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The Role of MRNIP in Chemoresistance

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The Role of MRNIP in Chemoresistance.

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Declaration

'I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

I confirm that I am submitting this work with the agreement of my Supervisor(s).'

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'Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Rwy'n cadarnhau fy mod yn cyflwyno'r gwaith hwn gyda chytundeb fy Ngoruchwyliwr (Goruchwylwyr)

Abstract

The maintenance of DNA and genome integrity is essential for life. A complex web of processes ensures faithful inheritance of genetic material. Among the myriad factors involved in regulation of these processes, the recently identified DNA repair protein MRNIP regulates the function of the core DNA repair nuclease MRE11. MRNIP-deficient cells are sensitive to multiple genotoxins, though in contrast, recent work reveals that MRNIP loss leads to resistance to the chemotherapies Gemcitabine and Cytarabine, which are distinct from other anti-neoplastic agents in being directly incorporated into the growing nascent DNA strand during replication. Furthermore, recent work suggests that phosphorylation of MRNIP at S217 may contribute to MRNIP function. The purpose of this project is to confirm and build on these findings to begin to elucidate the mechanisms via which MRNIP regulates chemosensitivity. We conducted a series of survival experiments in WT and MRNIP KO cancer cells following treatment with Gemcitabine, Cytarabine and two other nucleoside analogues. We conducted similar experiments using cells treated with the PARP inhibitor Olaparib, since MRNIP KO cells were previously demonstrated to be sensitive to this agent. We confirm that MRNIP loss causes resistance to Gemcitabine and PARP inhibitor sensitivity and demonstrate that the S217 residue is indeed functional in the regulation of Gemcitabine sensitivity. We also assessed classic markers of DNA damage in Gemcitabine and Olaparib treated MRNIP KO cells and confirmed the role of S217 in mediating MRNIP functionality in DNA damage modulation. We raised an antibody against the S217 phosphopeptide, although were unable to successfully validate the specificity of this antibody. We hypothesise that the phosphorylation of MRNIP S217 plays a role in MRNIP functionality, although it is beyond the scope of this project to understand this role and the importance it holds. In conclusion the results of the project suggest that MRNIP regulates chemosensitivity in an agent-specific manner and that the phosphorylation of MRNIP at S217 is important for MRNIP functionality.

Acknowledgements

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Abbreviations

AML	Acute Myeloid Leukemia
ATM	Ataxia Telangiectasia Mutated
BER	Base Excision Repair
BME	2-Mercaptoethanol
BSA	Bovine Serum Albumin
CLL	Chronic Lymphoid Leukemia
CRC	Colorectal Cancer
CTNA	Chain-Terminating Nucleoside Analogue
DAMPs	Damage-Associated Molecular Patterns
dCK	Deoxycytidine Kinase
dFdCTP	diFluorodeoxycytidine triphosphate (Gemcitabine)
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	Deoxyribonucleotide
DSB	Double Strand Break
DTT	Dithiothreitol
EC	Endometrial Cancer
FA	Fanconi Anaemia
F-ara-ATP	9-beta-D-arabinosyl-2-fluoroadenosine triphosphate
FBS	Foetal Bovine Serum
FWT	FLAG-Wild Type
HJ	Holliday Junction
HR	Homologous recombination
HTLF	Helicase Like Transcription Factor
ICL	Interstrand crosslink
K	Lysine
LS	Lynch Syndrome
MMR	Mismatch repair
MRN	MRE11-RAD50-NSB1
MRNIP	MRN Interacting Protein
NA	Nucleoside Analogue
NER	Nucleotide Excision Repair
NHEJ	Non-homologous End Joining
PAMPs	Pathogen-Associated Molecular Patterns
PARP	Poly-ADP-Ribose
PARPi	PARP inhibitor

PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PIKK	Phosphatidylinositol 3-kinase-related kinases
PRIMPOL	Primase and DNA-directed Polymerase
PRR	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
SMARCAL1	SWI/SNF subfamily A-like 1
SSB	Single Strand Break
TBS	Tris Buffered Saline
TLS	Trans Lesion Synthesis
TS	Template Switching
ZRANB3	Zinc Finger RANBP2-type Containing 3

1. Introduction

1.1 Causes and Statistics of Cancer.

Cancer is one of the leading causes of deaths worldwide, killing almost 10 million people each year (Gilbertson, 2011; Torre *et al*., 2015). There are many risk factors for cancer, such as smoking, obesity and poor diet, as well as genetic factors that determine cancer predisposition (Perrino *et al*., 2019; Torre *et al*., 2015). The most common cancers are female breast, colorectal, prostate and lung cancers (Ferlay *et al*., 2013; Siegel Mph *et al*., 2023). In 2012 in Europe, these cancers comprised half the European cancer burden, and represent a significant subfraction of the global cancer burden (Ferlay *et al*., 2013).

1.2 Hallmarks of cancer.

Although cancer is a genetically diverse disease, there are many hallmarks of cancer, which are similar for most cancer types (Hanahan, 2022; Hanahan & Weinberg, 2011). These were first described in a highly cited review published in 2000, which has since been updated and expanded twice in 2011 and 2022 (Hanahan, 2022; Hanahan & Weinberg, 2000, 2011). The hallmarks of cancer are divided into enabling characteristics and hallmark characteristics, with the general notion that enabling characteristics facilitate the development of hallmark characteristics (Hanahan, 2022). The confirmed enabling characteristics are tumour-promoting inflammation and genome instability and mutation, which allow cancers cells to adapt to their environment and favour beneficial traits (Hanahan, 2022; Hanahan & Weinberg, 2011). As more has been discovered about cancer etiology and pathogenesis, the hallmarks have been expanded to encompass improved understanding. Two emerging enabling characteristics include non-mutational epigenetic reprogramming and polymorphic microbiomes (Hanahan, 2022). Hallmark characteristics are traits that most cancer types display, which are beneficial for their survival. Many of these hallmarks facilitate cancer growth and proliferation (Hanahan, 2022; Hanahan & Weinberg, 2000, 2011), such as the ability to evade growth suppressors, a property that allows cancers to grow and avoid negative regulatory signalling networks that limit proliferation (Hanahan & Weinberg, 2011). Likewise, the ability to induce or access vasculature facilitates the generation of a blood supply to the tumour, and the consequent delivery of nutrients and other factors essential to sustain cell growth and viability (Hanahan & Weinberg, 2011). Most cancers also exhibit sustained proliferative signalling which allows the cancer to constantly grow even in the absence of normal extracellular signalling cues (Hanahan & Weinberg, 2011). Other hallmarks include the ability to resist cell death, replicative immortality, and activation of programmes of invasion and metastasis, which collectively allow the tumour to spread to other sites within the body (Hanahan and Weinberg, 2011). Two emerging hallmark characteristics include unlocking phenotypic plasticity, which allows cancer cells, differently from regular terminally differentiated cells to continue to

proliferate and grow, and senescent cells, which can allow changes in cell morphology, and allows cells to adapt to stressful environments, therefore making cancer cells more fit for survival (Saxena & Zou, 2022). These hallmarks are often associated with increased tumour survival, and are common to many different cancer types, they are therefore commonly targeted in cancer treatment strategies.

1.3 Genome instability in Cancer Pathogenesis.

Genome stability is essential for life, as DNA is the template for production of all protein and contains factors for the regulation of proliferation and transcription (Tubbs & Nussenzweig, 2017). Genome instability is defined as the presence of an elevated mutational rate, gross chromosomal instability, translocations, inversions, and/or repeat expansions (Tubbs & Nussenzweig, 2017). Although genetic alteration is the driving factor of evolution, mutations are commonly pathogenic, causing diseases such as cancer, which are often associated with age and the progressive accumulation of deleterious mutations (Maslov & Vijg, 2009; Tubbs & Nussenzweig, 2017). Most human cancers exhibit progressive genome instability, which is a crucial enabling factor in cancer pathogenesis. Loss of genome integrity is sometimes attributable to underlying genetic alterations that compromise DNA repair mechanisms, but in the vast majority of cancer cases, increased genome instability arises during pathogenesis via other mechanisms (Mateo *et al*., 2019; Chatterjee & Walker, 2017). Several cancers arise due to an increased mutational load driven by loss or impaired functionality of a genome maintenance factor, a case in point being the loss of functional *BRCA1* or *BRCA2* in subsets of ovarian, breast, pancreatic and prostate cancers (Rossing *et al*., 2019; Varol *et al*., 2018). *BRCA1* and *BRCA2* are important DNA repair factors that are crucial for the preservation of genome maintenance (Rossing *et al*., 2019; Varol *et al*., 2018).

The role of genome instability in chronic inflammation in cancer is currently under investigation. Genome instability-induced inflammation is thought to occur due to activation of the STING-cGAS signalling pathway, which can trigger a senescence-associated secretion of inflammatory cytokines, such as IL-6 (Barros *et al*., 2022; McNairn *et al*., 2019; Rodier *et al*., 2009). The immune system recognises threats to organism through Pattern Recognition Receptors (PRRs) and facilitates inflammatory responses (Cinat *et al*., 2021). Mechanisms to recognise possible threats include Pathogen-Associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Patterns (DAMPs) (Cinat *et al*., 2021; Jounai *et al*., 2012). While PAMPs recognise external pathogens, DAMPs recognise molecules from within cells and trigger an immune response (Cinat *et al*., 2021; Jounai *et al*., 2012). Although these are different mechanisms, the activation of the immune responses is similar, and recognition of these factors causes the immune system to trigger a response, such as phagocytosis,

cytotoxicity, or secretion of factors, in order to protect the organism against pathogens or damaged cells (Cinat *et al*., 2021). These changes affect the microenvironment and can promote tumour progression (Cinat *et al*., 2021). One important factor underpinning inflammation-driven tumour progression is the action of tumour-associated macrophages, which facilitate tumour survival and metastasis, as well as suppressing other anti-tumour components of the immune response (Lewis & Hughes, 2007).

1.3.1 Cancer Predisposition Syndromes.

Many germline mutations can predispose individuals to certain forms of cancer.

Heterozygous germline mutations in the tumour suppressors *BRCA1* or *BRCA2* can predispose the individual to breast and ovarian cancer, and this risk increases with age (Paul & Paul, 2014; Varol *et al*., 2018). *BRCA1* and *BRCA2* promote error-free DNA repair by Homologous Recombination (HR). When a heterozygous mutation carrier develops a mutation in the remaining functional *BRCA* allele, HR becomes ineffective. This is known as the double hit hypothesis (Chau & Wang, 2003; Paul & Paul, 2014; Varol *et al*., 2018). Eighty per cent of such mutations are point mutations (Varol *et al*., 2018). Not only does this cause DNA repair to be significantly impacted, but *BRCA* proteins are involved in transcriptional regulation and cell growth control (Varol *et al*., 2018).

Homozygous germline mutation of the *BLM* gene causes Bloom syndrome (De Voer *et al*., 2015; Ledet *et al*., 2019; Schayek *et al*., 2017). This is characterised by photosensitivity and growth deficiency, as well as cancer predisposition (De Voer *et al*., 2015; Ledet *et al*., 2019). The *BLM* gene encodes a RecQ DNA helicase, which is important in HR and in suppression of genetic exchange (De Voer *et al*., 2015; Ledet *et al*., 2019). The molecular function of *BLM* is DNA unwinding and 5’ long-range resection of Double Strand Breaks (DSB)-adjacent DNA to facilitate repair (Ledet *et al*., 2020). Loss of *BLM* functionality causes elevated sister chromatid exchange, which leads to an increase in genome instability, and an increased likelihood of cancer (Ledet *et al*., 2019). It is unclear whether heterozygous mutation carriers are at an increased risk for cancer (Ledet *et al*., 2019; Schayek *et al*., 2017).

Ataxia Telangiectasia is an autosomal recessive disease, characterised by progressive cerebellar degeneration, telangiectasia, immunodeficiency, premature aging, and cancer predisposition (Jackson, 1995; Rothblum-Oviatt *et al*., 2016). Homozygous mutation in the *Ataxia Telangiectasia, Mutated (ATM)* gene causes genome instability due to defective cellular signalling in response to DSBs (Rothblum-Oviatt *et al*., 2016). Individuals with heterozygous mutation are at an approximately 25% increased lifetime risk for cancer development, particularly lymphomas and leukemias in patients younger than 20, and breast, liver and

gastric cancers in older adults (Jackson, 1995; Rothblum-Oviatt *et al*., 2016). Individuals who have a heterozygous mutation in the ATM gene are generally not affected by the same symptoms as patients of the disease, but they are still at an increased risk of cancer, particularly breast with female carriers with a 2.3-fold increased risk, and gastrointestinal tract cancers (Jackson, 1995; Rothblum-Oviatt *et al*., 2016). Patients with a homozygous mutation are a 100 times more likely to develop cancer than age-matched controls (Thompson *et al*., 2005).

Fanconi Anaemia (FA) is a chromosomal instability disorder caused by germline mutations in any of the 23 *FA* genes (Nepal *et al*., 2017; Woodward & Meyer, 2021). The disease is characterised by early onset of aging, severe bone marrow failure and cancer predisposition (Nepal *et al*., 2017; Woodward and Meyer, 2021). Individuals with FA develop acute myeloid leukemia (AML) at a 700-fold higher incidence than the general population, and patients are also at a 50-fold higher risk of developing head and neck, oesophageal, anal and gastrointestinal cancer (Nepal *et al*., 2017). The *FA* genes encode for proteins active in the FA pathway (Nepal *et al*., 2017; Woodward and Meyer, 2021). This pathway is activated by DNA damage, usually as a result of DNA crosslinking, and acts to repair the lesion and maintain genomic stability (Kottemann & Smogorzewska, 2013; Nepal *et al*., 2017). The FA proteins have tumour suppressor roles; therefore, their loss leads to higher tumour incidence, even in patients who do not have the FA disease, since the incorrect repair of DNA crosslinks leads to genome instability (Kottemann & Smogorzewska, 2013; Nepal *et al*., 2017; Woodward & Meyer, 2021).

Lynch Syndrome (LS) is caused by germline mutations in one of four DNA mismatch repair (MMR) genes, with a prevalence of 1 in 200 (Seth *et al*., 2018). LS leads to cancer predisposition, particularly colorectal (CRC) and endometrial cancer (EC) (Biller, Syngal & Yurgelun, 2019; Seth *et al*., 2018). Three per cent of patients with CRC and 1.8% of patients with EC suffer from LS (Biller, Syngal & Yurgelun, 2019). LS also increases the lifetime risk of other cancers, such as ovarian, stomach, and pancreas (Biller, Syngal & Yurgelun, 2019). The underlying molecular phenotype is known as microsatellite instability, due to accumulation of DNA length altering mutations at microsatellites; these are used to screen individuals for the disorder (Biller, Syngal & Yurgelun, 2019). LS also causes a 100- to 1000-fold increase in mutation rate, causing genomic instability and an increased risk for cancer development (Seth *et al*., 2018). The disorder also causes a reduced susceptibility to apoptosis, as the MMR pathway plays an important role in recognising DNA damage and initiating apoptosis, again increasing the likelihood of cancer (Seth *et al*., 2018).

There are many more cancer predisposition syndromes, which lead to an increase in the likelihood of specific cancers, such as the PTEN hamartoma tumour syndrome, Li-Fraumeni syndrome, Cowden syndrome, Peutz-Jeghers Syndrome and many more (Correa, 2016; Yehia & Eng, 2021; Hanssen & Fryns, 1995; Wu & Krishnamurthy, 2022).

1.3.2 Acquired Mutations and Cancer.

Most cancers are caused by acquired mutations in specific genes, for example gain of function mutations in genes responsible for controlling cell proliferation.

The Ras gene family is the most frequently mutated in cancer (Moore *et al*., 2020). It is estimated that ~19% of patients with cancer have a mutation in a Ras gene (Prior *et al*., 2020). There are 3 Ras genes, *HRAS, NRAS, KRAS* (Prior *et al*., 2020). *KRAS* mutations are known to drive at least three cancer types: lung, colorectal and pancreatic cancer (Moore *et al*., 2020). Ras proteins transduce signals from cell surface receptors to the nucleus to drive growth-promoting transcriptional programmes (Moore *et al*., 2020). They control cell functions such as proliferation, survival, and differentiation (Moore *et al*., 2020; Prior *et al*., 2020). Ras becomes activated when bound to GTP and can activate a range of downstream targets (Moore *et al*., 2020; Prior *et al*., 2020). There are often distinct mutation patterns in specific cancer types in the Ras gene affected, leading to gain-of function mutations, which leads to Ras being more effective at binding GTP (Prior *et al*., 2020). Different mutations affect Ras to different extents, and differences in patient survival depend on the specific mutation present in the Ras gene (Prior *et al*., 2020).

The *TP53* gene is mutated in over 50% of human cancers (Lane & Lain, 2002; Mantovani *et al*., 2019; Zhang *et al*., 2020). p53 mutations are present in almost all types of human cancers (Zhang *et al*., 2020). p53 is a transcription factor, and an important tumour suppressor, which acts by binding to DNA and regulating target genes (Mantovani *et al*., 2019; Zhang *et al*., 2020). It has important roles in cellular functions such as apoptosis, cell cycle arrest, and DNA repair, through which it helps maintain a healthy genomic profile and suppress tumorigenesis (Mantovani *et al*., 2019; Zhang *et al*., 2020). The most common p53 mutations identified in cancer are missense mutations, which affect the DNA-binding ability of p53 (Zhang *et al*., 2020). However, p53 can also develop gain-of-function mutations, which cause the protein to become oncogenic and promote cancer progression (Dittmer *et al*., 1993)

1.4 DNA Replication, Damage, Repair and Cancer.

1.4.1 DNA replication.

DNA replication is essential for life (Ekundayo & Bleichert, 2019). High fidelity replication is a necessity, to preserve the stability of the genome (Ekundayo & Bleichert, 2019; Cortez, 2019). When errors occasionally arise, replication-coupled repair mechanisms are in place to correct these and maintain the integrity of the genome (Cortez, 2019). To combat these erroneous events, DNA replication is tightly controlled and regulated by many factors, ensuring that DNA replication only takes place once within a cell cycle to avoid polyploidy and aneuploidy (Ekundayo & Bleichert, 2019). DNA replication is also intricately linked to DNA repair (Hübscher, 2009). There are three stages of DNA replication: initiation, elongation and termination (Figure 1). Initiation is when DNA replication starts at discrete sites known as replication origins (Ekundayo & Bleichert, 2019). The replisome is assembled on the DNA in a bidirectional manner (Ekundayo & Bleichert, 2019). The elongation phase includes the movement of the replisome, which travels with the replication fork in both directions, synthesising DNA using the parental strands as templates (Ekundayo & Bleichert, 2019). Termination occurs when two converging replication forks meet, bringing DNA replication to an end (Dewar & Walter, 2017). At this stage the DNA replication machinery is disassembled, and the daughter strands are resolved (Dewar & Walter, 2017; Moreno & Gambus, 2020).

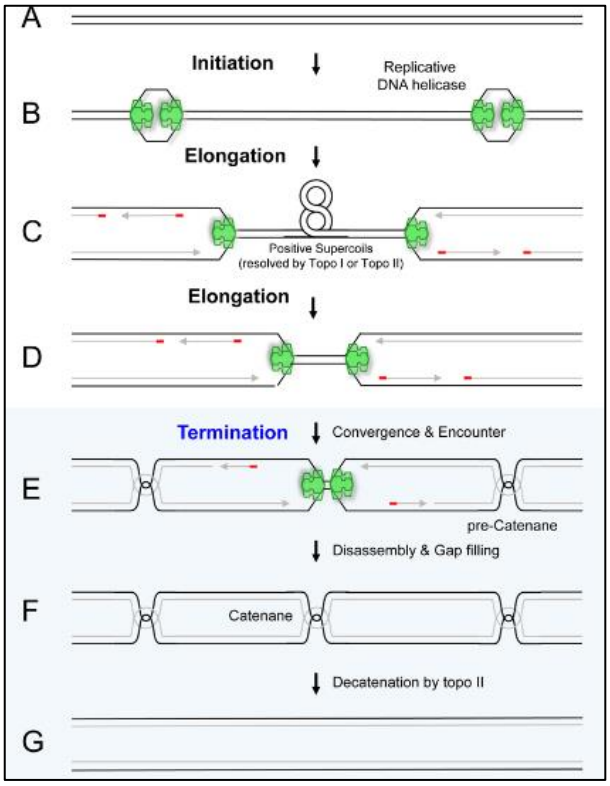


Figure 1- **DNA replication** (Dewar & Walter, 2017). Depiction of the stages of DNA replication. Initiation involves the beginning of DNA replication from discrete sites. Elongation involves the replisome moving along the DNA, synthesising new DNA using the parental strand as a template. Termination occurs when two replication forks converge and leads to the completion of DNA replication.

1.4.2 DNA Replication Stress.

Replication stress is known as any alteration to replication fork progression or fidelity (Saxena & Zou, 2022). DNA replication stress commonly leads to genome instability, which contributes to tumorigenesis (Saxena & Zou, 2022). There are many causes of replication stress, including direct barriers to the replication fork such as DNA lesions, regions that are difficult to replicate, depletion of nucleotide pools, and oncogene activation (Saxena & Zou, 2022). Dysregulated

DNA repair proteins can also contribute to DNA replication stress, as intermediates from these processes can act as barriers to the replication fork, interrupting DNA replication (Saxena & Zou, 2022).

1.4.3 DNA damage.

Maintaining the integrity of DNA is imperative for organismal survival (Carusillo & Mussolino, 2020). DNA damage is a frequent occurrence in most cells, and this damage has the potential to threaten genome integrity and cell viability, leading to cell death or cancer (Basu, 2018; Carusillo & Mussolino, 2020; Jackson & Bartek, 2009). Endogenous sources of damage include base misincorporation during DNA replication, formation of topoisomerase-DNA complexes, spontaneous base deamination and oxidative damage (Chatterjee and Walker, 2017). Exogenous damage can be caused by ionizing or ultraviolet radiation, exposure to toxins or environmental stresses and anti-cancer medicines (Chatterjee and Walker, 2017).

There are different forms of DNA damage (Jackson and Bartek, 2009). Single Strand Breaks (SSBs) arise commonly from processes in the cell such as topoisomerase malfunction (Jackson and Bartek, 2009). DSBs occur less frequently and are more detrimental to cell health as they are less easily repaired (Jackson and Bartek, 2009).

1.4.4 DNA damage markers.

DNA damage within the cell activates many pathways to efficiently repair the lesion and preserve genome integrity (Kuo & Yang, 2008). These processes often cause changes in surrounding molecules or the recruitment of a wide range of different proteins to the area of DNA damage (Kuo and Yang, 2008). As such, these changes in can be used as DNA damage markers to visualize sites and extent of DNA damage (Kuo and Yang, 2008).

An example of this is γH2AX. After DNA damage occurs, the histone H2AX is phosphorylated by ATM or another phosphatidylinositol 3-kinase-related kinase (PIKK) such as ATR or DNA-PK, depending on the nature of the lesion (Kuo and Yang, 2008). H2AX is a variant of the H2A protein family (Kuo and Yang, 2008). γH2AX phosphorylation is responsible for the localisation of DNA repair proteins to DNA damage sites (Kuo and Yang, 2008). γH2AX foci can be used as a DSB marker (Kuo and Yang, 2008).

Another marker used for assessment of the DNA damage response is *RAD51* (Wassing & Esashi, 2021). This is a protein involved in Homologous Recombination (HR) and is used as a marker of HR in immunofluorescence experiments (Wassing and Esashi, 2021). *RAD51* is loaded onto the DNA for initiation of HR, and it promotes the recombination process by allowing strand invasion of the resected ssDNA end of the DSB to use the sister chromatid as a template to repair the DNA lesion (Wassing and Esashi, 2021).

1.4.5 The replication stress response.

The cellular response to DNA replication stress varies dependent on the cause of the replication stress, but the overall goal is to restart replication to preserve genome stability (Gaillard *et al.,* 2015; Saxena & Zou, 2022). There are a range of important cell responses to replication stress: these include replication fork remodelling, activation of the S-phase checkpoint to prevent the cell from moving through the cell cycle with damaged DNA, and engagement of DNA repair or tolerance pathways to correct the lesion (Gaillard *et al.,* 2015; Saxena & Zou, 2022).

Fork remodelling is a way for the cell to regulate fork speed and allow time for DNA damage repair to take place, as well as allowing forks to bypass barriers (Berti *et al.,* 2020; Gaillard *et al.,* 2015; Saxena & Zou, 2022). There are many proteins which facilitate fork reversal including SWI/SNF subfamily A-like 1 (SMARCAL1), Zinc Finger RANBP2-type Containing 3 (ZRANB3), and Helicase Like Transcription Factor (HTLF) (Berti *et al.,* 2020; Saxena & Zou, 2022). *RAD51* is also required for fork reversal. REQ1 is responsible for resolving reversed forks, and is inhibited by PARP1 (Saxena & Zou, 2022). Reversed forks are protected by proteins such as *BRCA1*, *BRCA2* and *RAD51* to prevent DNA degradation mediated by the DNA repair nucleases MRE11 and EXO1 (Gaillard *et al.,* 2015; Saxena & Zou, 2022).

DNA damage Tolerance mechanisms allow lesions to be bypassed to ensure successful replication and allow the cell to progress through the cell cycle, despite challenges to replication fork progression (Saxena & Zou, 2022; Zeman & Cimprich, 2014). These mechanisms are highly evolutionarily conserved and are essential to ensure timely DNA replication within cells (Tirman, Cybulla, *et al*., 2021).

1.4.6 PRIMPOL.

One important DDT mechanism is repriming, which is performed by the Primase and DNA-directed Polymerase (PRIMPOL) (Saxena & Zou, 2022; Tirman, Cybulla, *et al.,* 2021). PRIMPOL levels in the cell are relatively constant throughout the cell cycle, however PRIMPOL binding to chromatin increases after treatment with genotoxic agents (Tirman, Cybulla, *et al*., 2021). PRIMPOL recruitment to chromatin following DNA damage is mediated by RPA (Guilliam *et al*., 2015; Tirman, Cybulla, *et al*., 2021). PRIMPOL possesses both primase and polymerase functions and is an important DNA damage tolerance factor (Tirman, Cybulla, *et al*., 2021; Wan *et al*., 2013). PRIMPOL generates new DNA primers after a DNA lesion on an undamaged template (Tirman, Cybulla, *et al*., 2021). PRIMPOL is a highly error-prone polymerase, with an error rate of ~10−4 errors per nucleotide incorporated (Keen *et al*., 2014; Tirman, Cybulla, *et al*., 2021). The processivity of PRIMPOL is usually limited to four nucleotides, as PRIMPOL has a self-limiting mechanism, where the ZnF domain of PRIMPOL

binds to ssDNA upstream of the catalytic core, and is linked by a flexible linker protein, limiting how far PRIMPOL can move along the DNA (Keen *et al*., 2014; Tirman, Cybulla, *et al*., 2021). Repriming by PRIMPOL after a DNA lesion leaves a ssDNA gap, which is filled in by post-replicative repair mechanisms (Saxena & Zou, 2022; Tirman, Cybulla, *et al*., 2021; Zeman & Cimprich, 2014).

1.4.7 The main DNA damage response pathways.

DNA repair processes are essential for responding to the constant DNA damage experienced by the cell (Chatterjee and Walker, 2017). Because DNA damage is detrimental to cell survival, a number of damage-specific mechanisms have evolved (Chatterjee and Walker, 2017). There are five main DNA damage response pathways which are active in different cell cycle stages (Chatterjee and Walker, 2017). BER is a mechanism that fixes small base lesions (Krokan & Bjørås, 2013). These do not significantly distort the structure of the DNA and are usually caused by deamination, oxidation or methylation (Krokan and Bjoras, 2013). This type of damage can be caused by spontaneous decay of the DNA, or environmental factors (Krokan and Bjoras, 2013). NER functions to remove bulky lesions from the DNA (Schärer, 2013). This form of damage can occur because of exposure to ultraviolet light or certain mutagens (Schärer, 2013). MMR is a mechanism used to correct mismatched nucleotides in the DNA to preserve genome stability (Fishel, 2015). This type of damage can occur because of polymerase misincorporations, or physical or chemical damage to nucleotides (Fishel, 2015). HR is a process used to repair breaks in the DNA and is particularly important when the DNA suffers a DSB (Wright *et al*., 2018). This mechanism allows the broken strand to invade and copy information from a homologous chromosome or sister chromatid to fix the DNA double strand helix with the correct information and maintain genome stability (Wright *et al*., 2018). NHEJ is a more direct DSB repair mechanism (Zhao *et al*., 2020). The ends of the breaks are processed, and then directly re-ligated, although this mechanism is considered error-prone compared to HR (Zhao *et al*., 2020).

1.4.8 Homologous recombination.

Homologous Recombination (HR) functions to facilitate the faithful repair of DNA DSBs (Orhan *et al*., 2021). NHEJ is active throughout the cell cycle, and repairs the majority of DSBs, NHEJ is, however an error-prone mechanism, and can lead to sequence alterations and chromosomal aberrations, which can increase cancer risk (Her & Bunting, 2018). HR competes with NHEJ and is active in both the S and G2 phases of the cell cycle, when homologous templates can be found in close proximity to the site of damage (Her & Bunting, 2018; Orhan *et al*., 2021). HR employs the sister chromatid, (or if this is unavailable, the homologous chromosome) as a template for accurate DNA repair (Orhan *et al*., 2021; Yamamoto & Hirasawa, 2022). DSBs can be lethal to a cell, HR is essential for cellular and organismal viability (Wright *et al*., 2018; Yamamoto & Hirasawa, 2022). The MRE11-RAD50-NBS1 (MRN) complex plays an important role in HR. This complex is involved in sensing DSBs. RAD50 is responsible for tethering the damaged DNA ends, while the exo- and endonuclease activities of MRE11 drive resection of break-adjacent DNA, which commits the cell to DSB repair by HR. As such, HR is also dependent on the phosphorylation of the MRE11 cofactor CtIP by Cyclin Dependent Kinases, short-range MRE11-dependent end resection, prior to bulk unwinding and resection by the nuclease DNA2 and the helicase BLM (Figure 2) (Her & Bunting, 2018; Wright *et al*., 2018). This process generates 3' overhangs on the DNA (Wright *et al*., 2018), following which the resultant ssDNA is coated with the single

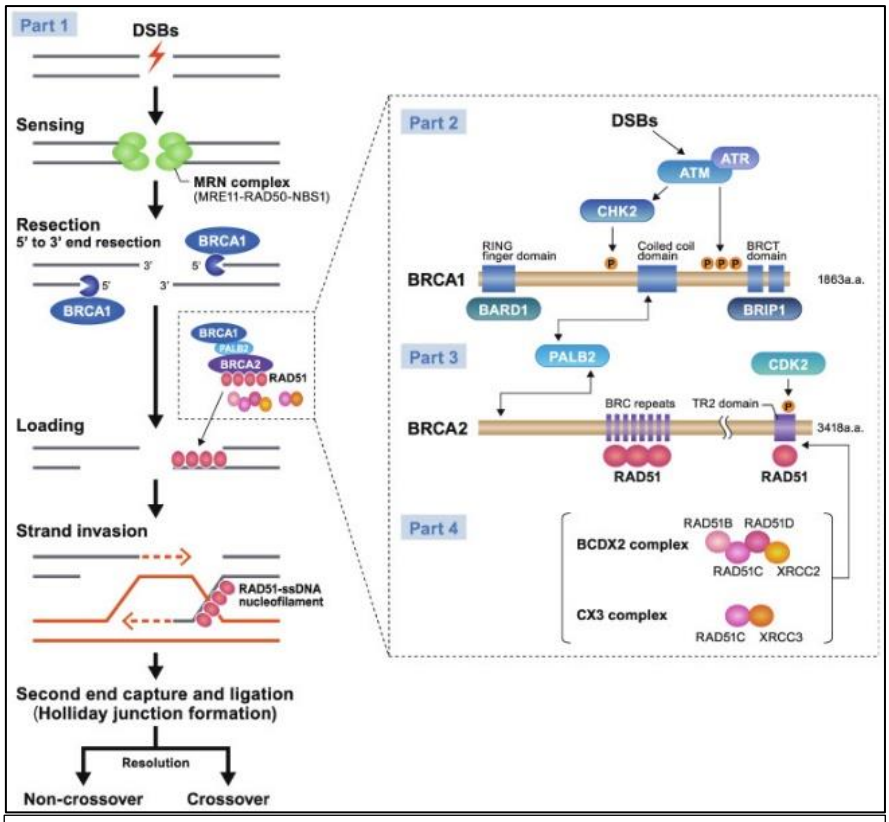


Figure 2- **Homologous Recombination** (Yamamoto & Hirasawa, 2022)**.** The process of HR is initiated by the MRN complex, which is involved in sensing DSBs and tethering damaged DNA ends for protection, followed by processing of the DNA ends to leave 3' overhangs. The ssDNA left is coated by RPA, before BRCA1 is recruited. Other factors are then subsequently recruited and RAD51 is loaded onto the DNA overhangs, leading to formation of nucleofilaments that facilitate strand invasion. Strand invasion leads to formation of a 4-way Holliday Junction, which is then resolved with or without crossover.

strand binding protein RPA (Figure 2). *BRCA1* is rapidly recruited to DSBs, where it promotes resection and the recruitment of other DNA damage response factors such as RAP80 and Abraxas (Yamamoto & Hirasawa, 2022). *BRCA2* mediates *RAD51* loading onto the resected DNA ends, forming nucleofilaments that facilitate strand invasion and homology searching (Yamamoto & Hirasawa, 2022). Post-strand invasion, homologous DNA is used as a template for DNA synthesis. This forms a 4-way DNA Holliday Junction (HJ), which can resolve with or without crossover by HJ resolvases or dissolvases (Yamamoto & Hirasawa, 2022). During this process the kinase ATM is also activated in an MRE11-dependent manner, driving DNA damage checkpoint activation.

1.4.9 DNA damage tolerance and Post-Replicative Repair.

Replication polymerases have high fidelity and accuracy, and as such they are prone to stalling when they encounter a lesion in the DNA (Branzei & Psakhye, 2016). This can be detrimental to the cell, as the timely replication of DNA is important to maintain cell viability. To complete replication, the cell must tolerate these lesions and continue replication, leaving the lesions to be repaired by a different mechanism (Branzei & Psakhye, 2016). The primary mechanisms of relevance to this thesis are Trans Lesion Synthesis (TLS) and Template Switching (TS) (Branzei & Psakhye, 2016).

TLS is considered error-prone, while TS is considered error-free, however TS can introduce errors when a homologous template is not available, and so dysregulation of either pathway can be detrimental to the cell (Branzei & Psakhye, 2016). The choice between TS and TLS is dictated by ubiquitination of Proliferating Cell Nuclear Antigen (PCNA) at lysine (K) site K164 (Bainbridge *et al.*, 2021; Branzei & Psakhye, 2016; Ler & Carty, 2022). Mono-ubiquitination is performed by the ubiquitin ligase RAD18 in a complex with the RAD6 ubiquitin-conjugating enzyme (Branzei & Psakhye, 2016; Branzei & Szakal, 2016; Ler & Carty, 2022). This mono-ubiquitination initiates interaction with TLS polymerases (Branzei & Psakhye, 2016). Poly-ubiquitination is again initiated by the RAD6/RAD18 complex, which then interacts with the UBC13-MMS2 complex to extend the mono-ubiquitin modification to poly-ubiquitin chains. Poly-ubiquitination of PCNA initiates TS (Bainbridge *et al.*, 2021; Branzei & Psakhye, 2016).

TLS is a mechanism to bypass DNA lesions at which a replicative DNA polymerase would stall (Chen *et al.*, 2022; Chun & Jin, 2010). This mechanism is considered error-prone and is performed by low-fidelity polymerases (Chun & Jin, 2010). These polymerases have less stringent active sites, allowing them to accommodate DNA lesions and continue replication (Chen *et al.*, 2022; Chun & Jin, 2010). In eukaryotic cells, TLS is carried out by Y-family polymerases, and these all

function at sites of DNA damage, with each specialising in repair or filling of different types of lesions (Chun & Jin, 2010). TLS occurs either directly at the replication fork or behind the fork at ssDNA gaps introduced by repriming DNA synthesis (Ler & Carty, 2022). TLS polymerases are recruited to sites of DNA lesions via mono-ubiquitination of PCNA (Ler & Carty, 2022). Although TLS is a way for the cell to maintain its ability to replicate DNA, this error-prone mechanism has been implicated in causing base misincorporations, causing mutations which can even drive cancer development or resistance to cancer therapy (Chen *et al*., 2022; Ler & Carty, 2022). Another mechanism via which TLS can contribute to anti-cancer drug resistance is through dysregulation of the TLS pathway, either by over-expression of regulatory proteins, such as RAD18 or through TLS polymerase levels themselves (Ler & Carty, 2022).

TS is a homology-dependent damage tolerance mechanism which uses a homologous sequence to bypass or repair a DNA lesion, either directly at the replication fork or via post-replicative repair (Ler & Carty, 2022). TS is initiated via poly-ubiquitination of PCNA and is preferentially active in early S-phase (Bainbridge *et al*., 2021; Branzei & Szakal, 2016; Ler & Carty, 2022). TS is considered error-free as it employs a homologous template and does not generally introduce errors into the DNA (Bainbridge *et al*., 2021; Ler & Carty, 2022). The sister chromatid junction that is formed must be resolved by BLM, TOP3α and RNI1/2 before replication can continue (Bainbridge *et al*., 2021).

While TLS can traverse a lesion or fill in gaps during post-replicative repair, TS is a mechanism of lesion bypass dependent on strand invasion event.

1.5 Cancer Treatment.

Decades of intensive research mean that most cancer patients can expect a course of treatment that will considerably extend their life, and in some cases be curative. Chemotherapy, radiotherapy and immunotherapy can be employed either as monotherapy, or as part of a broader combinatorial regimen (Amjad *et al*., 2023; DeVita & Chu, 2008).

1.5.1 Non-Targeted Cancer Medicines.

Radiotherapy has been widely used in cancer therapy since the 1960s, and chemotherapy was soon used in combination to reduce the likelihood of metastasis and recurrence (Amjad *et al*., 2023; DeVita and Chu, 2008). Traditional cytotoxic cancer therapies are not completely specific for cancer cells, and frequently cause serious side-effects such as immunosuppression, mucositis, nausea, vomiting, diarrhoea, infertility, nephrotoxicity, neurotoxicity and myelosuppression (Amjad *et al*., 2023; Zhang *et al*., 2022).

Traditional therapies inhibit cell proliferation, cancer growth and metastasis, and cause cancer cell death by many different mechanisms, such as by causing irreversible DNA damage,

stalling replication forks, targeting RNA or protein formation, or inducing apoptosis (Amjad *et al*., 2023; Zhang *et al*., 2022; Mokhtari *et al*., 2017). One treatment is rarely enough to see results, as only a fraction of cells will be affected, therefore repeated treatments are often required to see an effect (Amjad *et al*., 2023). Often, cancer cells develop drug resistance rapidly to repeated treatment, and so combination chemotherapy is used to combat this (Amjad *et al*., 2023).

1.5.2 Chain-Terminating Nucleoside Analogues.

Gemcitabine is a chemotherapy commonly used in the treatment of a wide range of solid tumours including ovarian, bladder, non-small-cell lung, pancreatic and breast cancer (Miao *et al*., 2019; Pandit & Royzen, 2022). It is a pyrimidine nucleoside analogue which is taken up by the cell via nucleoside transporters. Gemcitabine is then converted in the cell to its active triphosphate form (dFdCTP) by Deoxycytidine Kinase (dCK) (Miao *et al*., 2019; Mini *et al*., 2006). During DNA synthesis, dFdCTP competes with deoxycytidine triphosphate to be incorporated into the DNA (Figure 3) (Mini *et al*., 2006; Jones *et al*., 2014). Gemcitabine functions via masked-chain termination, (Figure 4) meaning once it is incorporated into the DNA, it allows addition of a limited number of nucleotides, leading to nucleases being unable to remove the Gemcitabine to allow continued DNA synthesis (Mini *et al*., 2006). Stalled replication forks can escalate to DSBs

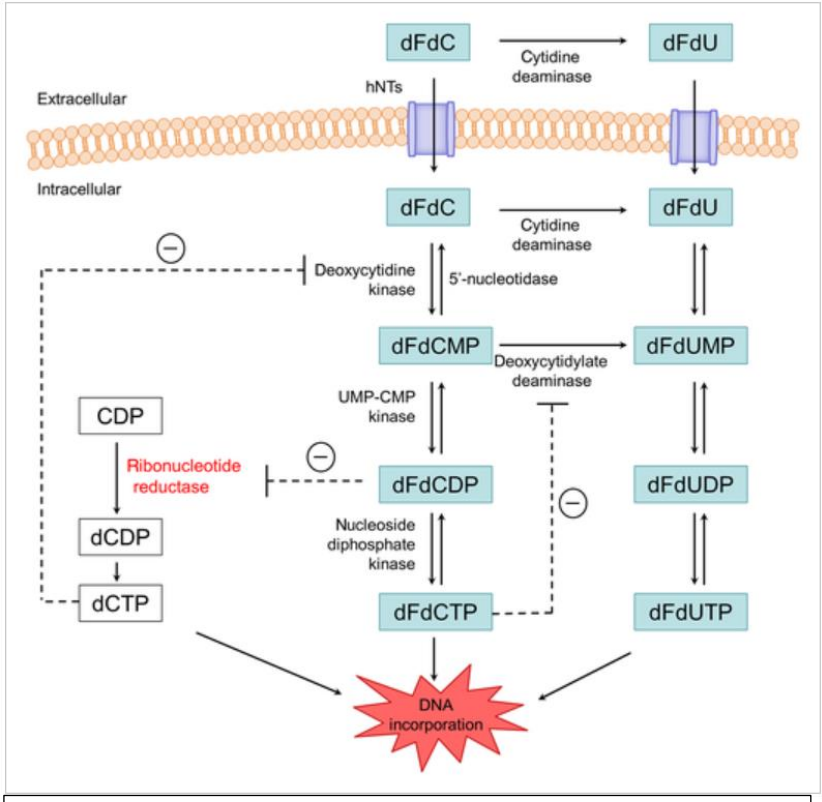


Figure 3- **Intracellular Metabolism of Gemcitabine.** (Derissen et al., 2018). Gemcitabine (dFdC) is taken into the cell by Human Nucleoside Transporters (hNTs). Once in the cell, Gemcitabine is phosphorylated by deoxycytidine kinase and converted to a monophosphate form (dFdCMP), which is then phosphorylated by pyrimidine nucleoside monophosphate kinase (UMP-CMP) to its diphosphate form (dFdCDP), before finally being phosphorylated by Nucleoside diphosphate kinase to its active triphosphate form (dFdCTP). This final triphosphate form is incorporated into the DNA, causing stalling of the replication fork.

upon further nucleolytic processing or collapse, which is toxic to the cell (Jones *et al*., 2014). Gemcitabine also inhibits ribonucleoside reductase, leading to depletion of cellular deoxyribonucleotide (dNTP) pools, causing inhibition of DNA synthesis and cell death (Mini *et al*., 2006). Gemcitabine is also thought to kill cancer cells indirectly by causing accumulation of HR intermediates, leading to DSB formation (Jones *et al*., 2014). When stalling of the replication fork occurs as a result of gemcitabine treatment, the HR factors *RAD51* and *BRCA2* are recruited to the chromatin at the site of DNA damage, as they are responsible for recovery of the replication fork by mediating restart (Jones *et al*., 2014). However, after gemcitabine treatment, these factors have been observed to inhibit fork progression and cause DSBs (Jones *et al*., 2014). This inhibition of DNA synthesis eventually leads to p53 independent apoptosis (Jones *et al*., 2014).

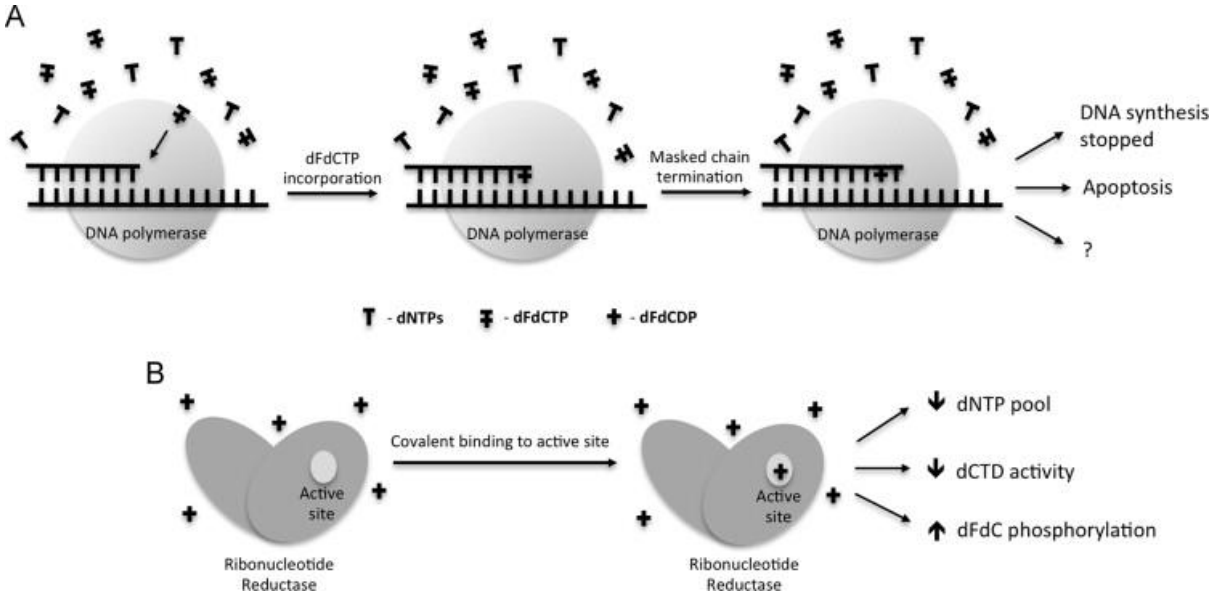


Figure 4- **Mechanisms of Action of Gemcitabine**. (De Sousa Cavalcante & Monteiro, 2014). Gemcitabine has different mechanisms of action. (A) The most important is its chain terminating activity, where dFdCTP gets incorporated into the DNA, only allowing the addition of limited nucleotides after, leading to masked-chain termination. (B) Gemcitabine also inhibits Ribonucleotide Reductase, leading to a disruption of the dNTP pool in the cell.

Cytarabine is a nucleoside analogue, used in the treatment of certain blood cancers (Chhikara & Parang, 2010; Faruqi & Tadi, 2022); (Löwenberg *et al*., 2011). Similar to gemcitabine, it is a pyrimidine analogue and is converted to its active triphosphate form in the cell where it then competes with cytidine for incorporation into genomic DNA (Faruqi and Tadi, 2023; Shepshelovich *et al*., 2015). The sugar moiety then prevents the DNA molecule from rotating, which in turn inhibits DNA replication (Faruqi and Tadi, 2023).

Fludarabine is currently the most commonly used purine analogue employed to treat Chronic Lymphocytic Leukaemia (CLL), although it also displays immunosuppressive activity (Lenz *et al*., 2004; Rodriguez, 1994). This drug is a derivative of adenine arabinoside monophosphate, an antiviral agent (Rodriguez, 1994). It is converted to the triphosphate form, 9-beta-D-arabinosyl-2-fluoroadenosine triphosphate (F-ara-ATP), in the cell and competes with deoxyadenosine triphosphate to be incorporated into genomic DNA (Lenz *et al*., 2004; Rodriguez, 1994). Fludarabine then leads to inhibition of DNA synthesis, leading to cell death (Lenz *et al*., 2004; Rodriguez, 1994). F-ara-ATP inhibits ribonucleotide reductase, DNA ligase and DNA primase (Lenz *et al*., 2004).

Clofarabine is a second-generation purine analogue (Tiley & Claxton, 2012; Zhenchuk *et al*., 2009). It is commonly used in the treatment of acute myeloid leukemia, particularly in older adults (Tiley & Claxton, 2012). This drug enters the cell and is converted to its triphosphate form, which is then incorporated into genomic DNA (Tiley and Claxton, 2012; Zhenchuk *et al*., 2009). Like gemcitabine, Clofarabine inhibits the action of ribonucleoside reductase, causing dNTP pool depletion, and inhibiting DNA synthesis, with the additional effect of increasing the likelihood of Clofarabine incorporation (Tiley and Claxton, 2012; Zhenchuk *et al*., 2009).

1.5.3 Alkylating Agents.

Cisplatin is a platinum-based chemotherapy used to treat cancers of the bladder, head and neck, lung, ovary, and testis (Dasari & Bernard Tchounwou, 2014; Makovec, 2019). Cisplatin induces cell death at least in part by introducing Inter-or Intrastrand Cross-links (ICLs) between Purine DNA bases (Dasari and Bernard Tchounwou, 2014). Once cisplatin enters the cell, it becomes activated by displacement of chloride ions with water molecules in the cytoplasm (Dasari and Bernard Tchounwou, 2014). The resulting product is a potent electrophile that binds to the N7 reactive center on purine residues (Dasari and Bernard Tchounwou, 2014). Cisplatin exposure also results in the generation of Reactive Oxygen Species (ROS), which also drives cell death (Dasari and Bernard Tchounwou, 2014). Cisplatin is also thought to interact with various other proteins within the cell including Protein Kinase C, which is important in signal transduction and cell regulation, and Mitogen-Activated Protein Kinase, which is important in signalling and controlling cell growth, although the interaction and consequences are still not completely understood (Dasari and Bernard Tchounwou, 2014). Despite its widespread use, the efficacy of platinum as an anti-cancer agent is limited due to the development of therapeutic resistance, and the inherent toxicity associated with use of this class of drug (Makovec, 2019). Cisplatin is known to cause nephrotoxicity and ototoxicity (Makovec, 2019; Pabla & Dong, 2008).

1.5.4 Precision Cancer Medicines.

The goal of many traditional cancer therapies is to kill cancer cells by causing DNA damage (Hosoya & Miyagawa, 2014). Within the cell, DNA damage is repaired to protect the cell, often causing the efficacy of cancer therapies to be affected by allowing the cell to survive (Hosoya & Miyagawa, 2014). However, due to specific DNA repair defects in specific cancers these can be exploited for treatment with DNA-damaging agents (Hopkins *et al*., 2022). The development of precision medicine has been made possible by the genomic era of medicine. The genetics of individual cancer can be used to develop treatment strategies to exploit specific changes to the cancer genome, employing synthetic lethal combinations to specifically target the cancer cells (Hopkins *et al.,* 2022; Hosoya & Miyagawa, 2014). An example of this is the development of Poly-ADP-ribose (PARP) inhibitors, which are used to treat cancers with DNA repair defects, such as treatment-refractory *BRCA*-deficient breast and ovarian cancer (Cortesi *et al*., 2021).

1.5.5 PARP Inhibitors.

PARP inhibitors are currently licensed for use in treatment of patients with *BRCA* deficient breast, ovarian, pancreatic or prostate cancers (Mateo *et al*., 2019). The potential of PARP inhibitors for clinical use was realised in 2005 in two publications, in which the specificity of *BRCA*-deficient cell sensitivity to PARP was first described (Bryant *et al*., 2005; Farmer *et al*., 2005).

There are 17 PARP enzymes, some of which are involved in DNA repair. PARP-1 is the main PARP enzyme involved in DNA repair in humans, with extensively studied roles in the Base Excision Repair (BER) pathway, which is responsible for repair of single stranded DNA breaks, and less studied roles in other repair pathways such as Nucleotide Excision Repair (NER) (Farmer *et al*., 2005; Pascal, 2018). PARP-1 also plays a role in DNA damage sensing and is involved in recruitment of other DNA repair proteins to the site of DNA breaks through the synthesis of poly (ADP-ribose) chains on target proteins (Farmer *et al*., 2005; Mateo *et al*., 2019; Pascal, 2018). After this occurs, auto-PARylation leads to PARP release from the site of action (Mateo *et al*., 2019).

Olaparib was the first PARP inhibitor to be approved for clinical use, and its targets include PARP1, PARP2, PARP3, and TNKS1 (Bruin *et al*., 2022). PARP inhibitors such as Olaparib function via at least two mechanisms (Farmer *et al*., 2005; Mateo *et al*., 2019). The first is the catalytic inhibition of PARP, which is the main known mechanism of Olaparib (Goulooze *et al.,* 2016; Lord & Ashworth, 2017; Mateo *et al.,* 2019). The other is by trapping PARP on the DNA (Lord & Ashworth, 2017; Mateo *et al.,* 2019). Heterozygous loss of PARP1 can cause severe deleterious effects, including a high rate of genome instability (Shao *et al*., 2023). Loss or inhibition of PARP function alone causes an increase in *RAD51* foci and sister chromatid

exchange but does not cause an increase in HR, suggesting that PARP is not directly involved in regulating HR, but that loss of PARP function does cause DNA lesions that are repaired by this pathway (Bryant *et al*., 2005; Farmer *et al*., 2005). *BRCA*-deficient tumours lack the ability to repair DNA lesions through HR, and so inhibition of PARP facilitates synthetic lethality to specifically target cancer cells with this genetic alteration (Bryant *et al*., 2005; Farmer *et al*., 2005). Cells with a homozygous loss of *BRCA1* display a 57-fold increase in sensitivity to PARP inhibitors compared to wild-type cells, and cells with a homozygous loss of *BRCA2* exhibit a 133-fold increase in sensitivity (Farmer *et al*., 2005). Whilst *BRCA*-deficient cells are sensitive to other DNA damaging agents, such as cisplatin, sensitivity is much less marked, at a 3-fold increase (Farmer *et al*., 2005). When PARP inhibition causes DNA damage in *BRCA*-deficient cells, DNA ends are not protected by HR proteins, leading to nascent DNA degradation, which is detrimental for the cell (Mateo *et al*., 2019). Because of a lack of HR, the damage caused is dealt with in erroneous ways (Bryant *et al*., 2005; Mateo *et al*., 2019). These cells depend on DNA repair mechanisms such as non-homologous end joining (NHEJ) which can lead to DNA fragmentation, ultimately leading to cell death (Mateo *et al*., 2019). Another possible source of sensitivity of *BRCA*-deficient cells to PARP inhibitors is the formation and failure to repair of post-replicative ssDNA gaps (Cong *et al*., 2021; Panzarino *et al*., 2021; Tirman, Cybulla, *et al*., 2021; Tirman, Quinet, *et al*., 2021). PARP inhibition inhibits fork-reversal, and as a result repriming by PRIMPOL is increased, which leaves ssDNA gaps behind the replication fork. *BRCA1* and *BRCA2* limit the prevalence of PRIMPOL and MRE11-dependent ssDNA gaps following Olaparib or Cisplatin treatment. Therefore, *BRCA* loss causes increased sensitivity to PARP inhibitors (Tirman, Quinet, *et al*., 2021). Some post-replicative repair mechanisms, such as the Trans-lesion Synthesis polymerase complex REV1-Polz have been shown to play a role in the repair of these gaps in HR-deficient cells (Taglialatela *et al*., 2021).

Resistance to PARP inhibitors is commonly observed in advanced disease. There are many proposed mechanisms for this (Lord & Ashworth, 2017). A possible mechanism is the loss of HR proteins 53BP1 or REV7, which leads to HR being restored in the absence of *BRCA* proteins, leading to rescue of the ability to resolve damage caused by PARP inhibition (Lord & Ashworth, 2017). Another possibility is the presence of a revertant mutation of the defective *BRCA* gene, so that function is at least partially restored, allowing compensation for the damage caused by PARP inhibition (Lord & Ashworth, 2017).

1.6 Background to the Investigation.

1.6.1 MRNIP.

The MRN complex plays an important role in DNA repair, via resection of damaged regions of DNA by MRE11. MRNIP interacts with the MRN complex, and has been demonstrated to

directly interact with MRE11, and limit its exonuclease activity. MRNIP prevents MRE11-dependent DNA degradation (Bennett *et al*., 2020). MRNIP also forms DSB-induced nuclear condensates, promotes MRN recruitment to DSBs, and drives DSB end-resection (Staples *et al*., 2016; Y. L. Wang *et al*., 2022). MRNIP plays an important role in MRE11 regulation during meiosis (Kazi *et al*., 2022; Lin *et al*., 2021). MRNIP depletion leads to impaired DNA repair and defective ATM-signalling, consistent with an established role for the MRN complex in ATM activation (Staples *et al*., 2016). The DNA repair and replication fork protection functions of MRNIP may modulate chemoresistance. Therefore, further studies which enhance understanding of the specific mechanisms of these functions would be beneficial to our mechanistic understanding of these functions.

1.6.2 Preliminary Studies.

Previously, work from the Staples laboratory suggests that MRNIP acts to prevent DNA damage and is rapidly recruited to sites of DNA damage (Staples *et al*., 2016). Ongoing studies in the Staples lab suggest that MRNIP not only plays a role in DNA repair mechanisms, but also in limiting post-replicative gaps in response to PARP inhibitor treatment. MRNIP loss causes sensitivity to the PARP inhibitor Olaparib. MRNIP depletion also leads to MRE11-dependent degradation of nascent DNA, which was hypothesised to be enhancing MRE11 removal of Gemcitabine. MRNIP KO cells were observed to be resistant to the nucleoside analogue Gemcitabine (Staples lab, unpublished data). The above observed phenotypes were also demonstrated to be PRIMPOL-dependent, as PRIMPOL-mediated repriming leads to ssDNA gaps in the nascent strand. Phosphorylation of MRNIP on the S217 site plays a role in MRNIP functionality in response to Gemcitabine treatment (Staples lab, unpublished data).

1.6.3 Focus of the Project.

The focus of the project is the further characterisation of MRNIP, and the exploration of MRNIP KO cell sensitivity to a wider range of chemotherapeutic agents.

Previous observations in the Staples lab demonstrated MRNIP-mediated Gemcitabine sensitivity and Olaparib resistance. This will be explored in the project via MTT assays, and by looking at DNA damage markers via indirect immunofluorescence. MRNIP KO cells have previously shown differential responses to other Nucleoside Analogues. This will also be explored through MTT assays during this project. The phosphorylation of the S217 site on MRNIP has been hypothesised to be important to MRNIP function. The validity of these results and further study into the role will also be explored through MTT assays and visualisation of DNA damage markers via indirect immunofluorescence during this project.

1.6.4 Questions the project will answer.

What is the role of MRNIP in mediating the cellular response to Gemcitabine or Olaparib?

What is the role of MRNIP in mediating the cellular response to other Nucleoside Analogues?

What role does the phosphorylation of MRNIP S217 site play in MRNIP-mediated responses to different chemotherapeutic agents?

2. Materials and Methods

2.1 Materials Used

2.1.1 siRNA

siRNA Target	**Target Sequence**
MRNIP	CAGGUUAUUAAUCGAGAUA
PRIMPOL	GAGGAAAGCUGGACAUCGA

Table 1- Target sequences of siRNA used in this thesis.

2.1.2 Antibodies

Antibody	Used for	Supplier	Source	Catalogue Number	Dilution used
53BP1	Western Blot	ABCAM	Rabbit	Ab21083	1:1000
BRCA1	Western Blot	GeneTex	Mouse	17F8	1:1000
FLAG	Western Blot	Merck	Mouse	SLCM4081	1:1000
GAPDH	Western Blot	Santa Cruz	Mouse	Sc-365062	1:1000
IdU/BrdU	Immunofluorescence	BD Biosciences	Mouse	B44	1:1000
MRNIP	Western Blot	Santa Cruz	Mouse	Sc-390012	1:2000
Phosphorylated S217 MRNIP	Western Blot	Eurogentec	Rabbit	ZGB22030	1:1000
PRIMPOL	Western Blot	Proteintech	Rabbit	29824-1-AP	1:1000
RAD51	Immunofluorescence	ABCAM	Rabbit	ab133534	1:1000
SMARCAL1	Western Blot	Santa Cruz	Mouse	Sc-376377	1:1000
γH2AX	Immunofluorescence	Merck	Mouse	05-636-I	1:1000

Table 2- Primary Antibodies used in this thesis.

Antibody	Used for	Supplier	Source	Catalogue Number	Dilution used
Alexa Fluor™ 488	Immunofluorescence (IdU)	Invitrogen	Goat anti-mouse	A11001	1:500
Alexa Fluor™ 594	Immunofluorescence (H2AX)	Invitrogen	Goat anti-mouse	A11005	1:500
Alexa Fluor™ 488	Immunofluorescence (*RAD51*)	Invitrogen	Goat anti-rabbit	A11008	1:500
Anti-mouse HRP	Western Blot	Cell Signalling	Horse	7076	1:3000
Anti-Rabbit HRP	Western Blot	Cell Signalling	Goat	7074	1:3000

Table 3- Secondary antibodies used for visualisation used in this thesis.

2.1.3 Solutions / Buffers

Description	Used for	Supplier	Content	Catalogue Number
Tris Buffered Saline (TBS)	Protein Pulldown Assay	Fisher	150 mM NaCl 50 mM Tris Base pH 7.4	BP2472-1
Tris Buffered Saline 10X (TBS)	Western Blot	N/A	200 mM Tris Base 1500 mM NaCl pH 7.6	N/A
Phosphate Buffered Saline (PBS)	Immunofluorescence	Sigma	2.7 mM KCl 2.7 mM KH_2PO_4 140 mM NaCl 8.1 mM Na_2HPO_4 anhydrous	D8537
RIPA Cell Lysis Buffer	Western Blot	N/A	25 mM Tris pH 7.5 150 mM NaCl	N/A

			1% NP-40 0.1% sodium dodecyl sulphate (SDS) 1% Sodium deoxycholate	
3% BSA in PBS	Immunofluorescence	N/A	0.9 g BSA 30 ml PBS	N/A
5% Milk in TBST	Western Blot	N/A	5 g milk 100 ml TBS 100 µl Tween	N/A
4% Paraformaldehyde	Immunofluorescence	Sigma-Aldrich	1.2 ml Formaldehyde 30 ml PBS	252549
Protein Pulldown Buffer	Protein Pulldown Assay	N/A	150 nM NaCl 3 mM KCl 25 mM Tris 10% Glycerol 1 mM DDT Protease/phosphatase inhibitor	N/A
NuPAGE™ MES SDS Running Buffer	Western Blot	Invitrogen	50 ml Concentrated stock Make up to 1 L Distilled water	NP0002
NuPAGE™ Transfer Buffer	Western Blot	Novex	50 ml Concentrated stock 20 ml methanol Make up to 1L Distilled water	NP0006-1
Tris Acetate SDS Running buffer	Western Blot	Novex	50 ml Concentrated stock Make up to 1 L Distilled water	LA0041
Triton X-100	Immunofluorescence	Fisher	N/A	BP151-100

Hydrochloric Acid	Immunofluorescence	Sigma-Aldrich	N/A	258148

Table 4- Solutions and buffers prepared and used in this thesis.

2.1.4 Other Materials

Description	Used for	Supplier	Catalogue Number
Halt Protease & Phosphatase Inhibitor Cocktail	Western Blot/ Protein Pulldown Assay	Thermo Fisher Scientific	78446
DAPI	Immunofluorescence	Sigma Aldrich	D9542
LDS	Western Blot	Novex	B0008
MRNIP pS217 Peptide	Protein Pulldown Assay	GenScript	Custom Made
MRNIP S217 Peptide	Protein Pulldown Assay	GenScript	Custom Made
Dynabeads M-280 Streptavidin	Protein Pulldown Assay	Thermo Fisher Scientific	11205D
HeLa Cell Nuclear Extracts	Protein Pulldown Assay	IPRACELL	CC012050
Dithiothreitol (DTT)	Protein Pulldown Assay	Thermo Fisher Scientific	R0861
2-Mercaptoethanol (BME)	Protein Pulldown Assay	Sigma Aldrich	M6250
MTT	MTT Assay	Thermo Scientific	L11939.06
Immu-Mount	Immunofluorescence	Thermo Fisher Scientific	9990402
Albumin Bovine Fraction V (BSA)	Immunofluorescence/ Western Blot	MELFORD	9048-46-8
Pierce™ ECL Western Blotting Substrate	Western Blot	Thermo Scientific	32106

NuPAGE™ 4-12% Bis-Tris Gel	Western blot of protein below 150 kDa	Invitrogen	NP0322BOX
NuPAGE™ 3-8% Tris-Acetate Gel	Western blot of proteins above 150kDa	Invitrogen	EA0378BOX

Table 5- Other materials used in this thesis.

2.2 Methods

2.2.1 Cell Culture

U2OS, HeLa and HCT116 cancer cell lines were cultured as adherent monolayers in Dulbecco's modified Eagle's medium (DMEM) (Supplier: Sigma Aldrich) supplemented with 10% foetal bovine serum (FBS), and maintained at 37°C, 5% CO_2 and 100% humidity.

2.2.2 siRNA Transfection

Four microlitres of Lipofectamine 2000 or Lipofectamine RNAiMAX were diluted in 100 µl serum-free DMEM. Specified siRNAs were made up in RNase-free water to a concentration of 100 µM and diluted to the preferred concentration in serum-free DMEM. The Lipofectamine and siRNA dilutions were then mixed 1:1 and incubated at room temperature. Following 20 minutes, the Lipo and siRNA mixture was added to the media to achieve a final siRNA concentration of 50 nM. Cells were lysed or fixed for processing after 48-72 hours incubation.

2.2.3 Immunofluorescence

30,000 cells were plated and grown on autoclaved glass coverslips in 24 well plates. After 24 hours, transfections and treatments were performed as indicated in individual experiments, according to the transfection protocol above. Cells were then fixed in 4% PFA in PBS for 10 minutes at 4°C and permeabilized in 0.5% Triton X-100 in PBS for 5 minutes at room temperature. Cells were blocked using 3% BSA in PBS for 30-60 minutes at room temperature. Cells were incubated with indicated primary antibody, made up in 3% BSA in PBS, overnight at 4°C. The following day, cells were incubated with indicated secondary antibody and DAPI, made up in 3% BSA in PBS, for 2 hours at room temperature. Between steps, cells were washed in PBS. Coverslips were mounted using Immu-Mount medium. Imaging was performed by fluorescence microscopy on a Zeiss LSM710 confocal microscope using 40X or 63X magnification. Images were analysed using Zen software.

2.2.4 Denaturing IdU Immunofluorescence

30,000 cells were plated and grown on autoclaved glass coverslips in 24 well plates. After 24 hours, cells were treated with a 100 nM dose of Gemcitabine. After 3 hours 40 minutes, 10 µM IdU was added to the experiment. After a further 20 minutes, cells were fixed in 4% PFA

in PBS for 10 minutes at 4°C. Cells were then permeabilized in 0.5% Triton X-100 in PBS for 5 minutes at room temperature. Cells were then incubated with 1.25M HCl. Cells were then blocked in 3% BSA in PBS for 45 minutes at room temperature. Cells were then incubated with a primary antibody recognising IdU in 3% BSA in PBS overnight (Table 2). The following day, cells were incubated with a secondary antibody for 2 hours at room temperature (Table 3). Between steps, cells were washed in PBS. Coverslips were mounted using Immu-Mount medium. Imaging was performed by fluorescence microscopy on a Zeiss LSM710 confocal microscope using 40X or 63X magnification. Images were analysed using Zen software.

2.2.5 Cell Lysis and Western Blot

Whole-cell extracts were prepared by lysing cells using RIPA buffer (Table 4) with added protease and phosphatase inhibitors at 1:100 dilution (Table 5). Gel electrophoresis was performed using NuPAGE gels (Refer to Table 5) and NuPAGE running buffer (Table 4) ran at 160V for 1 hour 20 minutes in Mini Gel Tanks from Invitrogen. Proteins were transferred onto PVDF membranes using NuPAGE transfer buffer (Table 4) at 30V for 1 hour in transfer tanks from Novex. Membrane was blocked for 30-60 minutes in 5% milk TBST and probed with primary antibodies overnight at 4°C and secondary antibodies for 2 hours at room temperature. Visualisation using Bio-Rad Chemi-doc imaging system was carried out.

2.2.6 MTT Assay

100,000 cells were seeded into wells of a 6-well plate and transfected with siRNA as indicated in individual experiments. After 24-48 hours, cells were Trypsinised, counted and 2000 cells per well were replated into 96-well plates. For experiments where no transfection was needed, 2000 cells per well were plated directly into 96 well plates. After a further 24 hours cells were treated with the compounds indicated in individual experiments. Plates were then incubated for an additional 48 hours before 1 mg/ml MTT was added for 2 -3 hours. Following this, plates were aspirated, and MTT was solubilised with the addition of 100 µl per well of DMSO, followed by shaking for 10 minutes to homogenise the solution. Readout of the plates was completed on a BioTek ELx800 plate-reader run on Gen5 software. Absorbance readout values for each dose were averaged and expressed as a percentage of the average of the untreated controls.

2.2.7 Immunoprecipitation Assay

One million WT, MRNIP KO HeLa cells expressing FLAG-WT or FLAG S217 were grown in 10 cm plates. After 24 hours, cells were lysed. FLAG M2 antibody-coated beads (Sigma) were washed 2 times with 1xTBST and incubated with whole cell lysates gathered from HeLa cells overnight at 4°C. Beads were then washed 4 times in 1xTBST containing protease and phosphatase inhibitor (Table 5), then 2XSDS loading buffer was added to the beads, and samples were boiled at 95°C for 2 minutes to remove the proteins from the beads. Gel electrophoresis was performed using a NuPAGE gel (Refer to Table 5) and NuPAGE running

buffer (Table 4) at 160V for 1 hour 30 minutes. Proteins were transferred onto PVDF membranes using NuPAGE transfer buffer (Table 4) at 30V for 1 hour. The membrane was blocked for 30-60 minutes in 3% BSA in TBST and probed with the phospho-S217 primary antibody overnight at 4°C and secondary antibody for 2 hours at room temperature. Visualisation using Bio-Rad Chemi-doc imaging system was carried out.

3. Results

The nuclease MRE11 has essential functions in DNA repair and the replication stress response and is known to degrade DNA at stalled replication forks (Kim *et al*., 2020). The Staples laboratory recently identified MRNIP as a regulator of MRE11 at stalled, reversed replication forks in response to hydroxyurea-induced replication stress (Bennett *et al*., 2020; Staples *et al*., 2016). Whether MRNIP has additional functions during chemotherapy-induced replication stress is currently under investigation. Preliminary findings indicate that MRNIP promotes Olaparib chemoresistance by limiting the prevalence of MRE11-dependent post-replicative ssDNA gaps formed consequent to PRIMPOL-mediated replication repriming. However, whether MRNIP displays similar functions in response to other replication stress agents is currently unknown. Here, the sensitivity of MRNIP KO cells to a range of Nucleoside Analogues: Gemcitabine, Clofarabine, Fludarabine and Cytarabine were tested. Given the role of PRIMPOL in the Olaparib response of MRNIP KO cells, and prior evidence that repriming occurs in response to Chain-Terminating Nucleoside Analogue (CTNA) treatment, it was hypothesised that PRIMPOL similarly modulates replication dynamics and cell fate in MRNIP KO cells treated with Gemcitabine (Kobayashi *et al*., 2016). PRIMPOL has also been suggested to incorporate Gemcitabine and Cytarabine, although whether PRIMPOL also incorporates Fludarabine and Clofarabine is untested (Díaz-Talavera *et al*., 2022; Tokarsky *et al*., 2017).

Prior mass spectrometry analysis conducted by the Staples laboratory identified phosphorylation of MRNIP at S217. Further studies in the laboratory identified a possible role for the phosphorylation of MRNIP S217 in modulation of chemoresistance (Staples lab, unpublished data). S217 is a proline directed phosphorylation site. As such, a number of proline-directed enzymes are candidate S217 kinases; Cyclin-Dependent Kinases (CDKs) 2, 3, 4, 6, and 8, ERK1/2, ERK5, p38, JNK and DYRK1. (Igarashi & Okuda, 2019; Pelech, 1995). Given that the majority of these kinases are either cell cycle phase-specific, or are responsive to stresses including DNA damage, it is possible that S217 phosphorylation facilitates cell cycle phase- or DDR-specific control of MRE11 via MRNIP. To better understand the role of S217 phosphorylation, further studies are required to uncover the kinase(s) and activating stimuli responsible.

The purpose of the investigation was to further explore the role(s) of MRNIP in cancer cells treated with nucleoside analogues or PARP inhibitors, to improve the current understanding of MRNIP function, with possible future implications for cancer therapy.

3.1 MRNIP loss leads to Gemcitabine resistance.

The first cancer therapy tested during this study was Gemcitabine. Previous studies in the Staples laboratory suggested that MRNIP KO cells are resistant to Gemcitabine treatment. To confirm this finding, an MTT assay was performed with Wild-type and MRNIP KO HeLa and HCT116 cell lines. Cells were treated with a varying dose of Gemcitabine for 72 hours, and the MTT assay was then used as a crude measure of cell survival (though the assay directly measures reduced NADH and NADPH generated during metabolism). MRNIP KO cells of both cell backgrounds displayed increased survival relative to their WT counterparts in response to Gemcitabine treatment (Figure 5A and B).

To assess the role of MRNIP in survival in another cell line in response to Gemcitabine treatment, MRNIP was depleted via siRNA transfection in the osteosarcoma line U2OS. Since previous work from the Staples laboratory suggested that replication repriming and fork reversal may be important processes with respect to the cellular response to Gemcitabine, MRNIP was depleted in PRIMPOL KO and SMARCAL1 KO U2OS. In contrast to the previous observations in MRNIP KO HeLa and HCT116 cells, MRNIP-depleted U2OS cells displayed increased sensitivity to Gemcitabine, relative to their WT counterparts. PRIMPOL KO cells displayed increased survival compared to WT cells, but MRNIP-depleted PRIMPOL KO cells displayed reduced survival, similar to MRNIP-depleted WT cells. Survival of SMARCAL1 KO cells was reduced compared to the WT cells, and this effect was unchanged by MRNIP depletion (Figure 6A). MRNIP depletion and PRIMPOL/SMARCAL1 status was monitored by Western blotting (Figure 6B-D). This experiment was performed once and required repetition to confirm the phenotypes observed.

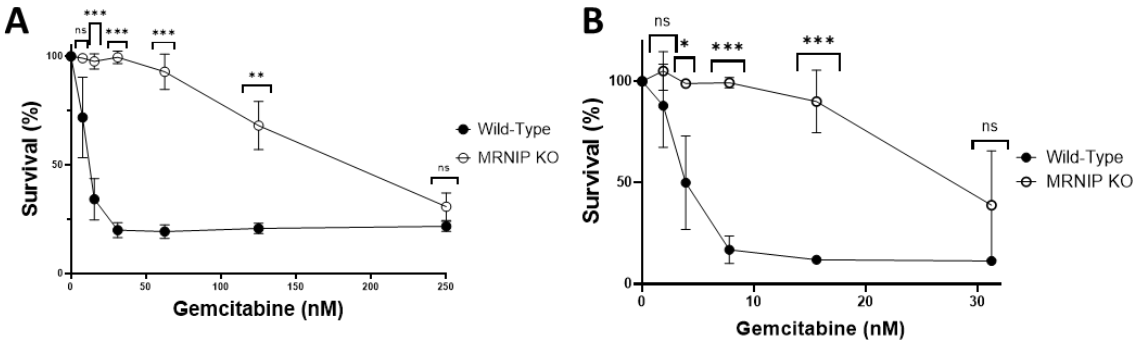


Figure 5 – ***MRNIP loss causes increased resistance to Gemcitabine treatment.***

*2000 cells/well were plated in 96 well plates. 4 wells were plated for each condition. Wild-Type and MRNIP KO (**A**) HeLa and (**B**) HCT116 cell lines were treated with the indicated concentrations of Gemcitabine. After 72 hours an MTT assay was performed, and the results were normalized to untreated controls. *p=<0.05 (N=3). The statistical significance was determined by unpaired t-test.*

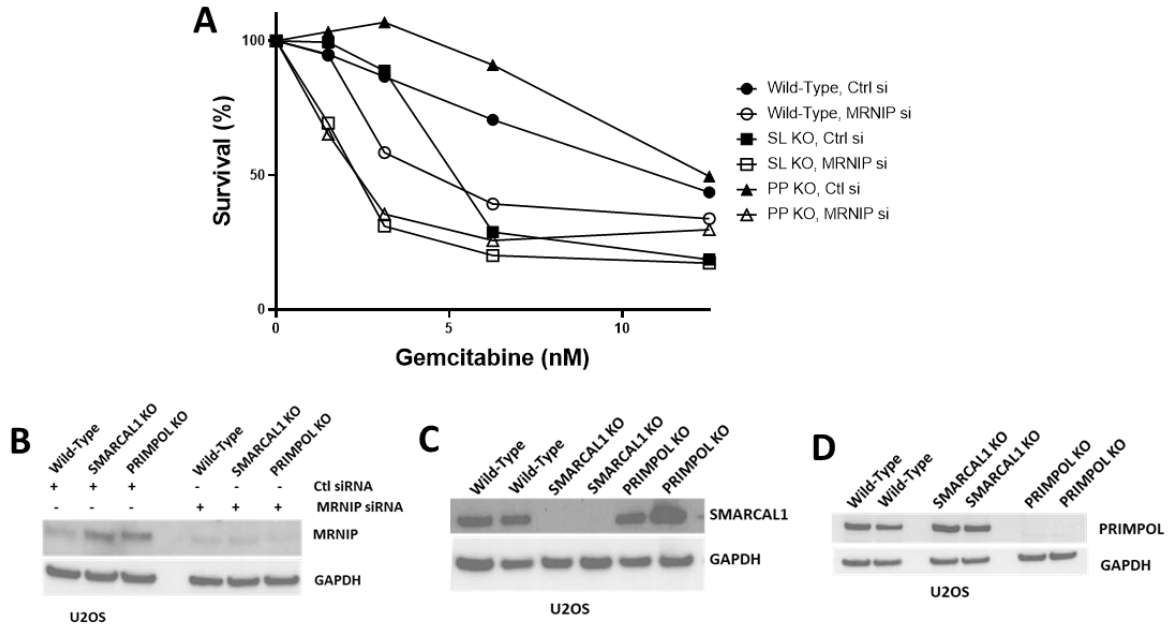


Figure 6 – ***The effect of MRNIP depletion on Gemcitabine chemosensitivity in U2OS cells.***

*2000 cells/well were plated. 4 wells were plated per condition. (**A**) Wild-Type and CRISPR derivative PRIMPOL KO and SMARCAL1 KO U2OS cells were transfected with Control or MRNIP siRNA. After 48 hours, cells were treated with the indicated concentration of Gemcitabine. After a further 72 hours, an MTT assay was performed, and the results were normalized to the untreated controls (N=1). (**B**) MRNIP depletion, (**C**) SMARCAL1 KO, and (**D**) PRIMPOL KO were confirmed by SDS-PAGE and western blotting of whole cell lysates.*

3.2 The effect of MRNIP loss on DNA damage markers in response to Gemcitabine treatment.

To develop our understanding of the role of MRNIP in the cellular response to Gemcitabine, indirect immunofluorescence experiments were undertaken to assess markers of DNA damage and recombination (*γH2AX* and *RAD51*, respectively). Cells were also depleted of PRIMPOL, to assess the potential role of replication repriming. Wild-type and MRNIP KO HeLa cells were grown on glass coverslips and transfected with Control or PRIMPOL siRNA, then stained with validated antibodies raised against *γH2AX* and *RAD51*. An increased basal frequency of *γH2AX*-positive and *RAD51*-positive cells were observed in untreated MRNIP KO cells relative to WT cells, in agreement with previous findings (Bennett *et al*., 2020; Staples *et al*., 2016). Reduced markers of DNA damage and recombination were observed in Gemcitabine-treated MRNIP KO cells, consistent with the observation that MRNIP KO cells are resistant to Gemcitabine. PRIMPOL depletion had no effect on the frequency of *γH2AX*-positive or *RAD51*-positive cells in the absence of Gemcitabine, though following Gemcitabine

treatment DNA damage in WT cells was ameliorated by PRIMPOL depletion. Conversely, depletion of PRIMPOL caused a slight increase in both markers in MRNIP KO cells treated with Gemcitabine, suggesting that repriming may exert opposing effects in the presence and absence of MRNIP (Figure 7A-C). MRNIP loss was confirmed by Western blotting (Figure 7D). However, this experiment must be repeated to ensure the validity of these initial observations.

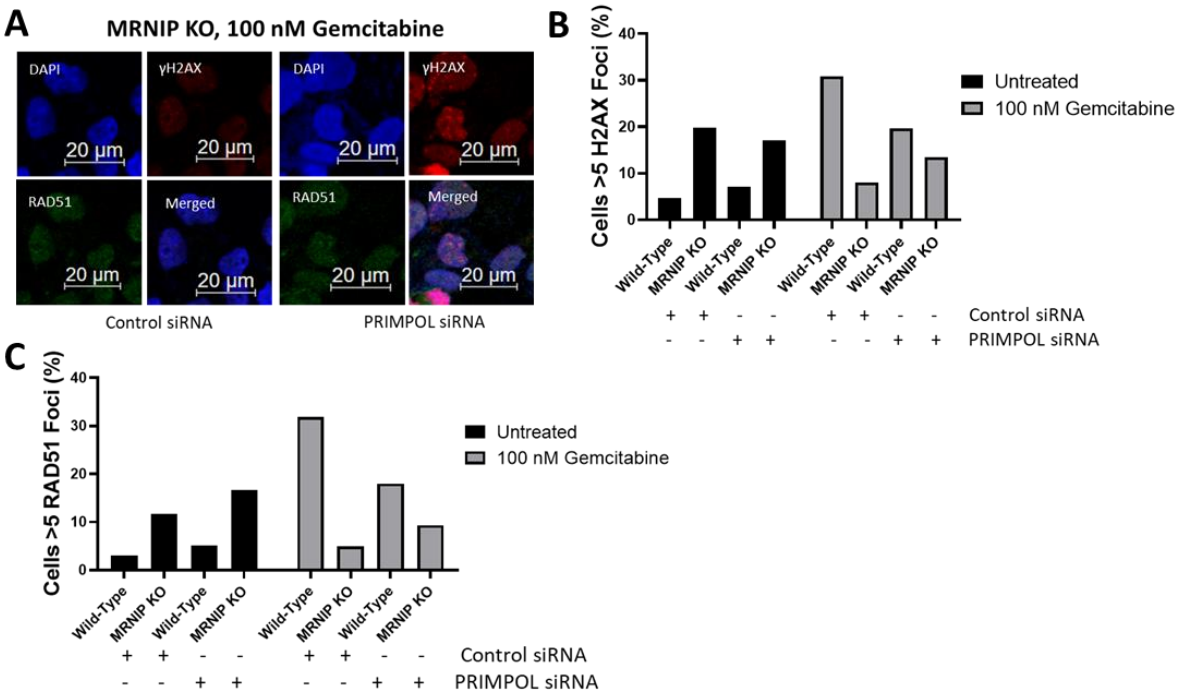


Figure 7 – ***The effect of PRIMPOL depletion on DNA damage markers in Gemcitabine-treated WT and MRNIP KO cells.***

*30,000 cells per well were plated. After 24 hours (**A**) Wild-Type and MRNIP KO HeLa cells were transfected with Control or PRIMPOL siRNA. After 48 hours cells were treated with the indicated doses of Gemcitabine. After 16 hours, cells were fixed and stained with an antibody recognising (**B**) γH2AX and (**C**) RAD51. Cells were counterstained with DAPI and visualised at 63X magnification and those containing more than 5 foci were counted as positive and expressed as a percentage of the total number of cells imaged. (**D**) MRNIP status was confirmed by SDS-PAGE and Western blotting of whole cell lysates. (N=1).*

The effect of Gemcitabine treatment on DNA replication in WT and MRNIP KO cells was assessed via denaturing IdU experiments. Cells were treated with 100 nM Gemcitabine, and then pulsed for 20 minutes with IdU to label nascent DNA. Cells were then fixed and stained with an anti-BrdU antibody that cross-reacts with IdU and visualised via indirect immunofluorescence. Gemcitabine treatment led to a marked reduction in the frequency of IdU-positive WT cells, although strikingly DNA replication in MRNIP KO cells was almost completely unaffected by Gemcitabine treatment (Figure 8A and B).

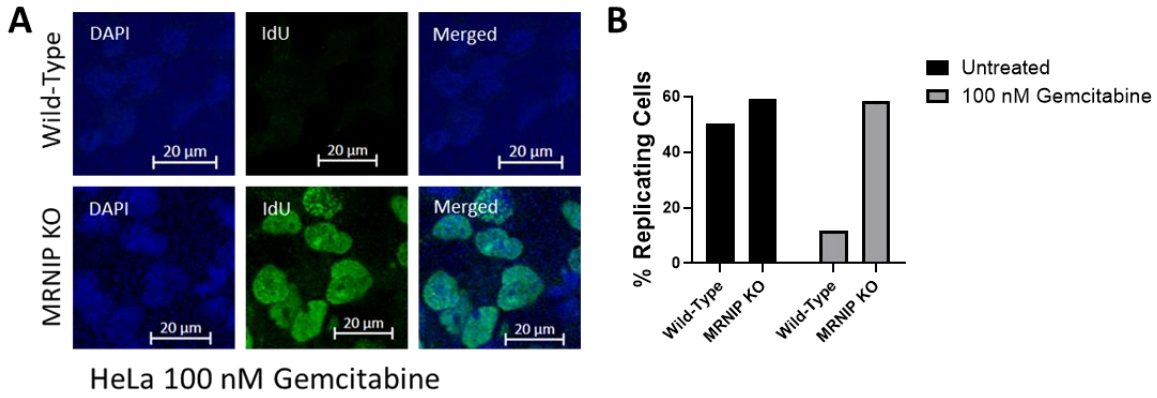


Figure 8 – ***The effect of MRNIP loss on DNA replication in response to Gemcitabine treatment.***

*30,000 cells per well were plated. After 24 hours (**A**) Wild-Type and MRNIP KO HeLa cell lines were treated with 100 nM Gemcitabine. After 3 hours 40 minutes, IdU was added to the cells. After a further 20 minutes, cells were fixed, permeabilized and DNA was denatured in HCl, before being blocked and stained with an antibody recognising IdU. Cells were counterstained with DAPI and visualised at 40X magnification. IdU-positive cells were counted and expressed as a percentage of the total number of cells imaged (**B**). (N=1).*

3.3. The role of MRNIP Serine 217 phosphorylation in the cellular response to Gemcitabine.

Previous mass spectrometry analysis conducted by the Staples laboratory demonstrated that MRNIP is phosphorylated on S217. An antibody against a phospho-peptide spanning the S217 site was raised (Eurogentec). To attempt to test and validate this antibody, MRNIP KO cells stably expressing doxycycline-inducible FLAG-WT or FLAG-S217A MRNIP were generated and whole-cell extracts derived from these lines were assessed by Western blotting with the p-S217 antibody. Extracts from WT and MRNIP KO cells were included as additional positive and negative controls. Unfortunately, no specific bands were observed in whole cell extracts (Figure 9A). To enrich MRNIP further and purify the cell extracts, FLAG-tagged WT or S217A MRNIP were immunoprecipitated using beads coated with anti-FLAG M2 antibody. A band of the correct molecular weight for the MRNIP protein was observed (Figure 9B). However, the band was also present in samples prepared from the unphosphorylatable FLAG-S217A MRNIP mutants. The blot was re-probed with FLAG antibody in attempt to validate the cells used, however this blot was unsuccessful and did not show any specific bands.

Previous observations suggested that phosphorylation of MRNIP at Serine 217 is important for function. To confirm this phenotype an MTT assay was performed with WT and MRNIP KO cells, and MRNIP KO cells expressing FLAG-WT MRNIP (FWT) or FLAG-S217A (S217A). Gemcitabine resistance was noted in MRNIP KO cells relative to WT cells, as previously observed. Expression of WT MRNIP in MRNIP KO cells restored Gemcitabine sensitivity,

though notably, expression of S217A MRNIP did not, suggesting that modification of this site is important in mediating the ability of MRNIP to drive chemosensitivity (Figure 10(A)). FLAG expression in the MRNIP KO + FLAG-WT or FLAG-S217A mutant cell lines was tested via Western Blotting of whole-cell lysates (Figure 10B).

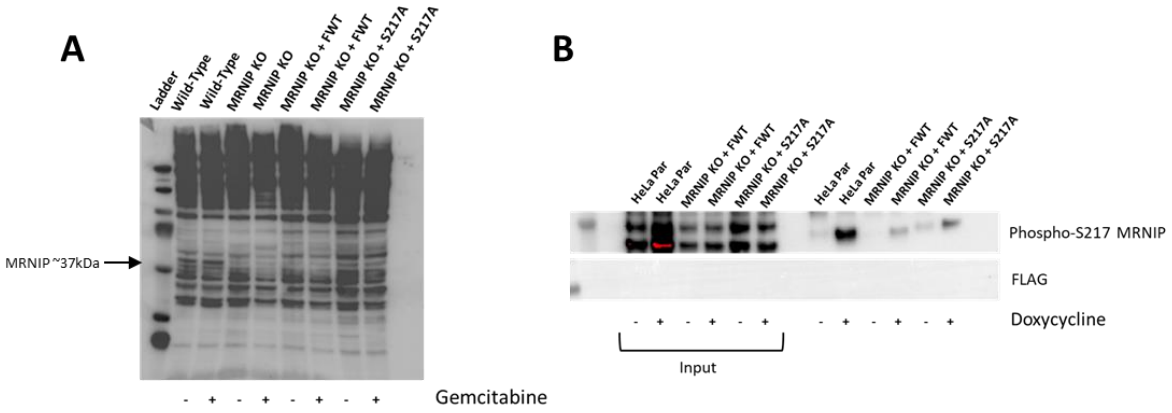


*Figure 9–**Testing of a polyclonal antibody raised against p-S217.***

*(**A**) Wild-Type and MRNIP KO HeLa cells, and MRNIP KO HeLa cells stably expressing FLAG Wild-Type MRNIP or FLAG-S217A MRNIP lines were treated with doxycycline, and 24 hours later were treated with 100 nM Gemcitabine for 16 hours. Cells were then lysed, and a Western Blot was performed using a phospho-S217 antibody. (**B**) Wild-Type, and MRNIP KO HeLa cells expressing FLAG WT or FLAG S217A were treated with doxycycline, and 24 hours later cells were lysed, and FLAG-tagged MRNIP was purified via immunoprecipitation with FLAG M2 beads. Eluates were probed with antibodies against p-S217 and FLAG.*

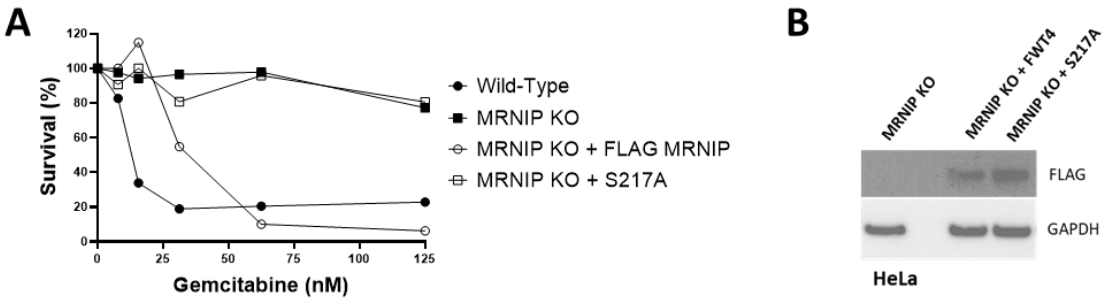


Figure 10– ***The role of Ser217 in the regulation of Gemcitabine resistance by MRNIP.***

*2000 cells/well were plated. 4 repeat wells were plated per condition. (**A**) Wild-Type and MRNIP KO HeLa cells, and MRNIP KO HeLa cells stably expressing FLAG Wild-Type MRNIP or FLAG-S217A MRNIP were treated with the indicated concentrations of Gemcitabine. After 72 hours an MTT assay was performed, and results were normalized to untreated controls. (**B**) FLAG expression was confirmed in FLAG-WT and FLAG-S217A cell lines by SDS-PAGE and Western blotting of whole-cell extracts. (N=1).*

To assess the impact of S217 phosphorylation status on DNA damage, immunofluorescent detection of the DNA damage and recombination markers *γH2AX* and *RAD51* were once again employed. MRNIP KO, MRNIP KO + FLAG-WT and MRNIP KO + FLAG-S217A HeLa cells were grown on glass coverslips and treated with 100 nM Gemcitabine for 16 hours. Cells were then fixed, stained with validated antibodies raised against *γH2AX* and *RAD51*, and visualised by indirect immunofluorescence. Expression of FLAG-WT but not FLAG-S217A MRNIP in MRNIP KO cells led to an increase in the frequency of *γH2AX*-positive cells, suggesting that S217 is required for MRNIP-mediated promotion of Gemcitabine-induced DNA damage (Figure 11A-C). In untreated cells, the frequency of *γH2AX*-positive cells did not vary significantly. The frequency of *RAD51*-positive cells in Gemcitabine-treated MRNIP KO cells and S217A cells was significantly reduced relative to FLAG-WT cells, supporting the observation that S217A mutants phenocopy MRNIP loss. In untreated MRNIP KO cells, expression of FLAG-WT MRNIP suppressed the basal levels of *RAD51*-positive cells, and this decrease was largely independent of S217 status.

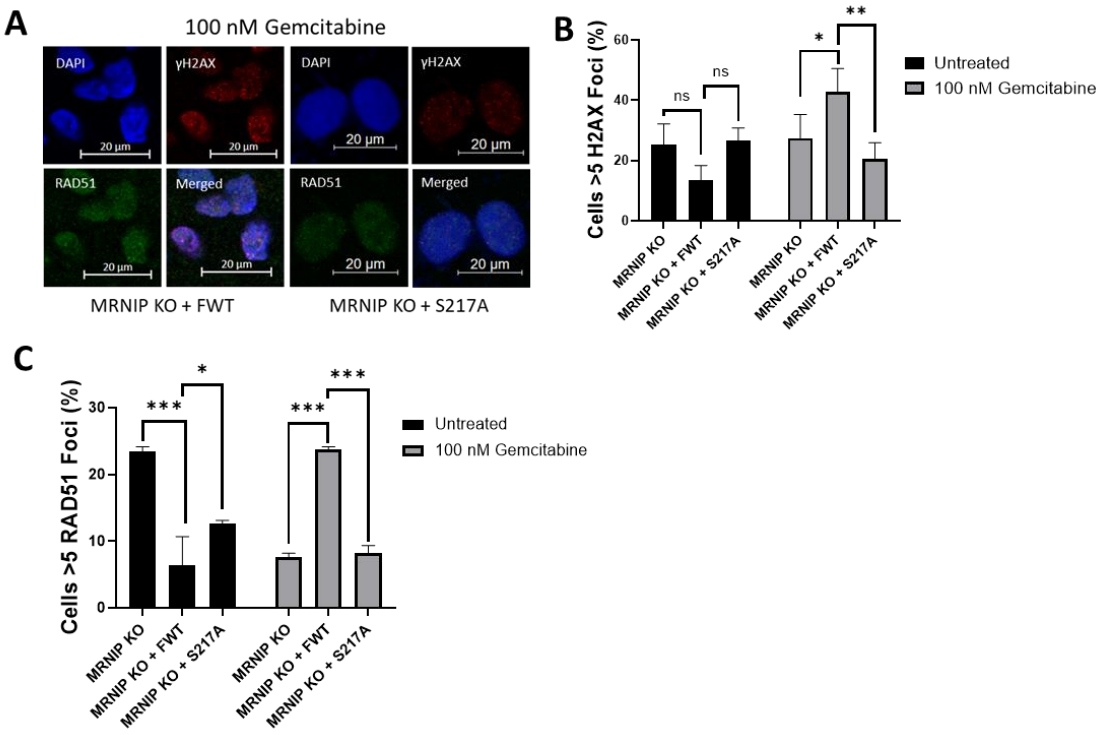


Figure 11- ***Gemcitabine-induced DNA damage is dependent on MRNIP Ser217 phosphorylation.***

*30,000 cells per well were plated. After 24 hours (**A**) MRNIP KO HeLa cell lines, and MRNIP KO cells stably expressing FLAG-tagged-Wild-type or S217A MRNIP were treated with 100 nM Gemcitabine. After 16 hours, cells were fixed and stained with antibodies recognising (**B**) γH2AX and (**C**) RAD51. Cells were counterstained with DAPI, visualised by indirect immunofluorescence, and those containing more than 5 foci were counted as positive and expressed as a percentage of the total number of cells imaged. *p=<0.05 (N=3). The statistical significance was determined using two-way ANOVA.*

3.4 The effect of MRNIP loss on the cellular response to Nucleoside Analogue treatment.

Spurred on by these interesting findings, the response of MRNIP KO cells to several other Nucleoside Analogues were tested. MTT assays were performed as a crude method to establish cell survival in response to treatment in response to varying doses of Clofarabine, Fludarabine, Cytarabine and Gemcitabine. Interestingly MRNIP loss resulted in chemosensitivity or chemoresistance, depending on the analogue tested (Figure 12). MRNIP KO cells were sensitive to Clofarabine and Fludarabine relative to their WT counterparts (Figures 10A and B). Conversely, MRNIP KO cells were resistant to both Gemcitabine as previously demonstrated, though large error bars in the Cytarabine assay precluded proper analysis (Figures 10C and D).

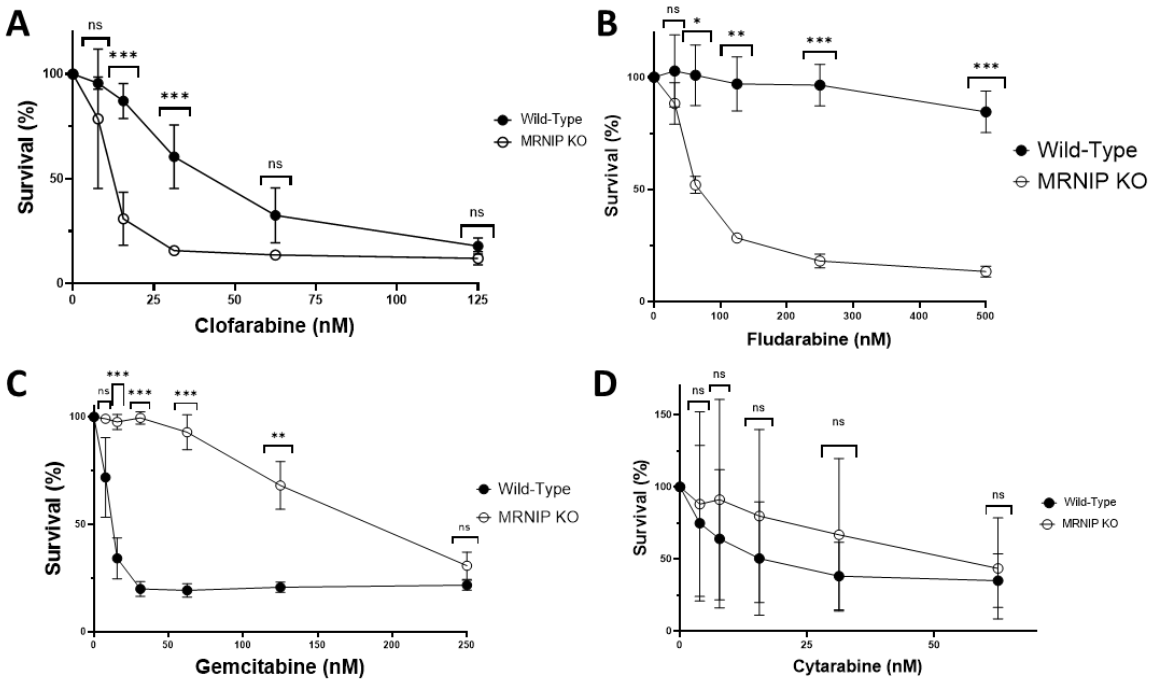


Figure 12 – ***The effect of MRNIP KO on HeLa cell survival in response to Nucleoside Analogue treatment.***

*2000 cells/well were plated in 96 well plates. 4 replicate wells were plated per condition. Wild-Type HeLa and MRNIP KO CRISPR derivative cell lines were treated with the indicated concentrations of (**A**) Clofarabine, (**B**) Fludarabine, (**C**) Gemcitabine or (**D**) Cytarabine. After 72 hours an MTT assay was performed, and results were normalized to untreated control. *p=<0.05 (N=3). The statistical significance was determined by unpaired t-test.*

PRIMPOL can incorporate Gemcitabine and Cytarabine into genomic DNA *in vitro* (Tokarsky *et al.*, 2017). The efficiency of PRIMPOL incorporation of Fludarabine and Clofarabine is unknown. To develop our understanding of the role of PRIMPOL in the cellular response to nucleoside analogue treatment, MTT assays were performed. WT and MRNIP KO HeLa cells were grown and transfected with a non-targeting control siRNA or an siRNA targeting PRIMPOL. Cells were then treated with varying doses of Clofarabine, Fludarabine, Cytarabine and Gemcitabine. PRIMPOL depletion had little effect on WT cells in response to Clofarabine treatment. However, MRNIP KO cells depleted of PRIMPOL displayed an increase in survival following Clofarabine treatment, however this may be due to poor baseline survival in MRNIP KO cells depleted of PRIMPOL in this experiment (Figure 13A). PRIMPOL depletion had little effect on WT or MRNIP KO cells in response to Fludarabine treatment (Figure 13B). WT cells displayed no phenotypic difference to PRIMPOL depletion in response to Gemcitabine treatment, while MRNIP KO cells depleted of PRIMPOL displayed a slight decrease in survival

at the highest dose tested – it is possible that this difference may be enhanced upon further dose escalation, though this requires additional experimentation (Figure 13C). PRIMPOL depletion had no significant effect on WT cells in response to Cytarabine treatment (Figure 13D). MRNIP KO cells depleted of PRIMPOL displayed a decrease in survival in response to Cytarabine, although this experiment requires further repetition.

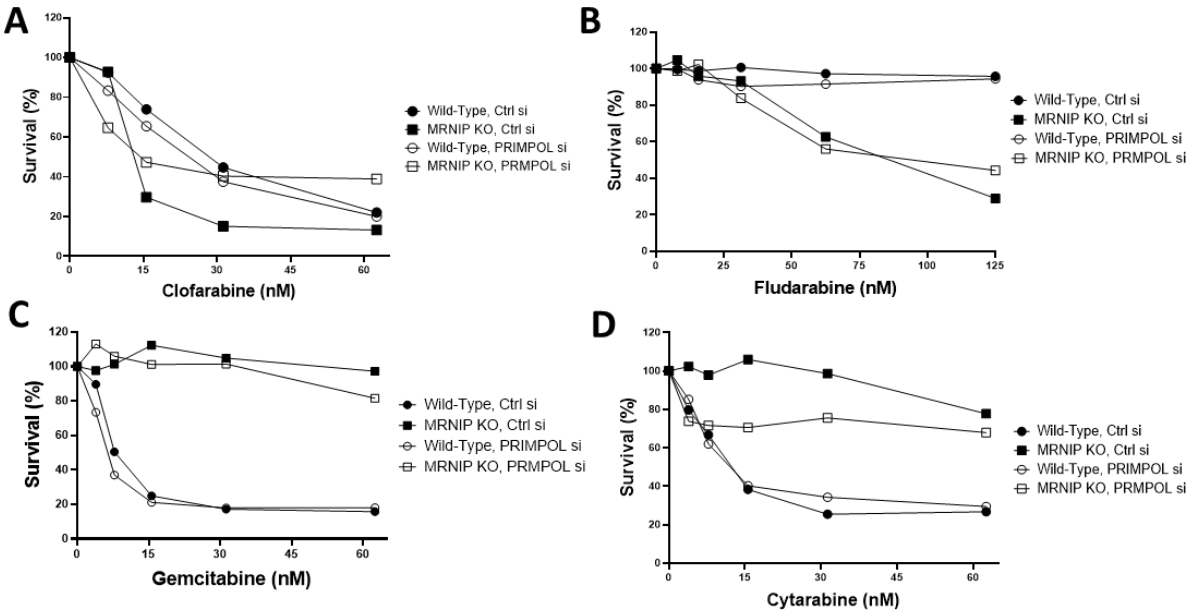


Figure 13– ***The effect of PRIMPOL knockdown on MRNIP KO HeLa cell survival in response to nucleoside analogues.***

*2000 cells/well were plated. 4 replicate wells were plated per condition. Wild-Type HeLa and MRNIP KO CRISPR derivative cell lines were transfected with Control or PRIMPOL siRNA. After 48 hours, cells were treated with the indicated concentrations of (**A**) Clofarabine, (**B**) Fludarabine, (**C**) Gemcitabine or (**D**) Cytarabine. After 72 hours an MTT assay was performed, and results were normalized to untreated controls (N=1).*

3.5 MRNIP loss leads to PARP inhibitor sensitivity.

Previous studies conducted by the Staples laboratory suggested that MRNIP KO cells are sensitive to Olaparib. To confirm this finding, MTT assays were conducted in Olaparib-treated WT and MRNIP KO HeLa and HCT116 cells. Cells were treated with varying doses of Olaparib. Both MRNIP KO HeLa and HCT116 cells displayed increased sensitivity to Olaparib treatment relative to their WT counterparts (Figure 14).

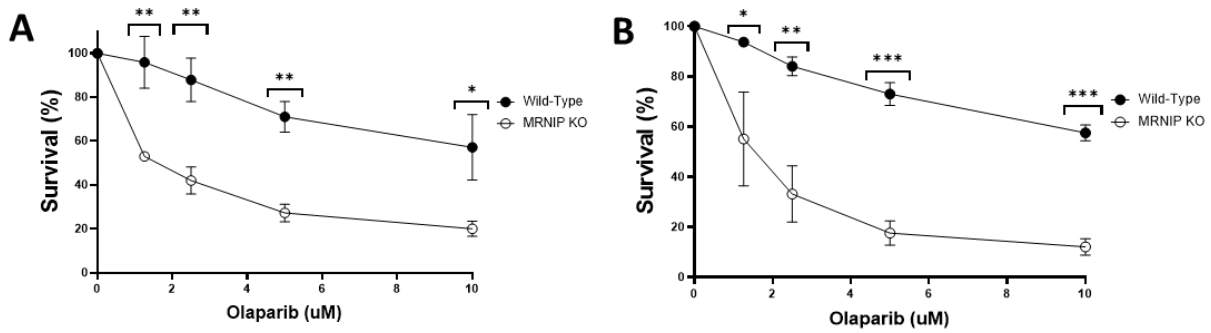


Figure 14– ***The loss of MRNIP causes sensitivity to PARP inhibition in cancer cell lines.***

*2000 cells/well were plated. 4 replicate wells were plated per condition. Wild-Type and MRNIP KO (**A**) HeLa and (**B**) HCT116 cell lines were treated with the indicated concentrations of Olaparib. After 72 hours an MTT assay was performed, and the results were normalized to the untreated controls. *p=<0.05 (N=3). The statistical significance was determined by unpaired t-test.*

3.6 The effect of MRNIP loss on DNA damage markers in response to Olaparib treatment.

To assess whether DNA damage was increased in MRNIP KO cells in response to Olaparib treatment, an indirect immunofluorescence experiment was conducted, to look at markers of DNA damage and recombination (*γH2AX* and *RAD51*, respectively). Cells were depleted of PRIMPOL to assess the potential role of replication repriming. WT and MRNIP KO HeLa cells were grown on glass coverslips and transfected with Control or PRIMPOL siRNA, then stained with validated antibodies raised against *γH2AX* and *RAD51*. Olaparib treatment led to an increase in *γH2AX*-positive and *RAD51*-positive cells in MRNIP KO cells, which was decreased by PRIMPOL depletion. MRNIP KO cells demonstrated an increased basal frequency of *γH2AX*-positive and *RAD51*-positive cells relative to WT cells in untreated conditions. Increased DNA damage was observed in Olaparib-treated MRNIP KO cells, consistent with our finding that MRNIP KO cells are sensitive to Olaparib treatment. PRIMPOL depletion had no effect on the frequency of *γH2AX*-positive or *RAD51*-positive cells in untreated conditions (Figure 15).

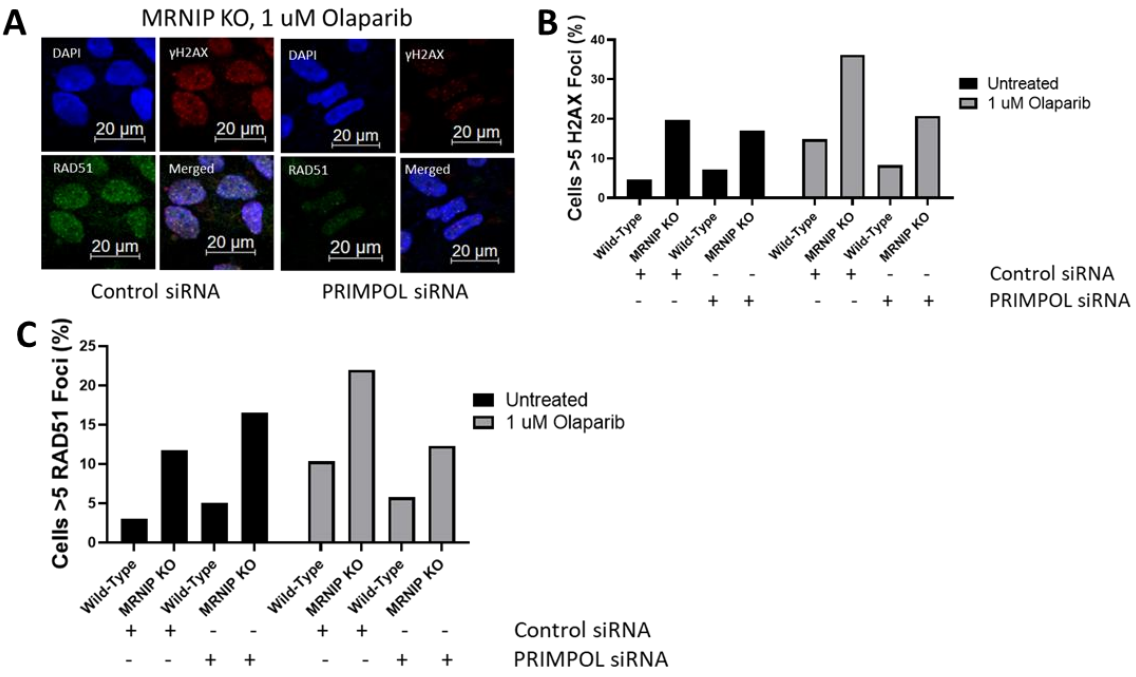


Figure 15– ***The effect of PRIMPOL depletion on DNA damage in response to PARP Inhibitor treatment.***

*(**A**) Cells were visualised via indirect immunofluorescence. 30,000 cells per well were plated. After 24 hours, Wild-Type and MRNIP KO HeLa cells were transfected with Control or PRIMPOL siRNA. After 48 hours cells were treated with a 1 µM dose of Olaparib. After 16 hours, cells were fixed and stained with an antibody recognising (**B**) γH2AX and (**C**) RAD51. Cells were counterstained with DAPI and those containing more than 5 foci were counted as positive and expressed as a percentage of the total number of cells imaged. (N=1).*

3.7 The role of MRNIP S217 phosphorylation in the response to PARP inhibitor treatment.

To understand whether phosphorylation of Serine 217 site on the MRNIP protein is important in mediating MRNIP function during the cellular response to Olaparib treatment, Olaparib-treated MRNIP KO, MRNIP KO expressing FLAG-WT or FLAG-S217A HeLa cells were stained with markers of DNA damage and recombination (*γH2AX* and *RAD51* respectively). Expression of FLAG-WT but not FLAG-S217A MRNIP in MRNIP KO cells led to a decrease in the frequency of *γH2AX*-positive cells, suggesting that S217 is required for MRNIP-mediated Olaparib resistance (Figure 16A and B). In untreated cells, the frequency of *γH2AX*-positive cells did not vary significantly between samples. The frequency of *RAD51*-positive cells in MRNIP KO cells and S217A cells was significantly increased relative to FLAG-WT cells, supporting the observation that S217A mutants phenocopy MRNIP loss (Figure 16C). In untreated cells, the frequency of *RAD51*-positive cells remained relatively constant.

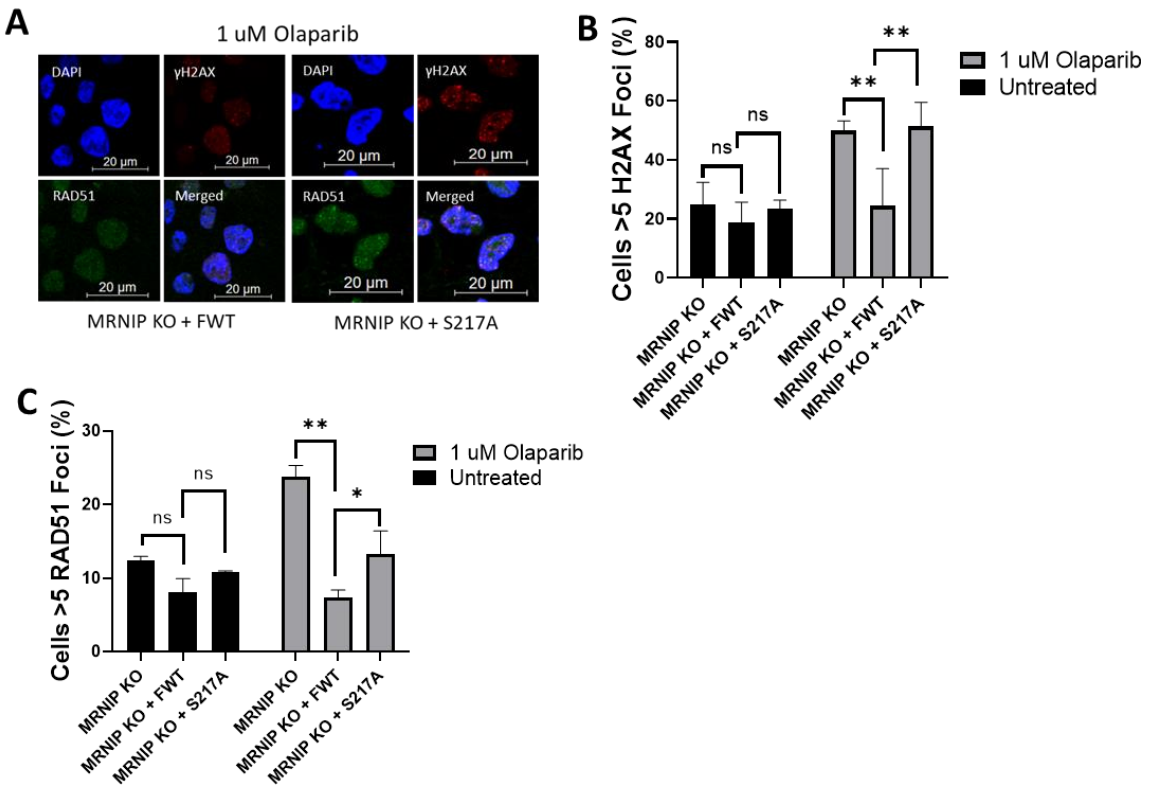
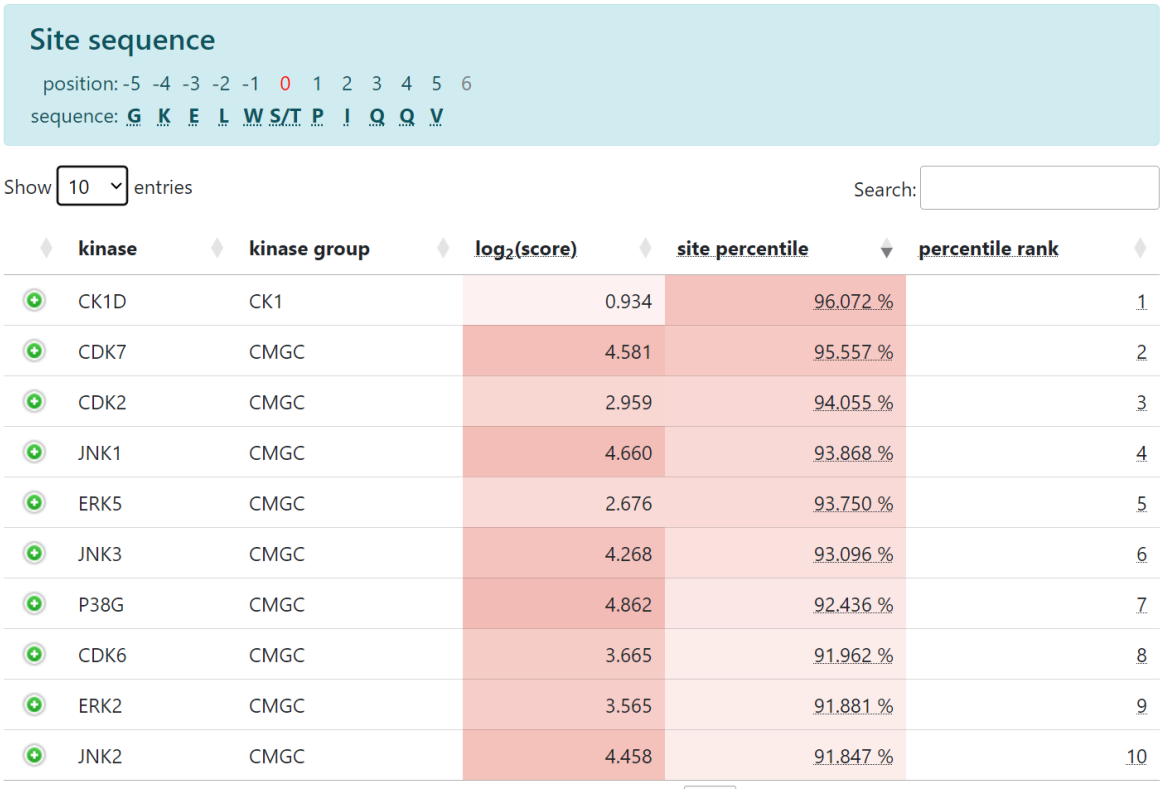


Figure 16- ***Serine 217 status modulates DNA damage markers in Olaparib-treated MRNIP KO cells.***

*30,000 cells per well were plated. (**A**) MRNIP KO HeLa cell lines, MRNIP KO stably expressing FLAG Wild-type MRNIP or S217A MRNIP were treated with 1 μM Olaparib. After 16 hours, cells were fixed and stained with antibodies recognising (**B**) γH2AX and (**C**) RAD51. Cells were counterstained with DAPI, visualised via indirect immunofluorescence, and those containing more than 5 foci were counted as positive and expressed as a percentage of the total number of cells imaged. *p=<0.05 (N=3). The statistical significance was determined using two-way ANOVA.*



Site sequence

position: -5 -4 -3 -2 -1 0 1 2 3 4 5 6

sequence: G K E L W S/T P I Q Q V

Show 10 entries Search:

	kinase	kinase group	log_2(score)	site percentile	percentile rank
	CK1D	CK1	0.934	96.072 %	1
	CDK7	CMGC	4.581	95.557 %	2
	CDK2	CMGC	2.959	94.055 %	3
	JNK1	CMGC	4.660	93.868 %	4
	ERK5	CMGC	2.676	93.750 %	5
	JNK3	CMGC	4.268	93.096 %	6
	P38G	CMGC	4.862	92.436 %	7
	CDK6	CMGC	3.665	91.962 %	8
	ERK2	CMGC	3.565	91.881 %	9
	JNK2	CMGC	4.458	91.847 %	10

Figure 17- ***Serine 217 possible kinase predictions*** (*Kinase Prediction*, n.d.)***.***

The sequence for Serine 217 site on the MRNIP protein was assessed using a kinase prediction software to see possible kinases acting on the site. A total of 303 results were discovered using the sequence with -5 and +5 amino acids either side of the site. The top 10 most likely kinases are displayed above in the table. The site percentile refers to how specific the kinase is likely to be to the predicted site.

3.8 Overview

As a whole, the results obtained suggest that MRNIP loss leads to resistance to Gemcitabine, suggesting that MRNIP mediates sensitivity to Gemcitabine in cancer cells. The increase of DNA damage markers in WT but not MRNIP KO cells treated with Gemcitabine supports this finding. The results also demonstrate that MRNIP loss causes sensitivity to Olaparib, suggesting that MRNIP mediates resistance to Olaparib in cancer cells. The results also demonstrate a role for MRNIP S217 phosphorylation in mediating MRNIP function and suggests this site is essential for MRNIP-mediated cellular responses to treatment with both Gemcitabine and Olaparib.

4. Discussion

The purpose of this study was to expand the understanding of the role(s) of the novel replication fork protection factor MRNIP in cells treated with replication stress-inducing genotoxic chemotherapies. MRNIP has been demonstrated to play a role in the cancer cell response to therapy, although further study is required to fully elucidate the functionality of MRNIP in the replication stress response. MRNIP is an established regulator of DNA repair and replication fork protection (Bennett *et al*., 2020; Staples *et al*., 2016). MRNIP stabilises stalled replication forks and limits the prevalence of ssDNA gaps formed during DNA replication stress (Bennett *et al*., 2020 and Bennett *et al*., under submission). *In vitro*, MRNIP interacts directly with MRE11, and acts to limit MRE11 exonuclease activity (Bennett *et al*., 2020). MRNIP has been demonstrated to remove Gemcitabine from genomic DNA (Boeckemeier *et al*., 2020), raising the possibility that altered MRE11 activity in MRNIP KO cells modulates Gemcitabine removal, or post-replicative processing of Gemcitabine-terminated nascent DNA ends. Here, the response of WT and MRNIP KO cancer cells were investigated following treatment with the nucleoside analogues Gemcitabine, Fludarabine, Cytarabine and Clofarabine, in addition to the PARP inhibitor Olaparib.

Gemcitabine is incorporated into nascent DNA during replication, leading to inhibition of DNA replication, cell cycle arrest and cell death (Miao *et al.,* 2019; Pandit & Royzen, 2022). Prior studies in the Staples laboratory demonstrated that MRNIP loss leads to Gemcitabine resistance. To confirm this phenotype, MTT-based growth assays were conducted in WT and MRNIP KO HeLa and HCT116 cell lines. Indeed, following Gemcitabine treatment, MRNIP KO cells of both backgrounds exhibited increased survival relative to their WT counterparts (Figure 5). There are potential issues with the use of the MTT reagent to assess cell viability, since MTT does not measure cell survival *per se*, but instead reacts with reduced NAD and NADP generated by metabolic activity. Clonogenic assays should be considered to confirm effects on cell survival. One possible explanation for the decrease in Gemcitabine sensitivity of MRNIP KO cells is the potentially enhanced ability of MRE11 to remove Gemcitabine from the nascent DNA in the absence of its negative regulator MRNIP, thus facilitating the continuity of DNA replication (Boeckemeier *et al.,* 2020; Oh & Symington, 2018; Staples *et al.,* 2016). Another possibility is that the absence of MRNIP leads to enhanced MRE11-dependent resection of post-replicative gaps following repriming, which is interesting in the light of recent evidence that the extent of resection at such sites licenses error-free gap-filling by template switching (Gyüre *et al*., 2023; T. Zhang *et al*., 2023). Similarly, PRIMPOL is also reported to incorporate Gemcitabine *in vitro* (Quinet *et al*., 2020; Tokarsky *et al*., 2017). At high Gemcitabine doses, PRIMPOL may be more likely to incorporate the NA into nascent DNA. This may lead to successive cycles of repriming and stalling, leading to the generation of short DNA fragments

peppered with ssDNA gaps. These short fragments would be unlikely to template switch to fill in the gaps generated, and this may delay ssDNA gap filling until G2, when TLS is the predominant mechanism (Kohn *et al*., 2009). As such, it is possible that CTNA incorporation and repriming suppresses TS. TLS can be a pathological process, in which mutagenesis occurs and may offer cancer cells opportunity to mutate and evolve (Gao *et al*., 2016). In MRNIP KO cells, it is possible that hyperactive MRE11 not only removes Gemcitabine, but also digests these short DNA fragments, allowing the cell to employ template switching as the predominant mechanism to repair post replicative gaps and continue DNA replication and survive. This is speculative and testing this hypothesis will require extensive experimental follow-up.

MRNIP was also depleted in U2OS cells via siRNA. PRIMPOL KO cells were used to assess the potential role of PRIMPOL in regulating Gemcitabine sensitivity. However, the findings contradicted previously observed phenotypes, as MRNIP-depleted cells were more sensitive to Gemcitabine relative to controls (Figure 6). One possible explanation for this discrepancy is that the observed phenotype is cell-type specific and is not universal for all cancer cell types. Another possible explanation is that the MRNIP siRNA used was not specific, causing increased cell death in these cells due to an off-target effect. MRNIP knockdown was confirmed by Western blotting, although it is possible that complete loss of MRNIP is required to reveal the Gemcitabine resistance phenotype. PRIMPOL KO cells demonstrated increased survival compared to WT cells treated with Gemcitabine, suggesting that repriming has a negative effect in WT cells. Although this experiment requires confirmation, it supports the hypothesis that repriming and stalling cycles could repress TS and thus impact survival. SMARCAL1 KO cells were also used, to test whether fork-reversal plays a role in the cellular response to Gemcitabine. Fork reversal is a mechanism often used by cells to protect nascent DNA and continue DNA replication in the presence of an obstacle, where the nascent strands are remodelled into a structure similar to a Holliday junction, and proteins such as *BRCA1*, *BRCA2* and *RAD51* limit resection of the DNA strands (Qiu *et al*., 2021; Tian *et al*., 2021; Zellweger *et al*., 2015). SMARCAL1 KO cells demonstrate a reduced survival in response to Gemcitabine therapy. This may represent a role for fork reversal in the Gemcitabine response – perhaps nucleolytic removal of Gemcitabine occurs at the end of the regressed nascent DNA arms of reversed forks, although limited data is available on this. This could be further assessed by depletion of the fork reversal factors ZRANB3, HTLF and FBH1 (Fugger *et al.,* 2015; C. E. Moore *et al.,* 2023; Poole & Cortez, 2017; Qiu *et al.,* 2021).

Failure to close repriming-dependent post-replicative gaps in a timely manner results in genome instability and reduced viability (Cong *et al*., 2021; Panzarino *et al*., 2021). To test

this hypothesis, the immunofluorescent detection of *γH2AX* and RAD51 was used as markers of DNA damage. PRIMPOL was depleted via siRNA to assess the role of repriming on Gemcitabine-induced DNA damage levels in WT and MRNIP KO cells. PRIMPOL depletion resulted in a decrease in DNA damage in WT cells treated with Gemcitabine (Figure 7). Conversely, in MRNIP KO cells, PRIMPOL depletion resulted in a moderate increase in DNA damage. This experiment was only performed once, and thus required confirmation. Nonetheless, the data is consistent with a model in which progressive cycles of repriming, Gemcitabine incorporation and fork stalling leads to excessive DNA gapping and inefficient post-replicative repair, which is relieved by excessive nucleolytic activity in MRNIP KO cells. Of course, other mechanisms are also possible, though none can be sufficiently explored via simple immunofluorescence experiments, that is limited in that the methodology does not directly assess effects on nascent DNA. To explore the mechanisms involved further, and test our hypotheses, one would conduct a series of S1 nuclease-linked DNA fibre assays in Gemcitabine-treated WT and MRNIP KO cells depleted of PRIMPOL or post-replicative gap-filling factors such as UBC13, which mediates TS, and RAD18 and REV3L which mediate TLS, allowing visualisation of which factors affect cell survival (Bellí *et al*., 2022; Hawks *et al*., 2023; Yang *et al*., 2015).

Many cancer therapies inhibit tumour growth at least in part by impeding DNA replication. Low doses of Gemcitabine are reported to effectively inhibit global DNA replication in cancer cell (Pandit & Royzen, 2022). To test the effect of Gemcitabine on DNA replication in MRNIP KO cells, a denaturing immunofluorescence experiment was performed, using a short pulse of the halogenated nucleotide analogue IdU to mark active replication sites. The percentage of MRNIP KO cells replicating following treatment with Gemcitabine was very similar to that of untreated cells, while WT cells treated with Gemcitabine displayed a significant reduction in replicating cells (Figure 8). This supports the hypothesis that MRNIP KO cells continue to replicate in the presence of Gemcitabine. This experimental finding might be predicted based on the prior observation that MRNIP KO cells continue to grow following Gemcitabine treatment, and indeed all Gemcitabine-resistant cell models might be expected to express this phenotype. However, additional work performed by the Staples laboratory implicates the TLS polymerase REV3L in suppression of the ssDNA and DNA damage in Gemcitabine-treated MRNIP KO cells, implying the presence of a specific resistance mechanism related to post-replicative gap filling. To explore the suspected connection between replication and MRNIP KO Gemcitabine resistance, a more in-depth study is required assessing the contribution of TLS polymerases and TS/TLS factors to Gemcitabine resistance, ssDNA gap suppression and cell survival in MRNIP KO models. Recent literature suggests UBC13 is important in repair of post replicative gaps via template switching during S phase, while TLS is the predominant

factor for repair during G2 phase (Hawks *et al*., 2023). This may suggest a cell cycle phase specific mechanism for MRNIP KO cell survival.

Mass spectrometry analysis suggests that MRNIP is phosphorylated at S217 (Staples laboratory, unpublished data). A polyclonal antibody against phospho-S217 was raised. A MRNIP-specific band was unable to be detected in whole cell extracts. Following target enrichment via immunoprecipitation of FLAG-tagged MRNIP, a band of the correct molecular weight was observed, although this band was present in all samples including the FLAG-S217A, which is unphosphorylatable (Figure 9B). Without a MRNIP KO sample as a control, it is difficult to determine whether the band observed is MRNIP, and the validity of this antibody is unknown. This experiment requires further optimisation to improve its validity, though more recent work by the Staples laboratory using FLAG-MRNIP-expressing lines has led to the successful validation of this antibody.

Previous observations in the Staples lab suggested a role for MRNIP Serine 217 phosphorylation in the replication stress response. These results demonstrated that relative to WT MRNIP, a S217 alanine substitution mutant does not promote Gemcitabine sensitivity when expressed in MRNIP KO cells (Staples laboratory, unpublished data). To confirm this phenotype, an MTT-based growth assay was carried out. As previously reported, MRNIP KO cells expressing FLAG-S217A MRNIP displayed a similar Gemcitabine resistance profile to MRNIP KO cells, while MRNIP KO cells expressing FLAG-WT MRNIP were sensitised to Gemcitabine to a similar extent to WT cells (Figure 10A). A weakness in this experiment is that the modification of S217 may not be phosphorylation, as it is possible that a different post-translational modification occurs at this site, such as ADP-ribosylation, acetylation or sulfation, or it is possible that serine substitution directly affects MRNIP function independently of post-translational modifications of this site. One method via which one might address this is via the use of a phosphomimetic mutant, with Serine217 replaced by the negatively charged aspartate or glutamate amino acids. Phenotypic comparison of cells expressing WT or S217D/E will confirm whether a negative charge at this site is important for MRNIP function and eliminate other possibilities. Initial attempts to generate this line consistently failed, suggesting that S217D/E expression may be cytotoxic. A more in-depth study is required to fully elucidate the functional importance of this phosphorylation site. An important first step would be to identify the kinase responsible for S217 phosphorylation. This could be accomplished via western blotting using a working phospho-S217 antibody following treatment with a range of kinase inhibitors. Given that S217 is a proline-directed phosphorylation site, initial studies would centre around inhibition of MAPKs and CDKs, against which numerous specific inhibitors exist. Analysis of the sequence adjacent to the S217 site of the MRNIP protein via kinase prediction software revealed a range of kinases theoretically capable of S217 phosphorylation, including

many MAPKs and CDKs (Figure 17) (*Kinase Prediction,* n.d.). CDK7 was among the highest likely candidates to phosphorylate S217, which is interesting because previous studies show that inhibition of CDK7 synergises with Gemcitabine treatment to decrease Gemcitabine resistance (Zeng *et al*., 2022). Interestingly, CDK2 downregulation has been shown to decrease Gemcitabine resistance in some pancreatic cancer backgrounds (Panebianco *et al*., 2021). Although these findings are not related to MRNIP, it may suggest a starting point of looking at CDK2 over CDK7 as a kinase, as this would correlate with the resistance pattern seen in MRNIP KO cells (Figure 10).

Furthermore, a series of experiments should be conducted to determine under which conditions S217 phosphorylation is stimulated. This could involve treatment with genotoxins such as Gemcitabine or Olaparib, as well as cell cycle block and release assays to assess S217 phosphorylation during cell cycle transit (Nocodazole, Thymidine or Lovastatin block).

The frequency of cells exhibiting the DNA damage markers *γH2AX* and *RAD51* were assessed via immunofluorescence in MRNIP KO cells expressing WT MRNIP or an alanine mutant of S217. Expression of FLAG-WT MRNIP in MRNIP KO cells led to an increase in DNA damage markers following Gemcitabine treatment. MRNIP KO cells expressing FLAG-S217A MRNIP displayed a similar level of DNA damage in untreated and Gemcitabine-treated conditions, similar to MRNIP KO cells (Figure 11). This suggests that S217 phosphorylation plays a role in regulating Gemcitabine-induced DNA damage. The rescue of the phenotype in cells expressing FLAG-WT MRNIP demonstrates that the effects seen are due to MRNIP loss, rather than an off-target CRISPR effect.

The cytotoxic mechanism of nucleoside analogues is not universal. While many NAs cause cancer cell death via incorporation into the DNA, other NAs act via different mechanisms (Jones *et al*., 2014; Miao *et al*., 2019; Pandit & Royzen, 2022; Robak *et al*., 2009). To test the response of MRNIP KO cell to NAs with different mechanisms of action, cells were treated with Clofarabine, Fludarabine and Cytarabine, and compared our findings to those observed following Gemcitabine treatment. Cytarabine acts in a similar way to Gemcitabine, in that it causes chain termination, although Cytarabine does not share the RNR inhibition function of Gemcitabine. Cytarabine is incorporated into the nascent strand of the DNA, where it inhibits DNA replication, leading to cell cycle arrest and cell death (Faruqi & Tadi, 2022; Marcogliese & Yee, 2017). MRNIP KO cells are resistant to both Gemcitabine and Cytarabine (Figure 12C and D). Although in this experiment, the differential survival of WT and MRNIP KO cells is not statistically significant, the data shows a trend of resistance to Clofarabine. This finding needs

to be confirmed, and the assay optimised to improve reproducibility. However, other work performed in the laboratory confirms that MRNIP KO cells are resistant to both Cytarabine and Gemcitabine. Given that Cytarabine does not inhibit RNR, this work suggests that the resistance of MRNIP KO cells to Gemcitabine is related to its chain termination activity, and not its ability to inhibit RNR. Clofarabine and Fludarabine have different mechanisms of action than Gemcitabine and are less efficiently incorporated into nascent DNA, instead acting via inhibition of DNA polymerase and RNR. (Lech-Maranda *et al*., 2009; Robak *et al*., 2009; Zhenchuk *et al*., 2009). This leads to disruption of cellular nucleotide pools, causing fork stalling and cell death. Clofarabine and Fludarabine are more potent RNR inhibitors than Gemcitabine. The sensitivity of MRNIP KO cells to Clofarabine and Fludarabine suggests that MRNIP promotes resistance to nucleotide pool disruption (Figure 12A and B). This is supported by previous observations in the Staples lab demonstrating that MRNIP KO cells are sensitive to hydroxyurea, a potent RNR inhibitor (Agrawal *et al*., 2014; Bennett *et al*., 2020).

Following previous observations that repriming has a possible role in modulation of Gemcitabine sensitivity in WT cells, other NAs were tested as well. PRIMPOL was depleted in HeLa cells, and treated cells with the NAs Clofarabine, Cytarabine and Fludarabine, as well as Gemcitabine as a control experiment (Figure 13). The results were not conclusive, with PRIMPOL depleted samples displaying a similar phenotype to controls. Clofarabine-treated WT cells depleted of PRIMPOL displayed a mild reduction in survival relative to controls (Figure 13A). This may suggest that repriming is beneficial for the cell following Clofarabine treatment. MRNIP KO cells depleted of PRIMPOL display an increase in survival relative to controls. Although this result appears interesting, these observations may be due to poor growth of cells following depletion of particular targets. Given that all samples are normalised to untreated controls for each siRNA used, drastically altered baselines values are likely to affect the range of attainable values following genotoxic challenge. The significance of the result is difficult to determine and requires confirmation. Fludarabine chemosensitivity was unaltered by PRIMPOL depletion (Figure 13B). This could be a consequence of partial PRIMPOL depletion, highlighting the importance of confirmation of target depletion via Western Blotting and the use of positive controls. Gemcitabine-treated cells also displayed similar survival regardless of PRIMPOL depletion (Figure 13C). Further experiments are required to test the role of PRIMPOL in CTNA resistance in MRNIP KO cells, including the use of a positive control for successful disruption of PRIMPOL functionality.

Olaparib is a PARP inhibitor (PARPi). PARP enzymes play many distinct roles within the cell, and the functions of PARP1 include SSB repair, Okazaki fragment processing and replication fork reversal. Therefore, by inhibiting PARP, all these functions are affected, leading to increased generation of ssDNA gaps, elevated frequency of SSBs and increased replication

fork progression. Previously in the Staples laboratory, results demonstrated that MRNIP KO cells are sensitive to treatment with Olaparib. To confirm this phenotype, an MTT based assay was employed to measure WT and MRNIP KO HeLa and HCT116 cell survival following Olaparib treatment. MRNIP KO cells of both backgrounds displayed reduced cell survival relative to their WT counterparts (Figure 14). A weakness in this experiment is that under normal circumstances, PARP enzymes use NADH in the cell for ribosylation of ADP (Navas & Carnero, 2021; Nosseri *et al.*, 1994; Shockett & Stavnezer, 1993; L. Wang *et al.*, 2022). An MTT assay is based on the cellular ability to reduce MTT through NAD-dependent oxidoreductases, and therefore PARP inhibition may alter the NADH and NADPH levels, and thus the ability of the cell to metabolise MTT, causing the results to be less representative of survival alone (Mills & Allison, 1990; Surin *et al.*, 2017; L. Wang *et al.*, 2022). To mitigate this, clonogenic survival assays should be employed, as they directly measure cell survival. In *BRCA*-deficient cells, PARPi causes an accumulation of ssDNA gaps (Simoneau *et al*., 2021). PARPi also leads to ssDNA gaps by inhibiting Okazaki fragment processing, and also inhibits the repair of SSBs through inhibition of PARylation of target proteins to recruit DNA repair factors to sites of SSBs (El-Khamisy *et al.*, 2003; van Wietmarschen & Nussenzweig, 2018). SSB accumulation has been shown to cause cell death, due to the inherent instability of ssDNA and the possibility of conversion of SSBs to DSBs during later stages of the cell cycle (Bryant *et al*., 2005; Cong *et al*., 2021; Farmer *et al*., 2005; Panzarino *et al*., 2021). Furthermore, clustered SSBs can be treated as DSBs by the cell. These factors may be of relevance in MRNIP KO cells. However, these may cause different problems in MRNIP KO cells, such as hyperactive MRE11 preventing timely closure of post-replicative ssDNA gaps, leading eventually to cell death (Goulooze *et al.*, 2016; Javle & Curtin, 2011; Murai *et al.*, 2012; Panzarino *et al.*, 2021). Persistent DNA gaps may be filled by erroneous mechanisms in later stages of the cell cycle, such as TLS. Although TLS is a DNA repair mechanism, the error-prone nature may introduce mutations into the genetic code.

DNA damage markers were surveyed to assess whether MRNIP loss leads to an increase in DNA damage in response to Olaparib treatment. MRNIP KO cells treated with Olaparib demonstrated an increase in DNA damage relative to untreated cells (Figure 15). In WT cells, the increase in DNA damage was less marked. This suggests that MRNIP KO cells suffer increased DNA damage in response to Olaparib treatment relative to WT cells. Although this finding agrees with the observation that MRNIP KO cells exhibit reduced survival in response to Olaparib treatment, it does not explain the underlying mechanism, and again this requires a more in-depth study. DNA fibre assay experiments would once again be beneficial, as they would allow visualisation of the progress of the replication fork and may demonstrate the problems which arise in MRNIP KO cells as a result of Olaparib treatment. MRNIP KO cells treated with Olaparib and depleted of PRIMPOL displayed a decrease in DNA damage relative

to controls, suggesting a role for repriming in the damage caused. This increase in DNA damage may be consequent to the altered processing of repriming-induced ssDNA gaps in MRNIP KO cells. Damage levels in WT cells remained relatively constant from untreated to treated cells whether depleted of PRIMPOL or not, suggesting that in WT cells repriming does not lead to excessive DNA damage. This experiment again requires confirmation before any final assumptions can be made, but may indicate that repriming is a beneficial mechanism (as expected), unless key gap protective or nuclease regulatory factors are lost.

Given the role of Serine 217 phosphorylation in Gemcitabine chemoresistance, the role of S217 in Olaparib-treated cells was explored, again employing immunofluorescence to examine the prevalence of the DNA damage markers γH2AX and *RAD51*. MRNIP KO cells expressing FLAG-S217 alanine and FLAG-WT MRNIP were treated with Olaparib. FLAG-S217A cells displayed an increase in DNA damage following Olaparib treatment, similar to the MRNIP KO cell response to PARP inhibition. Cells expressing FLAG-WT MRNIP displayed similar levels of DNA damage in treated and untreated samples (Figure 16). This supports the earlier hypothesis that S217 site phosphorylation is important for MRNIP function. Though this is again limited in what it demonstrates mechanistically, as the experiment is confined to DNA damage levels, it does show that the function of the S217 site phosphorylation is not specific to the Gemcitabine response and does play a broader role in MRNIP function. Figure 11C and Figure 16 C display different baseline levels of RAD51 foci in MRNIP KO cells as well as the FLAG-WT MRNIP cells. This is a source of error in the data and may suggest the results are not significant. However, it is possible this discrepancy is due to the stock of cells used, as some may have a higher level of baseline damage due to their storage or passage level. It is also possible that the staining was more successful on one experiment than the other, meaning RAD51 foci were more visible and able to be counted. The significance of this difference requires further examination to determine the validity of the results.

4.1 Future Experiments

Due to time constraints faced during this project, many experiments could not be conducted, and there are many future experiments to be considered. As the accuracy of MTT assays to determine cell survival in response to treatment is questionable, in any future experiments conducted, MTT assays would be confirmed with clonogenic survival assays. Another important aspect would be to confirm all the current experiments by conducting repeats, to assess the reproducibility of each result. The exploration of the role of repriming in cellular response to Gemcitabine would be instrumental in understanding the mechanism by which MRNIP KO cells are resistant. This could be conducted by more MTT or clonogenic assay experiments using siRNA to knockdown PRIMPOL. Another focal point of future directions of the project would be the study of the significance of the S217 site phosphorylation on MRNIP

function. This would include perfecting the use of the phosphorylated S217 antibody by optimisation of Western Blotting or Immunoprecipitation experiments, or employment of a new, more accurate antibody, to show a clear phosphorylated MRNIP band. This would be followed by an experiment with a panel of kinase inhibitors to determine which is/are responsible for S217 phosphorylation and understand how the role of any kinases identified fits with the cellular response to different cytotoxic treatments. The investigation of how MRNIP cells replicate in the presence of Gemcitabine is another area which requires further study. This would be done via loss of function experiments targeting TLS polymerases and other TLS/TS factors. Further studies on different nucleoside analogues could also be conducted, including more survival assays to understand whether repriming has a role in the cellular response to these agents.

5. Conclusion

The purpose of the investigation was to explore the functions of the MRNIP protein in cellular response to various chemotherapeutic agents. Previous work on MRNIP in the Staples lab demonstrated that loss of MRNIP causes resistance to Gemcitabine but sensitises certain cancer cell lines to Olaparib treatment. Survival assays completed during this project confirm these findings, and assays assessing DNA damage markers suggest a protective role for MRNIP. The findings demonstrate that MRNIP status alters chemosensitivity in a lesion specific manner and suggests that a more in-depth study of the role of MRNIP in cancer is warranted. Due to time constraints of the project, there are many future experiments that were not able to be completed, which would be beneficial to further the understanding of MRNIP function. One of the main areas to explore would be to assess the role of repriming in the cellular response to Gemcitabine. To combat the unreliability of MTT assays faced during this project, confirmation of the results via clonogenic survival assays would be an important factor. Many of the experiments conducted also require confirmation, which would increase their reliability and credibility. Another direction of the project would be to explore the phosphorylation of MRNIP S217 in more detail, and the role of the MRNIP kinase in regulating the replication stress and DNA damage responses.

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