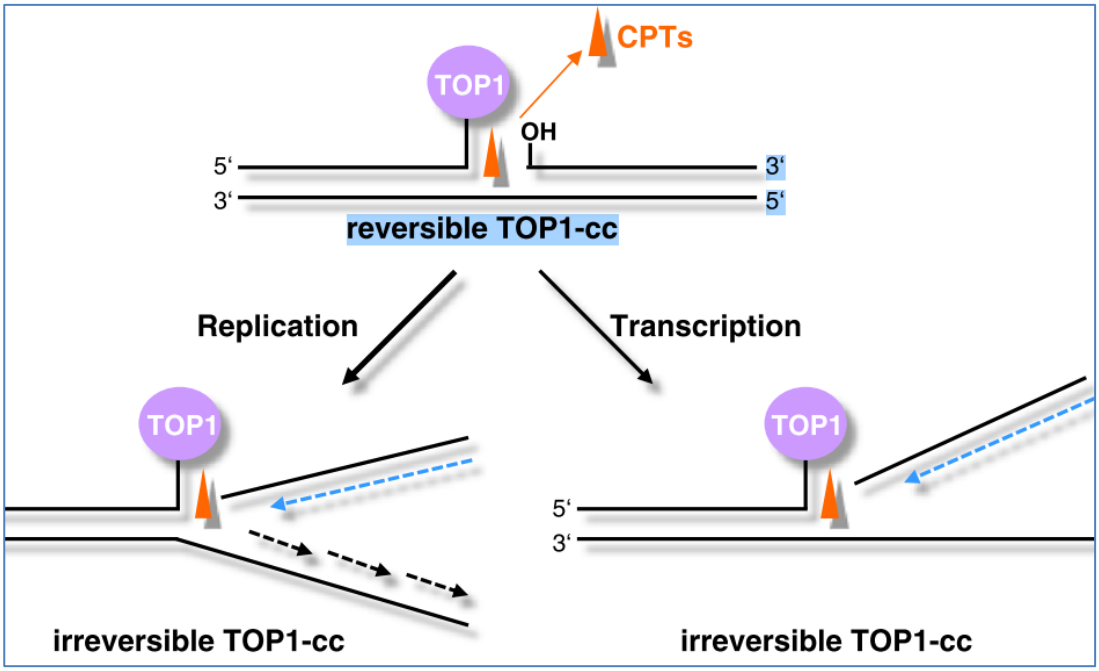

Figure 7.1: CPT Induced TOP-cc Complex Stabilisation. Reversible Top-cc complex is able to spontaneously resolve in the absence of replication or transcription, however replication run off can lead to double strand breaks or stalled replication forks which require enzymatic resolution (Pommier et al., 2008).

Persisting Top-cc complexes induce γH2AX activation in replicating cells and have been shown to cause powerful inhibition of further DNA synthesis (Foster et al., 2011). This reduction in DNA synthesis has been shown to be in part due to physical replication block by trapped topoisomerases, as well as S-phase checkpoint activation caused by CHK1 and CHK2 phosphorylation (Shao et al., 1999). Experiments where replication is prevented using the polymerase inhibitor aphidicolin have shown that γH2AX and checkpoint activation only occur during active replication in the presence of CPT (Furuta et al., 2003). Similarly, when CPT is removed from tissue culture media, the equilibrium between free and trapped topoisomerase begins to revert leading to the eventual resumption of replication and transcription, except in cells lacking functional CSA and CSB TC-NER factors which fail to resume transcription following CPT treatment (Desai et al., 2003). Inhibition of CHK1 has also been shown to induce sensitivity to CPT by preventing the activation of the S-phase checkpoint which ultimately causes greater DNA damage due to continued DNA synthesis (Facchinetti et al., 2004; Shao et al., 1997).

Several mechanisms by which cells survive trapped Top-cc complexes and remove the lesion have been proposed. Firstly, replication fork reversal which forms complex quaternary

structures similar to Holliday junctions have been shown to occur in response to trapped topoisomerase enzymes. These Holliday-like structures are resolved by BLM/TopIIIβ heterodimers which function as part of the RecQ helicase system (Hickson, 2003). Secondly, 5' endonucleases such as the XPF-ERCC1 and MUS81-EME1 heterodimers have been shown to cleave splayed arm structures that occur at Top-cc induced stalled replication forks (Pommier, 2003; Regairaz et al., 2011). This 5'endonuclease attack causes the collapse of the structure into DSBs which cause the phosphorylation of ATM leading to S-phase checkpoint activation (Shao et al., 1997, 1999) (Fig.7.2).

Trapped Top1 has also been shown to be targeted for proteosomal degradation via polyubiquitination (Tomicic and Kaina, 2013). Proteolytic degradation of the topoisomerase enzyme leaves behind the covalently trapped tyrosine residue, with a polypeptide tail of varying length, creating the lesion similar to the artificial complex used in *in vitro* models termed 3'-tyrosyl-DNA (El-Khamisy et al., 2007). 3'-tyrosyl-DNA is a substrate for the DNA repair enzyme tyrosyl-DNA-phosphodiesterase (Tdp1) which was discovered in 1999 (Pouliot et al., 1999). Tdp1 cleaves the covalent bond between the remaining tyrosine residue and the 3'end of the DNA which creates a 3'phosphate end which is further processed by the phosphatase enzyme hPNK which creates the 3'-OH needed for re-ligation (Fig.7.2) (Das et al., 2014). The Tdp1 enzyme which also removes covalently bound DNA glycosylations is highly conserved, and homozygous loss of function mutations (H493R) leads to the hereditary DNA repair disease SCAN1 (spinocerebellar ataxia with axonal neuropathy 1) (Rass et al., 2007). SCAN1 presents during childhood and is characterised by increasing neurosensory and motor impairment with declining cognitive function (Fam et al., 2012). Individuals with SCAN1 are most commonly heterozygous for an H493R point mutation which has been shown to render cultured cells from these patients highly sensitive to CPT, PARP inhibitors and ionising radiation but not the topoisomerase II poison etoposide, or bleomycin (Das et al., 2014; El-Khamisy et al., 2007). Tdp1 has been shown to form a subunit of a larger complex with XRCC1, PARP1, polymerase β and ligase III. The relatively recently identified protein aprataxin has also been shown to interact with the XRCC1 complex (Clements et al., 2004; Moreira et al., 2001). Cell lines with *XRCC1* and *PARP1* mutations have been shown to be sensitive to CPT, and loss of function of aprataxin causes AOA1

(ataxia with oculomotor apraxia 1) which is the most common form of ataxia in Japan which also induces CPT sensitivity (Rass et al., 2007; Tomicic and Kaina, 2013).

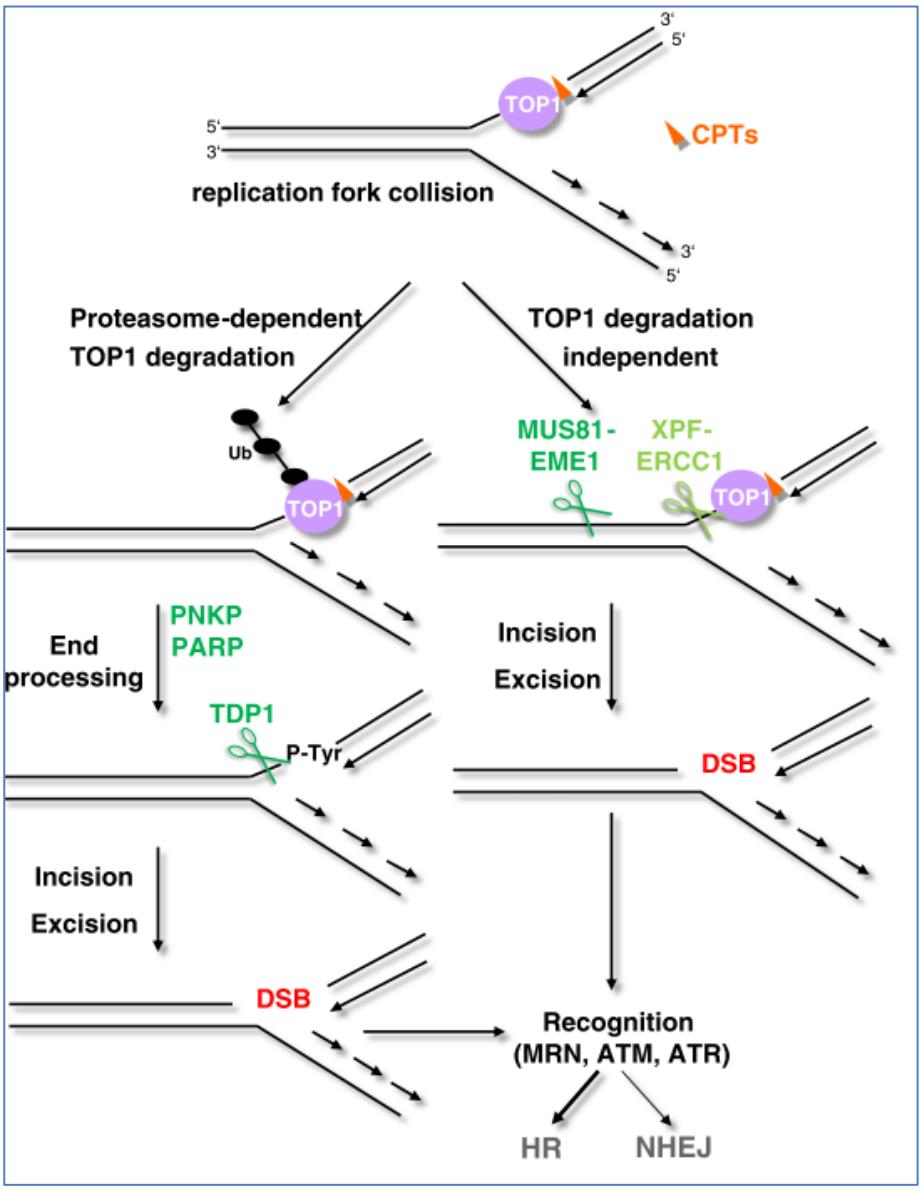

Figure 7.2: Removal of Trapped Topoisomerase I from DNA. The removal of trapped topoisomerase from replication or transcriptional substrates by either Tdp1 following proteolytic degradation, or endonuclease attack by XPF-ERCC1 or MUS81-EME1 resulting in DSB formation (Tomicic and Kaina, 2013).

Due to the multiple repair mechanisms that have been shown to remove trapped topoisomerases, several groups have shown that deficiency in one or more of the pathways results in dramatically increased CPT sensitivity. For example, PARP inhibition in cells from SCAN1 patients has been shown to super-sensitise cells to CPT (Das et al., 2014). Similarly, Tdp1 deficiency in combination with CHK1 inhibition dramatically increases CPT sensitivity suggesting that Tdp1 is the most prominent factor in the removal of trapped topoisomerase

enzymes in the absence of effective S-phase checkpoint activation (Pouliot et al., 1999). Deficiency in the endonuclease Mre11 has also been shown to increase sensitivity to CPT in cells lacking Tdp1 (Liu et al., 2002). Mre11 which is part of the larger MRN complex has 3'-flap endonuclease activity which has been shown to be able to cleave trapped topoisomerase substrates (Fig.7.3). Additionally, *S. pombe* cells lacking Rad50 and Mre11 nuclease activity have been shown to be sensitive to CPT (Hartsuiker et al., 2009). As many colorectal cancers have been shown to carry Mre11 mutations, and the fact that most treatment regiments for these cancers involve CPT derivatives, it is essential to understand the role of both Mre11 and Tdp1 in topoisomerase removal (Foster et al., 2011; Stracker and Petrini, 2011).

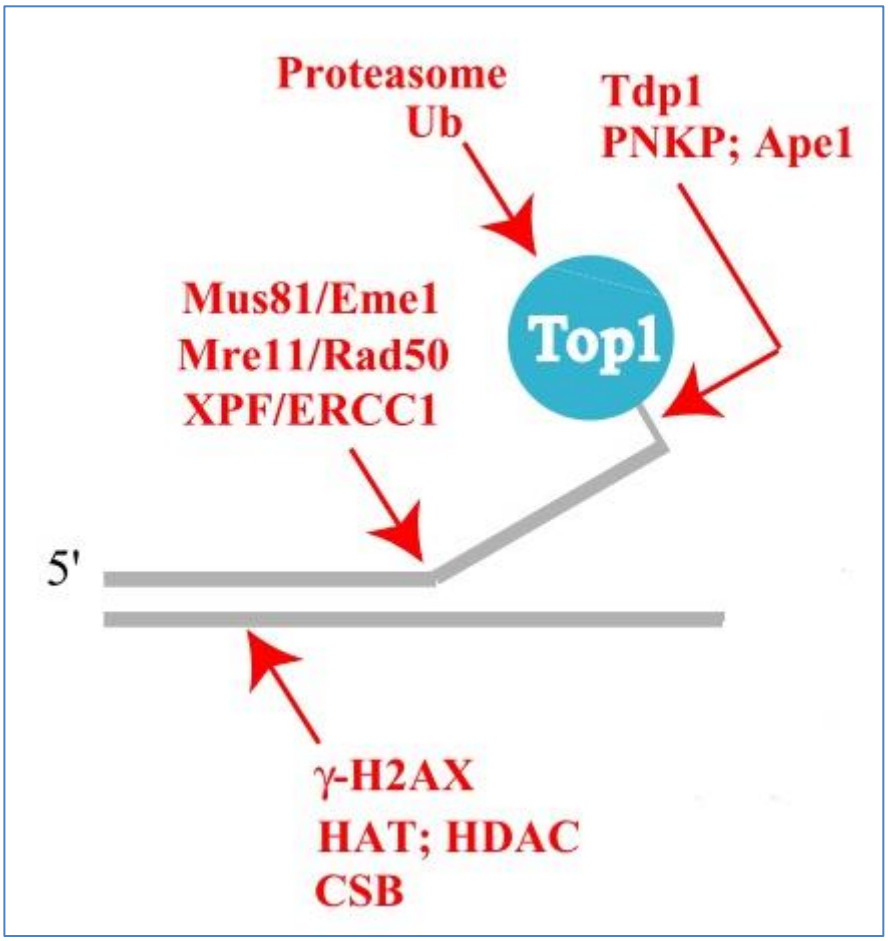

Figure 7.3: Topoisomerase Removal by 3'Flap Endonucleases. The removal of trapped topoisomerase by either proteolytic attack followed by Tdp1 removal, or by the action of several 3'-flap endonucleases, MUS81, XPF, or Mre11 (Tomicic and Kaina, 2013 modified).

MRN Complex

The highly conserved MRN complex has been shown to have roles in both DNA damage signalling and DNA repair in response to DSBs, as well as telomere maintenance, meiosis and DNA replication (Kavitha et al., 2010; Lee and Dunphy, 2013). The MRN complex is made up of the subunits Mre11, Rad50 and Nbs1, from which it gets its name. The core of the MRN complex consists of two Mre11 and two Rad50 proteins which bind each other to form a hetero-tetramer, and one Nbs1 protein molecule which interacts with Mre11 (Lamarche et al., 2010). Mre11 and Rad50 share homology with the *Escherichia coli* proteins SbcD and SbcC which lacks a Nbs1 homolog, whereas in the yeast model *Saccharomyces cerevisiae*, the Nbs1 homolog XRS2 functions as the signalling kinase (Krogh et al., 2005).

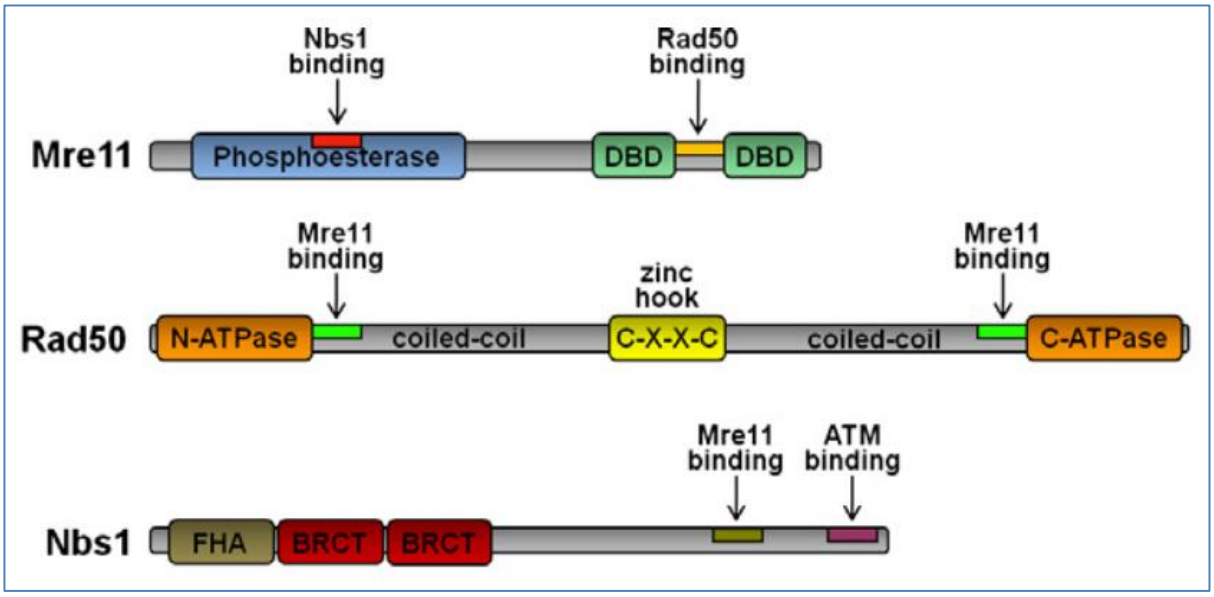

Figure 7.4: MRN Subunit Domain Structure. Schematic showing multiple domains of Mre11, Rad50 and Nbs1 (Lamarche et al., 2010 modified).

As shown in Figure 7.4, Rad50 has two coiled-coil domains separated by a Zinc hook. Rad50 is responsible for DNA binding and has been shown to be able to bind to the ends of DSBs. This is achieved by the N-terminal and C-terminal Walker motifs that associate together in the 3D protein structure to form a ATP-dependent DNA binding cassette which has been shown to partially unwind dsDNA at DSB sites (de Jager et al., 2001). The symmetry of the Rad50 molecule allows for the binding of two Mre11 molecules at the junction between the DNA binding domain and the central coiled-coil domain, which forms the heterotetramer $Mre11_2Rad50_2$ (Lamarche et al., 2010). It has previously been reported that the ATP hydrolysis ability of the Rad50 protein controls the affinity and duration of DNA binding (Hopfner et al., 2000).

The Nbs1 subunit of the MRN complex is thought to have no direct DNA repair activity, however it is involved in the signalling of DNA damage (Kavitha et al., 2010). Within the 76 kDa Nbs1 protein, the N-terminal FHA domain is responsible for interacting with several DNA repair factors which have a phosphorylated threonine residue within the Ser-X-Thr motif (Williams et al., 2009a). DNA repair factors which share this phosphorylation site include the *S. pombe* CtIP homologue, Ctp1 and the p53 binding protein Mdc1 (Lloyd et al., 2009). Similarly, the adjacent BRCT domain is responsible for interacting with phosphorylated checkpoint machinery proteins (Lamarche et al., 2010). Much of what is known about Nbs1 functions has been learnt from studies using patient cell lines that have hypomorphic Nbs1 mutations resulting in the condition Nijmegen breakage syndrome (NBS) (Digweed and Sperling, 2004). Research using patients lacking the C-terminal Mre11 binding domain has shown that Nbs1 binding is essential for the nuclear import of the Rad50/Mre11 complex, and without which Nbs1 can be nuclear localised, but Rad50 and Mre11 can not (Desai-Mehta et al., 2001). The main role of Nbs1 within the MRN complex is the recruitment of ATM to sites of DSBs. Several SQ motifs within the central region of the Nbs1 structure have been shown to be phosphorylated by ATM and in turn recruit and cause the auto phosphorylation of ATM itself (Buis et al., 2008; Lee et al., 2013). Due to this co-activation between Nbs1 and ATM, it is thought that the MRN complex has both damage sensing and damage signalling roles (Uziel et al., 2003).

The Mre11 subunit of the MRN complex is responsible for the nuclease function. It has been shown that Mre11 has both 3'-5' ssDNA and 3'-5' dsDNA exonuclease activity, as well as ssDNA endonuclease activity which targets junctions between ssDNA and dsDNA (Krogh et al., 2005). As shown in Figure 7.4, Mre11 has a highly conserved manganese/magnesium dependent phophodiesterase motif which forms the nucleolytic ability of the protein, as well as two DNA binding domains at the C-terminus. Mre11 was shown to be an essential gene in higher eukaryotes, where gene knockouts in mice models cause embryonic lethality (Buis et al., 2008). Conditional knockout models using DT40 cells also show the essential role of Mre11 where cells rapidly accumulate double strand breaks and die following induction (Yamaguchi-Iwai et al., 1999). However similarly to Nbs1, much of what is known about the Mre11 nuclease has been learnt using cell lines derived from patients with hypomorphic mutations in the protein which leads to the condition ATLD (Taylor et al., 2004). Of the

various cell lines generated from ATLD patients with Mre11 truncations, no mutations have been identified in the phosphodiesterase motif, however most truncations result in the inability of the expressed Mre11 protein to bind Rad50 (Buis et al., 2008).

Several Mre11 point mutant cell lines have been generated to study the specific role of the phosphodiesterase motif and separate its function from widely used ATLD derived models. Several highly conserved residues have been targeted and modified in the Mre11 *S. pombe* homolog (formerly known as Rad32) such as D25A, which is located in the phosphodiesterase motif, exon 1. Also created in *S. cerevisiae*, Mre11 D25A strains were shown to be less sensitive to ionising radiation than Mre11Δ, showed a partial ability to bind Rad50 *in vitro*, and was proficient in NHEJ, however this point mutation causes telomere truncation (Furuse et al., 1998; Lewis et al., 2004a). Mre11 D56N and H125N mutants, however, show even less ionising radiation sensitivity compared to Rad32 D25A and have telomeres of normal length (Llorente and Symington, 2004; Moreau et al., 2001). Mre11 point mutants D25A, D56N and H125N in *S. pombe*, correspond to human Mre11 residues D16A, D65 and H129 respectively (Takata et al., 2005; Tomita et al., 2003).

Mre11 has been shown to be recruited to stalled replication forks and initiate resection to form 3'-overhang structures that are required for Rad51 loading which ultimately controls homologous recombination (Jones and Petermann, 2012; Jones et al., 2014; Thompson et al., 2012). Due to the exonuclease activity of Mre11 and its reported presence at stalled replication forks, it is likely that Mre11 has a role in the processing or removal of nucleoside analogues. Several studies have utilised the CHK1 inhibitor MK-8776 to investigate the role of Mre11 in response to stalled replication, as well as using the Mre11 inhibitor mirin (Lee et al., 2013; Matsumoto et al., 2013). Mirin has been shown to inhibit the exonuclease activity of Mre11 and prevent the MRN mediated activation of ATM (Dupré et al., 2011). As previously mentioned, Mre11 can produce ssDNA at sites of stalled replication forks, and the inhibition of CHK1 increases RPA and yH2AX phosphorylation. However, when Mre11 is inhibited by mirin, the increase in RPA and yH2AX activation, as well as ATM activation seen in MK-8776 treatment is prevented (Thompson et al., 2012). It has therefore been suggested that Mre11 nuclease activity is detrimental for the repair of stalled replication forks when CHK1 is inhibited. This is thought to be due to the lack of BRCA2 and Rad51 recruitment caused by CHK1 inhibition that leads to over resection by Mre11 which

produces long ssDNA that is a substrate for MUS81 mediated replication fork collapse (Jones et al., 2014; Montano et al., 2013). This has been shown via the comet assay where fewer DSBs are detectable in MK-8776 treated cells when Mre11 is inhibited (Thompson et al., 2012).

Although many studies have used ATLD patient cell lines to study Mre11, specific nuclease deficient point mutated cell lines have not been widely reported in higher eukaryotes, especially in relation to nucleoside analogues and CPT induced topoisomerase DNA damage. Work presented here uses Mre11 point mutant DT40 cell lines, D60N and H129N (comparable to *S. cerevisiae* D56N and H125N) to investigate the requirement of Mre11 nuclease activity in resistance to nucleoside analogues and topoisomerase I poisons. These Mre11 mutant cell lines were generated by targeted integration of homologous constructs containing a puromycin resistance cassette, which replaced the wild type *Mre11* allele in an Mre11 +/- background by Dr Ellen Vernon. These two mutant *Mre11* alleles have been shown to be both endo and exonuclease deficient, whereas the Mre11 H68S mutant is only deficient in exonuclease activity (Williams et al., 2009b). Results suggest that Mre11 D60N and H129N are the most sensitive to nucleoside analogues gemcitabine and cytarabine compared to control, as well as CPT; however, results are further complicated by an apparent haploid insufficiency in Mre11+/- controls which also may affect the mutant cell lines which were created from the heterozygous cell line.

This work builds on unpublished data from our lab which has shown that in the model organism *S. pombe*, Mre11 point mutants D65N and H134S (equivalent to higher eukaryotic D60N and H129N) are sensitive to the nucleoside analogues gemcitabine and cytarabine, as well as camptothecin. The *S. pombe* Mre11 mutants D65N and H134S also show a deficiency in the removal of gemcitabine from genomic DNA as measured by LC-MS/MS as discussed in Chapter 1, potentially indicating that Mre11 is able to removal incorporated gemcitabine from newly replicated DNA.

Results

Firstly, sensitivity to several nucleoside analogues and topoisomerase poisons was measured by colony forming assays. As DT40 cells are a suspension cell line, this was performed using semi-solid methylcellulose RPMI media as described in Chapter 2.

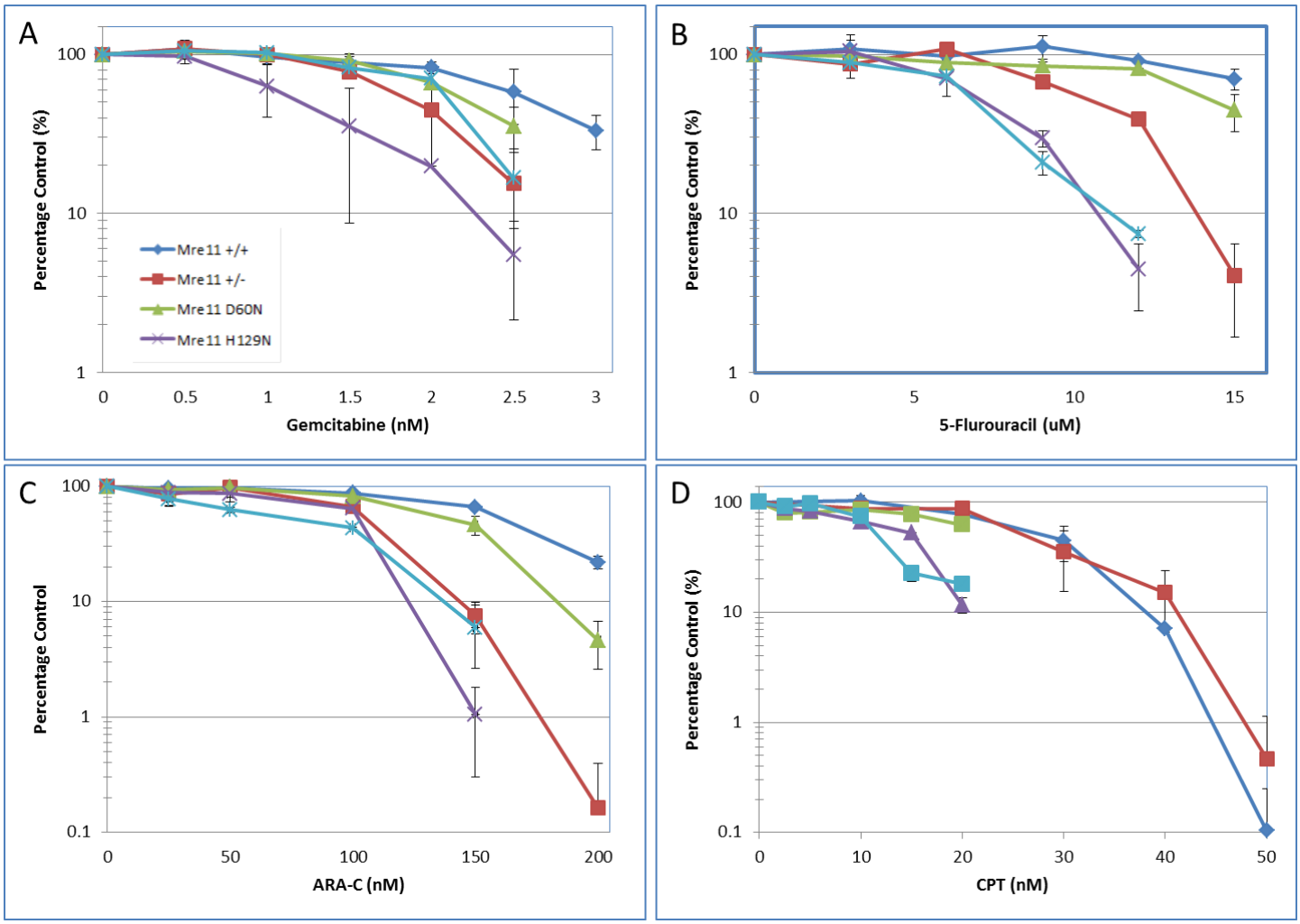

Figure 7.5: Mre11 Nuclease Deficiency Induces Sensitivity To Nucleoside Analogues and Camptothecin. Mre11 clone sensitivity data as assayed by colony formation following 8-10 days continuous exposure to (**A**) gemcitabine (**B**) 5-FU (**C**) ARA-C, (**D**) CPT. Error bars indicated standard deviation of at least 3 independent repeat experiments.

As seen in Figure 7.5, Mre11 D60N and H129N show increased sensitivity to the nucleoside analogues gemcitabine, cytarabine and 5-flurouracil compared to Mre11 +/+. However the increased sensitivity when compared to Mre11 +/- controls is less. This is not the case when Mre11 mutant cell lines are treated with the topoisomerase I poison camptothecin where wild type and Mre11 +/- cells show similar sensitivity. Mre11 D60N and H129N fail to grow

in concentrations of camptothecin above 20 nM whereas several colonies were present at 50 nM with Mre11 +/+ DT40 and Mre11 +/- cells.

Mre11 protein levels were then checked via western blotting to determine whether the levels of Mre11 was equal in both the Mre11 D60N and H129N mutants, and to what extent heterozygosity in the Mre11 +/- cell line effects its levels compared to the Mre11 +/+ DT40 cell line.

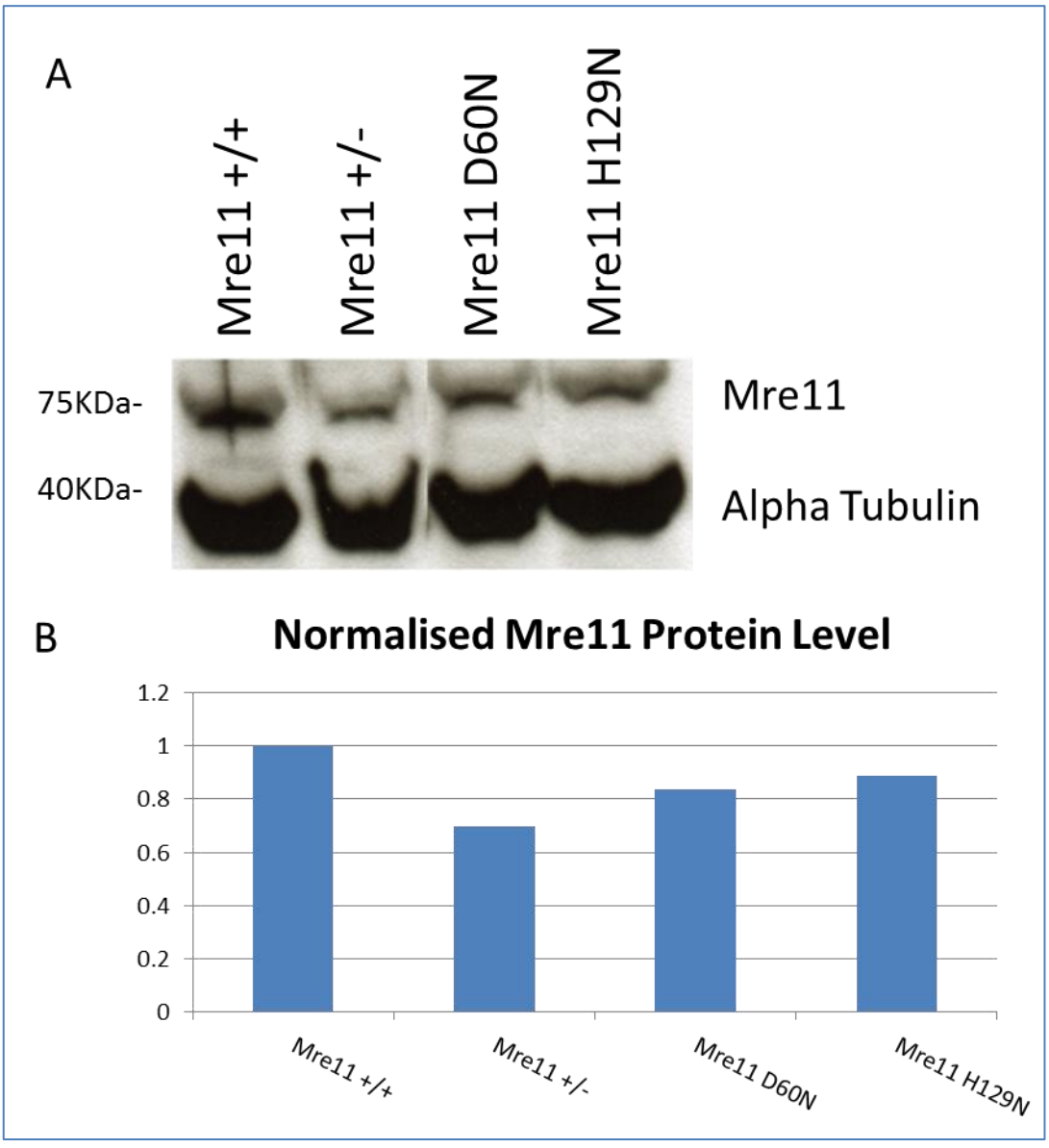

Figure 7.6: Mre11 Expression in DT40 Clones. (A) Western blot showing Mre11 and tubulin loading control. (**B**) Normalised Mre11 signal intensity relative to Mre11 +/+ as determined by area under the peak analysis via ImageJ.

As expected, the Mre11 +/- cell line had the lowest Mre11 protein levels due to its heterozygosity. Targeted integration of both Mre11 D60N and H129N constructs seems to

partially restore expression compared to Mre11 +/+ DT40 cells, however as this was only tested once, it is not possible to accurately assess Mre11 protein levels comparatively. Normalisation of the Mre11 levels (Fig.7.6B) was performed via area under the peak analysis using imageJ software where maximum pixel density was extrapolated for both tubulin loading controls and the detected Mre11 band. The ratio between the two were calculated in Mre11 +/+ DT40 cells then compared to Mre11 clones.

Due to the difference between the Mre11 +/- and Mre11 +/+ sensitivity to nucleoside analogues, it was suggested that the low Mre11 levels seen in the heterozygous cell line maybe responsible for the nucleoside analogue sensitivity seen in the Mre11 +/- cell line. Therefore potentially indicating that Mre11 has both structural and enzymatic activity in dealing with various DNA damage lesions that result from nucleoside analogue treatment, whereas only the nuclease activity is required for CPT removal. It may also be the case that low levels of wild-type Mre11 limits the rate of lesion repair in an apparent gene dosage effect, potentially leading to inappropriate repair pathways taking over. To further investigate this hypothesis, colony formation experiments were repeated using the topoisomerase II poison etoposide which is thought to be repaired via NHEJ with only a structural role for the MRN complex. To accurately assess the sensitivity of the Mre11 D60N and H129N cell lines to nucleoside analogues and camptothecin, comparisons to the Mre11 +/- cell line need to be made due to how the mutants were generated.

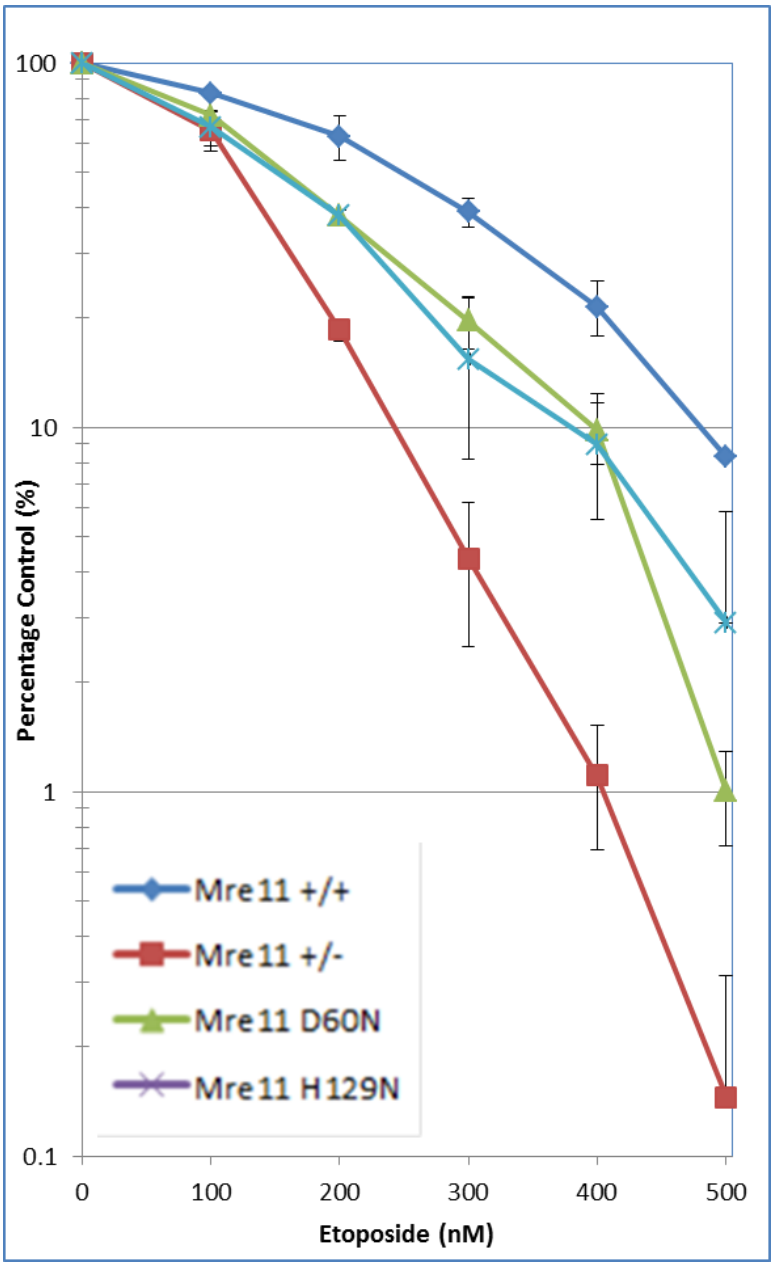

Figure 7.7: Mre11 +/- Cell Line is Sensitive to the Topoisomerase II Poison Etoposide. DT40 Mre11 clone sensitivity to etoposide as measured by colony formation. Error bars indicate standard deviation of at least 3 independent repeat experiments.

As shown in Figure 7.7, the heterozygous Mre11 +/- cell line is sensitive to the topoisomerase II poison etoposide compared to Mre11 +/+ Control as well as the nuclease deficient Mre11 clones. This result indicates that haploid insufficiency may play a part in sensitivity to topoisomerase II poisons and nucleoside analogues, but not to topoisomerase I poisons to which the Mre11+/- cell line showed no sensitivity to compared to Mre11 +/+. It therefore maybe the case that low levels of wild type Mre11 is detrimental in etoposide induced DSB repair, potentially due to a requirement for Mre11 to interact with Ku70/80 in

NHEJ in order to facilitate repair. However, the presence of a nuclease deficient Mre11 only induces intermediate sensitivity to etoposide, potentially due to the catalytically inactive Mre11 protein blocking access to the lesion for other NHEJ machinery.

Based on the observed sensitivity of Mre11 nuclease deficient cells to camptothecin (Fig.7.5), and the reported role of MRN in trapped topoisomerase I removal (Regairaz et al., 2011), we wanted to investigated whether Mre11 D60N and H129N mutants are sensitive to CPT due to difficulty in S-phase progression and replication. Slow resolution of trapped DNA-topoisomerase intermediates caused by Mre11 nuclease deficiency could potentially slow down DNA replication. Flow cytometry experiments were performed to test S-phase progression in the presence of CPT in Mre11 +/+ and Mre11 mutant cell lines.

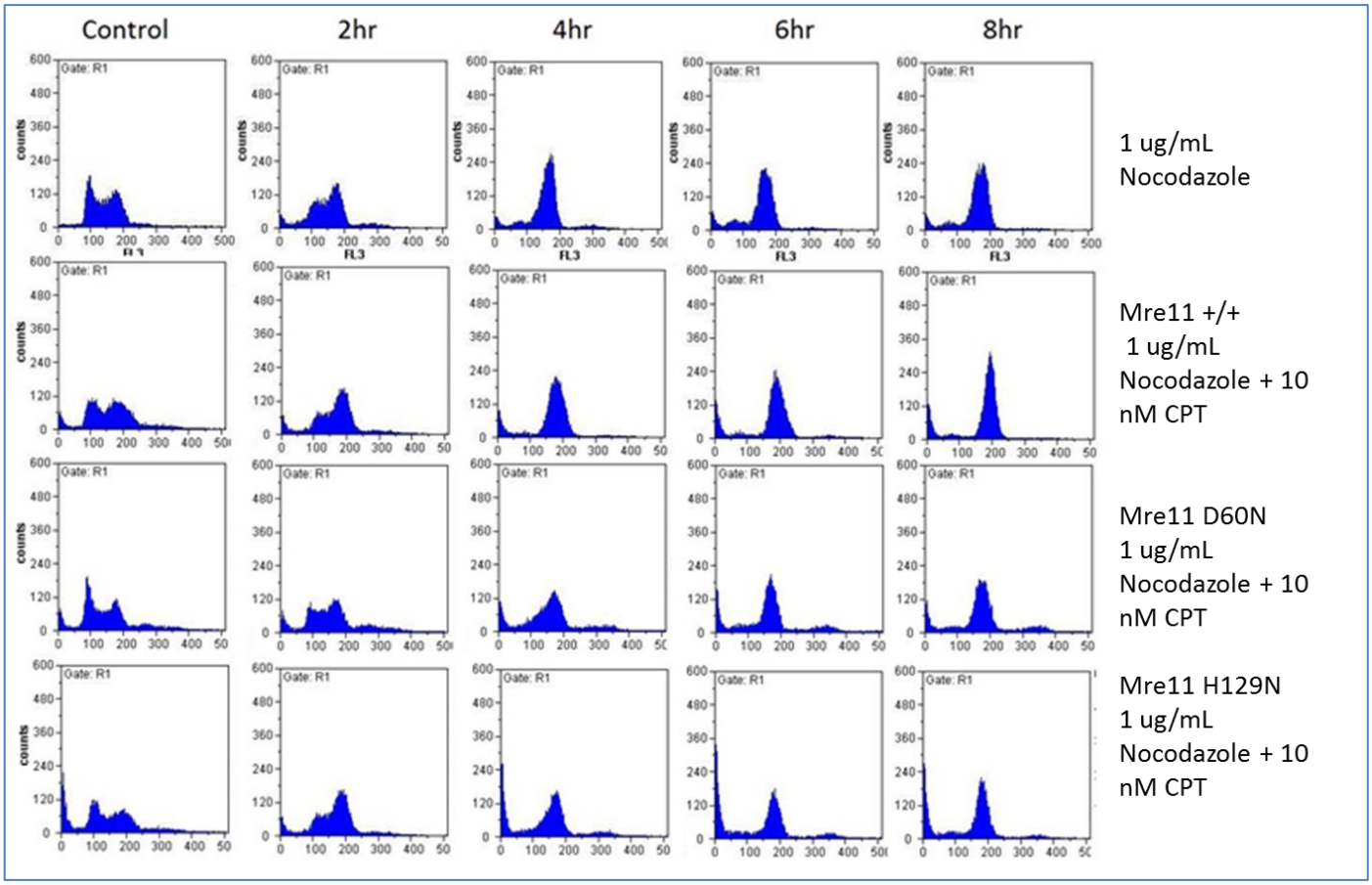

Figure 7.8: Mre11 Nuclease Deficiency Slows S-phase In The Presence Of CPT. DT40 Cell cycle flow cytometry stained with 2.5 ug/mL propidium iodide with 1 ug/mL nocodazole (top) or 10 nM CPT with nocodazole (lower).

As shown in Figure 7.8, nocodazole prevents cell division in DT40 cells caused by inhibition of spindle formation which causes DT40 cells to accumulate in G2/M after 4-6 hours. In the case of Mre11 +/+ DT40 cells, the presence of 10 nM CPT does not delay the G2/M

accumulation seen after 4 hours in the nocodazole only treated control. However, in the case of Mre11 D60N and H129N point mutants, a significant proportion of cells are still in S-phase after 4 hours. S-phase completion does not occur until 6 hours where the G2/M peak is at its narrowest. This result therefore indicates that Mre11 nuclease activity is required during S-phase in the presence of CPT, and without efficient Mre11 activity, replication slows. This experiment was then further expanded with asynchronous DT40 cultures incubated continuously in the presence of 20 nM CPT, including the Mre11 +/- cell line.

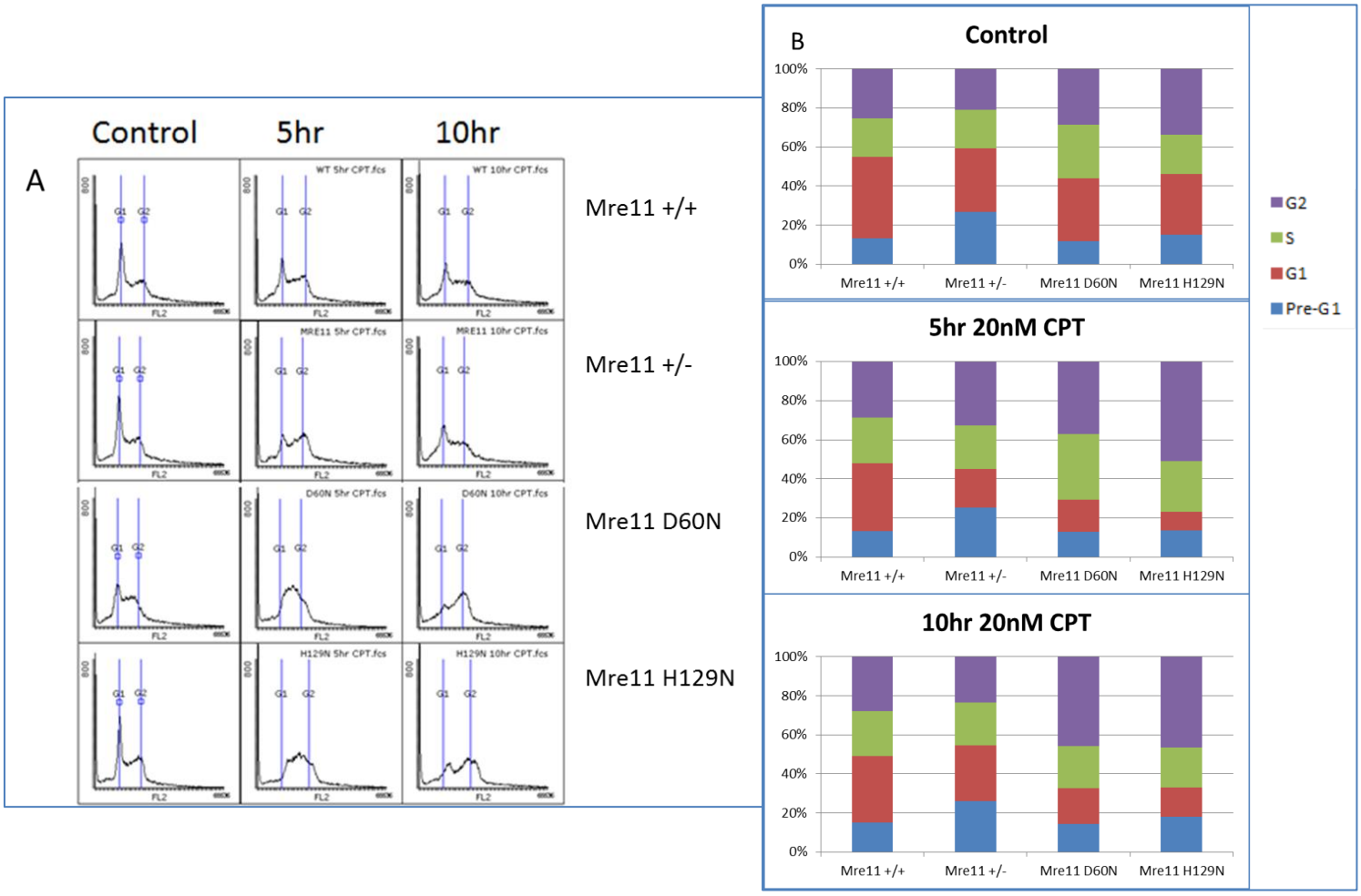

Figure 7.9: CPT Causes G2/M Arrest in Cells Deficient in Mre11 Nuclease activity. (**A**) DT40 asynchronous cultures incubated in the presence of 20 nM CPT, time points measured at 5 and 10 hours after CPT exposure. (**B**) Quantification of cell cycle phase following CPT incubation at indicated time points based on G1 and G2 peak position gating.

Under constant 20 nM CPT exposure, Mre11 +/+ and Mre11 +/- cell lines show very minor cell cycle profile changes (Fig.7.9). However, in the case of Mre11 D60N and H129N cell lines, S-phase accumulation is seen after 5 hours, and G2/M arrest occurs after 10 hours.

Similarly to Figure 7.8, this result indicates that Mre11 nuclease activity is required to progress efficiently through S-phase in the presence of CPT. G2/M arrest in these nuclease-deficient cell lines is consistent with G2/M checkpoint activation potentially indicating the presence of DSBs.

As Mre11 nuclease-deficient DT40 cells are sensitive to CPT as well as showing a slight sensitivity to nucleoside analogues, the cell cycle progression experiment was repeated under conditions of constant incubation with 10 nM gemcitabine and 250 nM cytarabine in order to investigate what effect Mre11 nuclease deficiency has on S-phase progression in the presence of nucleoside analogues.

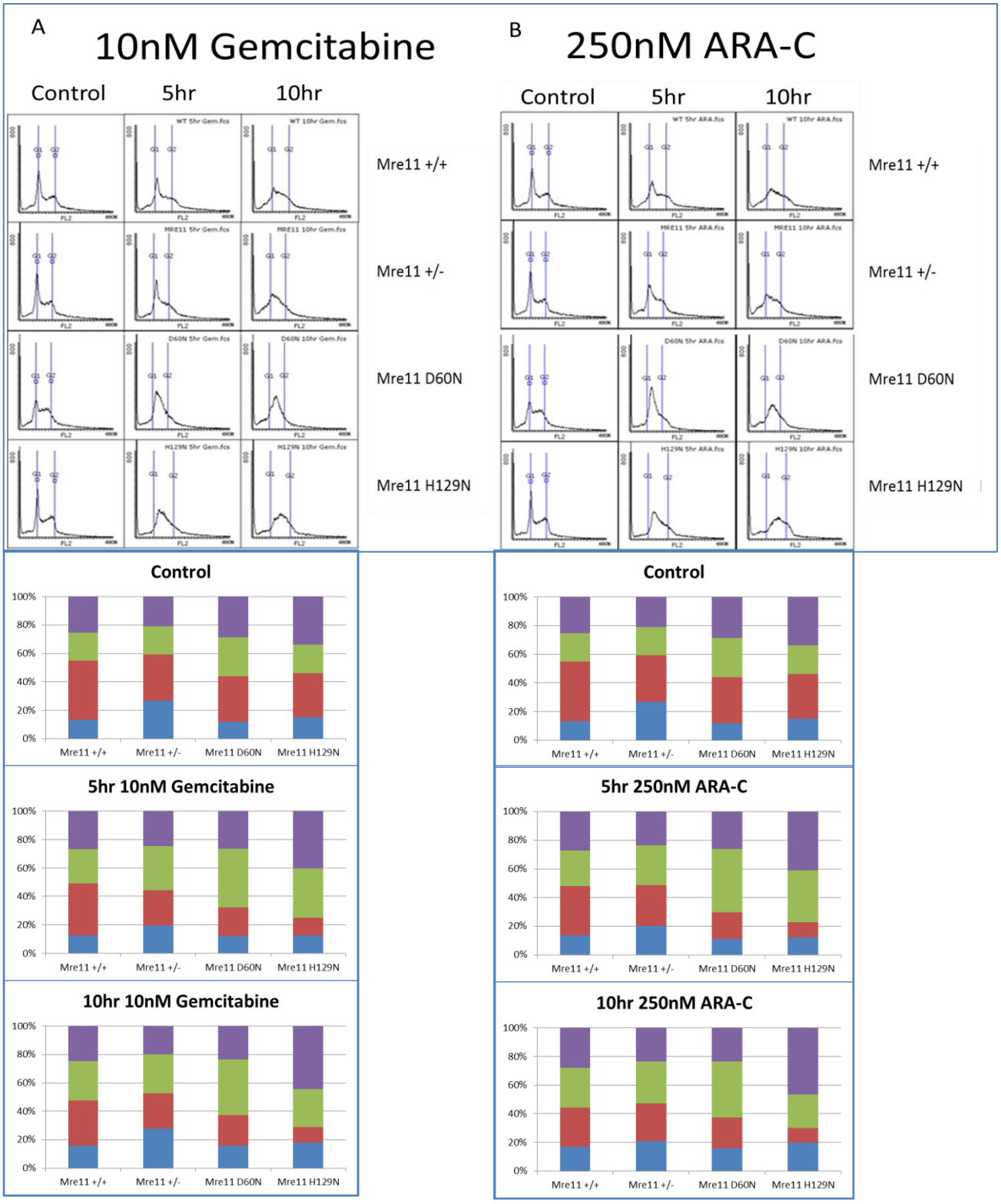

Figure 7.10: Gemcitabine and Cytarabine Cause Intra-S-phase Accumulation in Mre11 Nuclease Deficient Cell Lines. DT40 asynchronous cultures incubated in the presence of 10 nM gemcitabine (**A**) or 250 nM cytarabine (**B**), time points measured at 5 and 10 hours after nucleoside analogue exposure. Lower panels indicate percentage quantification of cell cycle phases over 5 and 10 hours based on G1 and G2 peak position gating.

As shown in Figure 7.10, both Mre11 D60N and H129N mutant cell lines time dependently accumulate in S-phase in response to gemcitabine and cytarabine. Mre11 +/+ and Mre11 +/- cell lines show slight S-phase delay, however to a lesser extent than Mre11 D60N and H129N. Intra-S-phase accumulation seen in these two mutants suggests that Mre11 nuclease activity is required for progression through S-phase in the presence of nucleoside analogues.

Discussion

It has been widely reported that the MRN complex has several key roles including DSB repair, telomere maintenance and DNA damage signalling (Lamarche et al., 2010; Rupnik et al., 2008). However, the specific roles of the Mre11 nuclease in repairing replication-associated DNA damage lesions is less well characterised. The use of cell lines with Mre11 truncations from patients with ATLD provides a limited tool for the study of the Mre11 nuclease due to the complication of dissociation from Rad50, and potential remaining nuclease activity. It is therefore necessary to study the specific nuclease functions of Mre11 using point mutated nuclease deficient alleles. Use of nuclease deficient Mre11 alleles containing amino acid substitutions in highly conserved residues allows for the investigation of lesion specific DNA repair with far greater control than with ATLD patient cell lines.

Work presented in this chapter uses the DT40 cell line with several Mre11 nuclease deficient point mutants which have been previously described in the *S. cerevisiae* Mre11 homolog (Previously Rad32) (Krogh et al., 2005; Lewis et al., 2004b; Llorente and Symington, 2004). It has been reported that Mre11 nuclease-deficient cells are less sensitive to ionising radiation than Mre11Δ, or ATLD human cells (Llorente and Symington, 2004). However, the specific amino acid substitution appears to give an additional layer of complexity as $Mre11^{Rad32}$ D25A seems to be more sensitive to radiation than $Mre11^{Rad32}$ D56N and H125N. the Mre11 D25A mutant is thought to prevent its interaction with Rad50 increases its sensitivity to radiation compared to other nuclease deficient alleles (Lewis et al., 2004c). Using the Mre11 D60N and H129N nuclease deficient DT40 cell lines, we aimed to investigate the role of the Mre11 nuclease in the resistance to camptothecin and nucleoside analogues and relate results back to published data from other groups (Hartsuiker et al., 2009).

DT40 clones were first tested for sensitivity to nucleoside analogues gemcitabine, cytarabine, and 5-flurouracil, as well as the topoisomerase I poison camptothecin by colony forming assays using semi solid media (Fig.7.5). Mre11 D60N and H129N are consistently more sensitive than DT40 Mre11 +/+ cells to nucleoside analogues, 5-FU and CPT. However, Mre11+/- cells are more sensitive to nucleoside analogues than Mre11 +/+ but show no increased sensitivity to CPT. This therefore suggests that Mre11 has both a nuclease role and is required structurally; potentially by stabilising lesions allowing other factors to be recruited, in response to nucleoside analogues but potentially only required in a nuclease capacity in response to topoisomerase removal. Due to the reported parallel activity between Tdp1, XPF-ERCC1 and Mre11 in resolving trapped topoisomerase enzymes (Pommier, 2003), this result is consistent as the other removal mechanisms are able to takeover when Mre11 expression is reduced. To further investigate this hypothesis, Mre11+/- cells were tested for sensitivity to the topoisomerase II poison etoposide which has been shown to predominately be repaired by NHEJ but with only a structural interaction role for MRN involvement (Hande, 1998; Kavitha et al., 2010; Montecucco and Biamonti, 2007). Mre11 +/- cells showed highest sensitivity to etoposide in colony forming assays which further suggests that reduced Mre11 expression induces sensitivity to some DNA lesions such nucleoside analogue damage and etoposide induced double strand breaks, but not to CPT (Fig.7.7).

Based on the unique phenotype of Mre11 D60N and H129N cell lines in response to CPT which shows sensitivity where the Mre11 +/- cell line does not, cell cycle progression experiments were performed in order to investigate the ability of Mre11 nuclease-deficient cell lines to undergo replication in the presence of CPT (Fig.7.8). The spindle stabilising drug nocodazole was used here to arrest cells in G2/M. nocodazole allows for the assessment of S-phase progression by preventing cells from entering a second cell cycle. Mre11 +/+ DT40 progressed to G2/M completely after 4 hours at the same rate as controls in the presence of 10nM CPT, whereas Mre11 D60N and H129N were delayed in complete replication until the 6 hour time point. This result, combined with G2/M accumulation seen in asynchronous cultures (Fig.7.9) suggests that Mre11 Nuclease activity is required for Top-cc repair and in the absence of nuclease activity, S-phase progression is slowed and G2/M accumulation occurs which suggests the presence of double strand breaks (Furuta et al., 2003). The

MRE11+/- cell line shows near identical cell cycle profile after CPT incubation compared to Mre11 +/+ with no evidence of G2/M accumulation which further suggests that Mre11 is required in a competent nuclease capacity, not merely structurally in response to CPT.

Based on the apparent difference in Mre11 nuclease requirements between Top-cc removal and nucleoside analogue resistance (Fig.7.6), cell cycle progression experiments were also performed using the nucleoside analogues gemcitabine and cytarabine. Although Mre11 +/- cell line shows nucleoside analogue sensitivity in colony formation assays, there is relatively little difference in cell cycle profiles following incubation with both nucleoside analogues compared to wild type. Whereas both Mre11 D60N and H129N show pronounced time dependent Intra-S-phase accumulation. This therefore implies that the nuclease function of Mre11 is in fact required for efficient S-phase progression in response to nucleoside analogues. It is therefore possible that Mre11 has multiple roles in processing nucleoside analogue induced stalled replication forks, both enzymatically and structurally possibly in conjunction with CHK1 activation and recruitment. Nocodazole S-phase progression experiments were not performed for gemcitabine and cytarabine as they were for CPT treatment (Fig.7.8) due to time constraints. This experiment may help to investigate the results seen in figure 7.10 and potentially show that wild type and MRE11 +/- cell lines are able to complete S-phase in the presence of nucleoside analogues where Mre11 D60N and H129N cell lines are not.

It has been shown that CHK1 inhibition by MK-8776 in combination with Mre11 depletion (either by siRNA or using ATLD cell lines) reduces DNA damage caused by MK-8776 both in terms of yH2AX and detectable double strand breaks (Thompson et al., 2012). However in the presence of efficient ATR-CHK1 signalling, Mre11 nuclease activity seems to be required for survival. This implies a potential model for nucleoside analogue removal where CHK1, and proteins it recruits to stalled replication forks such as Rad51 and BRCA2, control the Mre11 mediated removal of incorporated nucleoside analogues and prevent excessive resection which has been shown to reveal a substrate for Mus81 mediated fork collapse (Daboussi et al., 2002; Forment et al., 2011; Parsels et al., 2010; Regairaz et al., 2011). Where Mre11 expression is low such as in Mre11+/- cell lines, this endonuclease action maybe slowed or performed by another enzyme which induces the sensitivity seen in the Mre11 +/- cell line compared to wild type (Fig.7.6).

This work reveals that Mre11 nuclease activity is required for cell survival, and efficient S-phase progression following treatment with the Top1 poison CPT, and cells expressing low amounts of wild type Mre11 show no increased sensitivity to CPT. However, in response to the nucleoside analogues gemcitabine and cytarabine, cells with low Mre11 levels (Mre11 +/-) are similarly sensitive compared to nuclease deficient Mre11 D60N and H129N cells.

Chapter 8: Discussion

In order to overcome resistance to chemotherapeutic attack seen in cancer treatments, it is essential to first understand the intricate mechanisms of DNA repair which often mediate the removal of damage induced by most chemotherapy drugs. If the factors which support cell survival following drug treatments can be identified, more targeted and successful treatment regimens can be utilised once any given cancers specific mutation spectrum is deduced. The nucleoside analogue gemcitabine is used in the treatment of pancreatic and lung cancers which can often become refractory to drug treatments. It is therefore essential to build on the current understanding of nucleoside analogue mediated replication arrest and attempt to understand which factors are required for successful cell survival.

Work in this thesis has identified a requirement for several of the NER factors in survival following nucleoside analogue treatment, and shown that in their absence cells become sensitive to both gemcitabine and cytarabine. Furthermore this has been inferred to replication fork stability by the use of NER deficient cell lines which were shown to have an innate sensitivity to both CHK1 and ATR inhibition, and when combined with nucleoside analogues, and the replication stalling agent hydroxyurea, this inhibition becomes considerably more cytotoxic. This work has built on previous unpublished data from the lab which showed that in *S. pombe* cells lacking, XPA^{Rhp14}, $XPC^{Rhp41/42}$ and $ERCC1^{Swi10}$ are sensitive to nucleoside analogues, however differences between *S. pombe* data and human cells have been found, in that the absence of XPG results in sensitivity to nucleoside analogues, whereas XPG^{Rad13} does not. Additionally *S. pombe* data showed that XPF^{Rad16} cells are not sensitive to gemcitabine, whereas in human cells XPF deficiency results in a mild sensitivity. These differences between *S. pombe* and human results may be caused by protein-protein interactions that are necessary for gemcitabine resistance/removal that exist in human cells, which do not in lower eukaryotes, or different redundant nucleases or pathways that differ between the two organisms.

The NER-deficient cell lines from XP patients collected for use in this study were first tested for sensitivity to the drug 4NQO which produces lesions which have been shown to be repaired by NER (Dollery et al., 1983; Waters et al., 1992). It was shown that all of these cell

lines were sensitive to 4NQO in a predictable and well published manner which relates to both their complementation group phenotype severity and their published UDS percentage following UV irradiation. As many of the NER deficient cell lines used in this study showed different sensitivity severities to 4NQO and nucleoside analogues; for example XP-C cells which are relatively resistant to 4NQO but highly sensitive to gemcitabine, and XP-F cells which are sensitive to 4NQO but relatively resistant to gemcitabine, it was deduced that nucleoside analogue sensitivity seen in the absence of NER factors is unlikely to be caused by the mechanism proposed for as the classical model of NER.

The gemcitabine removal assay described in Chapter 1 which showed that in *S. pombe* cells lacking XPA^{Rhp14}, $XPC^{Rhp41/42}$ and $ERCC1^{Swi10}$, have a deficiency in gemcitabine removal as measured by LC-MS/MS relative to a cytosine isotope was attempted several times in human XP cell lines throughout this work. Due to technical treatment concentrations and time issues, no consistent data was generated. It is therefore not possible to directly link NER factors to gemcitabine removal based on this work. However, based on the *S. pombe* data, and results presented here, it is possible that several NER factors that have no direct endonuclease activity, such as XPA and XPC assist in the removal of nucleoside analogues from replication forks by other nucleases.

Several studies have shown the cellular response to gemcitabine in a similar manner to results shown here. Gemcitabine causes rapid and persistent yH2AX activation which occurs in a pan-cellular manner rather than the traditional idea of yH2AX foci which occur at the sites of DSBs (Shi et al., 2001). This pan-cellular yH2AX activation has been proposed to be in response to stalled replication forks as it is also seen in treatment with hydroxyurea which induces fork stalling by dNTP depletion (Elvers et al., 2012; Huang et al., 1991; Shi et al., 2001). For this reason we chose to investigate the role of NER factors in resistance to nucleoside analogues with a specific focus on the replication fork, and the signal kinases that control its speed and stability.

The roles of the ATR-CHK1-Cdc25A S-phase checkpoint are becoming increasingly expanded from cell cycle arrest to include replication damage lesion stabilisation. Although CHK1 is known to prevent further origin firing by the stabilisation of Cdc25A, it is also becoming known for recruiting factors to the sites of stalled replication forks in conjunction with ATR

(Elvers et al., 2012; Mohni et al., 2014; Warmerdam et al., 2010). Following replication stalling, it has been shown that ATR becomes activated, assisted by ATRIP, due to long patches of ssDNA which occur ahead of the stalled polymerase as it becomes uncoupled from the replicative helicase (Bomgarden et al., 2006; Schwab et al., 2013). Activated ATR in turn phosphorylates CHK1 at position S345 which in turn triggers the autophosphorylation of CHK1 at S296 (Parsels et al., 2011b). ATR and CHK1 then in turn bring about the inhibition of further origin firing and the recruitment of replication fork binding proteins such as claspin, Rad51 and BRCA1, as well as maintaining the phosphorylation of RPA which is bound to ssDNA ahead of the replication fork (Trenz et al., 2006). This process is thought to stabilise the fragile replication fork and protect it from collapsing (Fig.8.1).

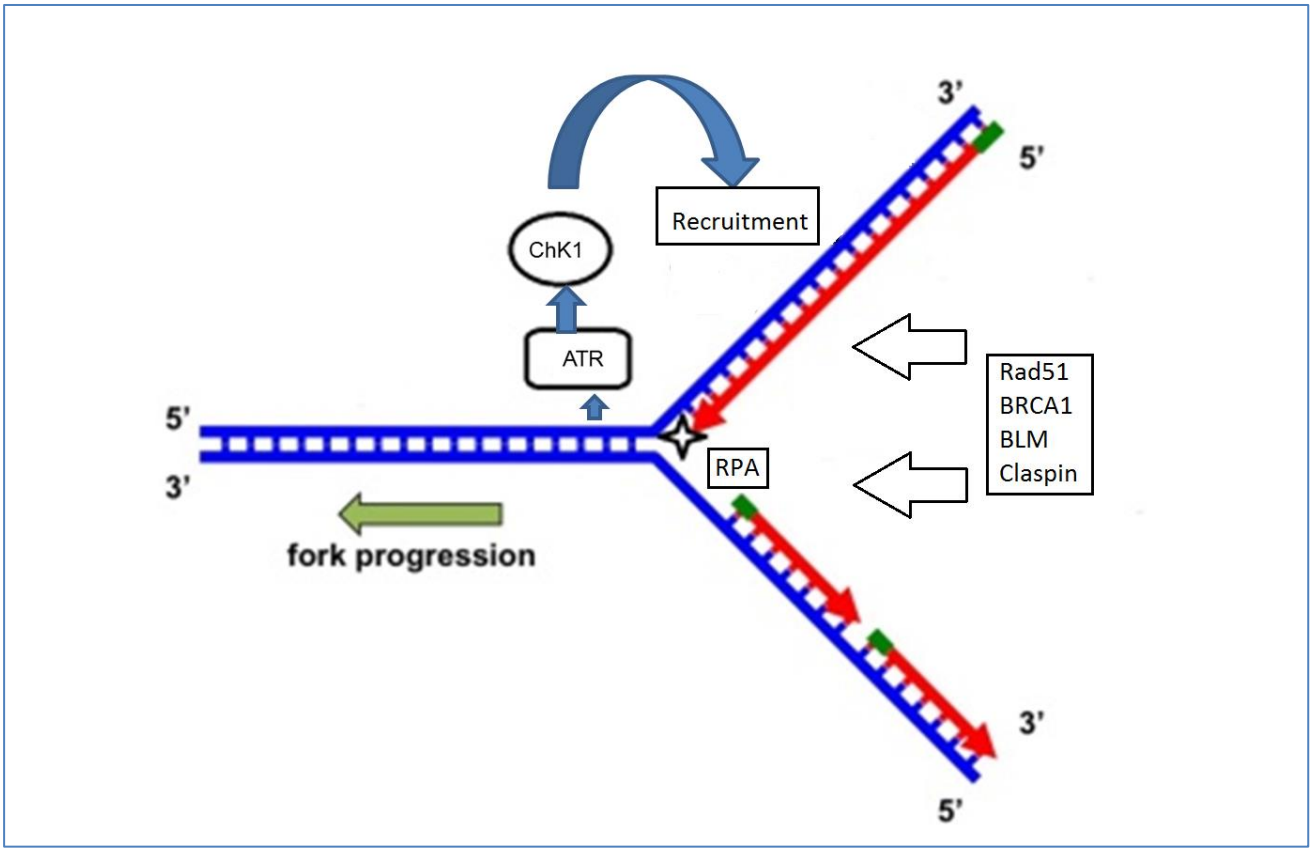

Figure 8.1: ATR and CHK1 Activation Leads to Replication Fork Stabilisation. Following replication fork stalling by nucleoside analogues indicated by black star, ATR and CHK1 rapidly activate and bring about the recruitment of factors such as BLM, BRCA1 and RPA.

The inhibition of both ATR and CHK1 have been shown to sensitise cells to several replication stalling lesions caused by cisplatin, hydroxyurea or gemcitabine (Montano et al., 2013; Thompson et al., 2012). Inhibition of both of these kinases prevents the recruitment of stabilising factors which protect the replication fork and also prevents the inhibition of

further origin firing which would exacerbates DNA damage (Montano et al., 2013; Prevo et al., 2012). The CHK1 inhibitor MK-8776 has been shown to increase DSBs at stalled replication forks due to the absence of protecting proteins which allows nucleolytic attack to occur by MUS81 or MRE11 (Fig.8.2) (Karnitz et al., 2005; Rawlinson and Massey, 2014).

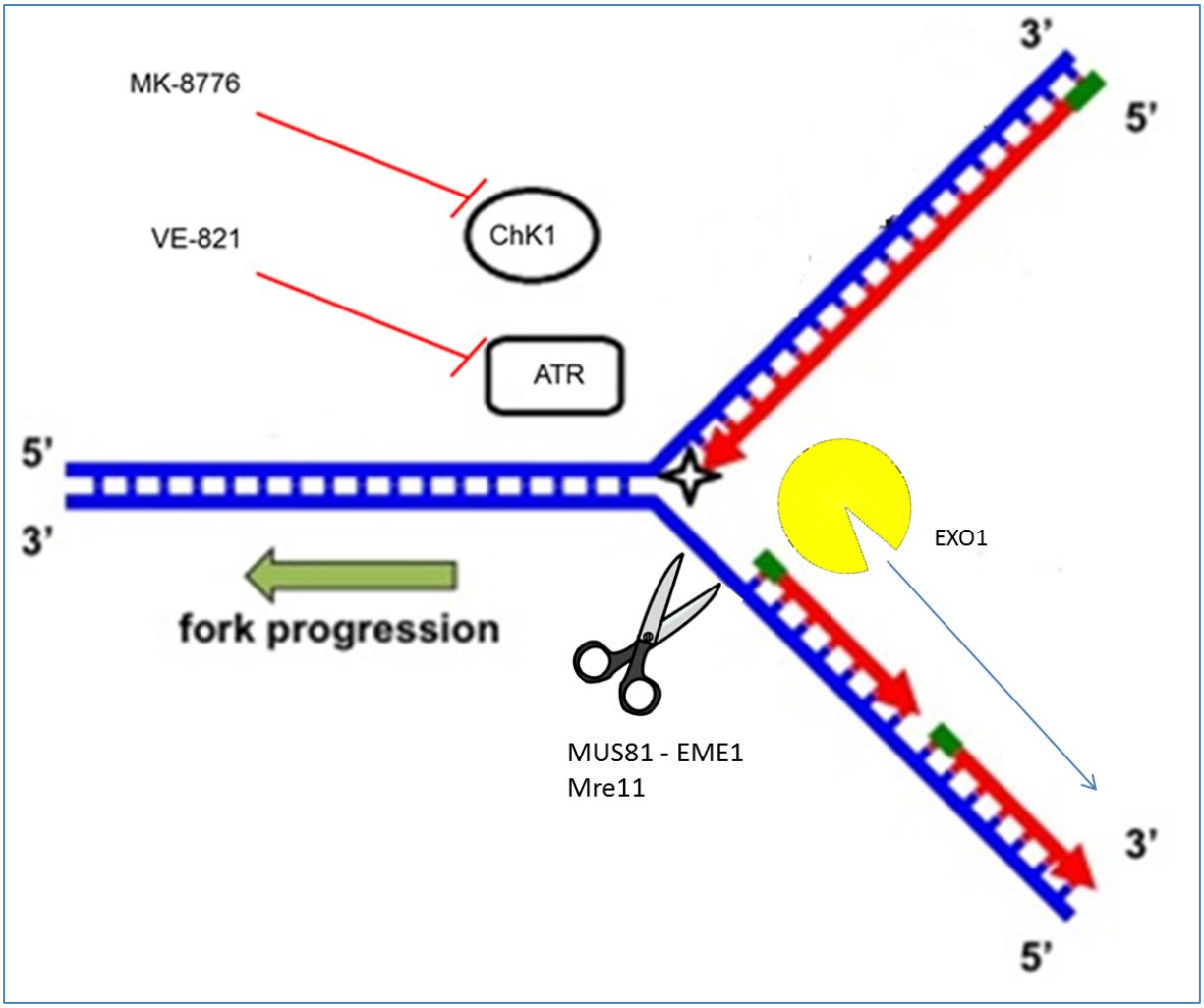

Figure 8.2: ATR and CHK1 Inhibition Prevents Replication Fork Protection. Both VE-821 and MK-8776 prevent replication fork stabilisation which results in replication fork collapse following EXO1 5'-3' Okazaki fragment removal and MUS81 or Mre11 strand cleavage.

Work in this thesis has shown that NER deficient cell lines from XP patients are sensitive to both ATR and CHK1 inhibition which suggests that NER factors maybe in part helping to stabilise replication forks, either by protecting from nucleolytic attack or by an unreported interaction with S-phase signalling kinases, or assisting in the removal of nucleoside analogues from the replication fork. The effect of CHK1 inhibition with MK-8776 seems to be greater than inhibition of ATR in producing cytotoxicity in XP deficient cell lines, particularly affecting XP-A, XP-C and XP-G complementation groups. As both XPA and XPC

have direct DNA binding activity, their role in replication fork stabilisation may be structural which may aid other factors in binding. This could also be the case for the sensitivity produced by MK-8776 in XPG deficient cell line XPCS1RO, however the lack of 3' endonuclease activity may be involved.

Inhibition of both ATM and DNA-PK failed to increase sensitivity of both wild type and NER deficient cell lines to nucleoside analogues. As no effect with these inhibitors was observed in MRC5 cells, it is unlikely that defective ATM or DNA-PK signalling is involved with the sensitivity seen in NER deficient cell lines.

The addition of the nucleoside analogues gemcitabine and cytarabine, as well as hydroxyurea in combination with CHK1 or ATR inhibition in NER-deficient cell lines powerfully increased their sensitivity as measured by colony formation, and annexin V antibody binding. However, in cells treated with 4NQO, only ATR inhibition resulted in an increase in cytotoxicity. These observations suggest that the effect of CHK1 inhibition combined with NER deficiency is unique to replication stress inducing drugs. ATR signalling is known to be important in nucleotide excision repair which correlates with the sensitisation seen following 4NQO and VE-821 treatment in XP cell lines (Li et al., 2011).

Taken together, the results of this thesis suggest that NER factors play a role in replication fork stability separate from their role in NER, which involves a requirement for the S-phase signalling cascade. Nucleoside analogue induced replication fork stalling seems to require several NER factors to prevent cell death, and inhibition of either ATR or CHK1 increases the instability produced by the lack of NER factors. The absence of XPA, XPC and XPD produces the highest sensitivity when CHK1 inhibition is combined with gemcitabine treatment, and the XPF deficient cell line XP2YO which is relatively resistant to gemcitabine is sensitised by the addition of MK-8776 and VE-821. Conversely, XPG and CSA deficient cell lines although sensitive to CHK1 inhibition alone, show the lowest increase in sensitivity when combined with gemcitabine, but are sensitised when the gemcitabine treatment is combined with VE-821. This therefore suggests that unlike the other NER factors, XPG and CSA have roles in stabilising replication forks that are ATR dependent, not CHK1 dependent. Additionally, work here using DT40 cell lines (Chapter 7) has shown that the Mre11 nuclease is also required for resistance and S-phase progression in the presence of nucleoside analogues.

The Mre11+/- cell line expressing lower levels of the Mre11 protein (as detected by western blot, Fig.7.6) also showed an increase in sensitivity to nucleoside analogues revealing a possible gene dosage effect in gemcitabine resistance. Combining Mre11 and NER results presented here, it is possible that several NER proteins are involved in stabilising nucleoside analogue induced stalled replication forks that requires efficient ATR-CHK1 signalling, which may assist in the removal of incorporated gemcitabine by Mre11. In the absence of efficient ATR-CHK1 signalling or NER factors, replication forks that have been blocked maybe inappropriately degraded by Mre11 or Mus81.

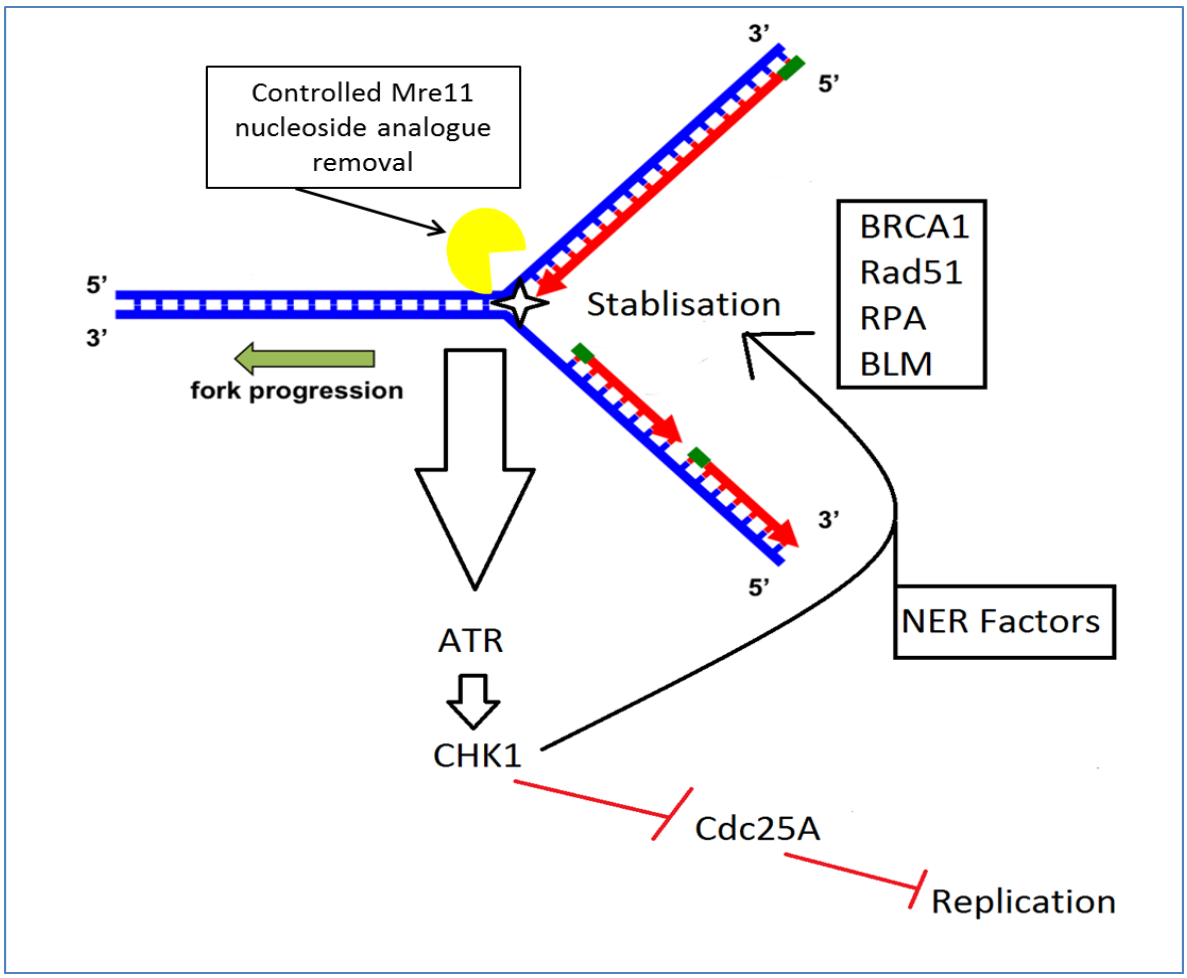

Figure 8.3: Replication Fork Stabilisation in Response to Nucleoside Analogues Requires ATR-CHK1 Signalling and NER Factors. Suggested model showing potential NER factor recruitment by ATR or CHK1 following nucleoside analogue (star) induced replication fork stalling, leading to replication fork stabilisation by BRCA1, RPA and Rad51 subunits, allowing for appropriate Mre11 mediated resection.

To further investigate and validate the observations seen in this thesis, CRISPR/Cas9 knockout models maybe generated to produce an isogenic comparison of cells lacking NER factors. This would remove the potential complications of residual activity of the truncated

NER proteins seen in XP patient cell lines, particularly in relation to the XPF cell line XP2YO which contains a point mutated allele (Thr556Ala) that may contain have residual activity and ERCC1 binding ability (Ahmad et al., 2010). The use of XP patient cell lines in this thesis represents a significant limitation as minor differences in protein levels, DNA repair factor genetic sequence and rate of growth may all vary. Using CRISPR/Cas9 knockout technology will also allow for the generation of cell lines lacking multiple NER factors. This will allow for the separation of potentially different roles of each NER factor to determine whether one, or more mechanisms may be involved. Targeted integration of point mutations into NER factors in isogenic cell lines may also be useful in accessing the specific residues and domains required for nucleoside analogue treatment survival.

The inhibitors VE-821 and MK-8776 are in clinical trials for use in combination with several different chemotherapeutics and different cancers. However results in this study provide a potential enhancement in effectiveness if gemcitabine is combined with either of these inhibitors in cancers that are positive for NER factor loss of function mutations which are common in a variety of tumour types (Hoeijmakers, 2001; Roberts and Gordenin, 2014). Increasing the effectiveness of gemcitabine and other nucleoside analogues with CHK1 and ATR inhibition in cancers mutated in NER factors may help to provide an increased survival rate for pancreatic and lung cancers which are treated with gemcitabine that currently have a comparatively low rate of survival.

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