INVESTIGATING THE ROLE OF NSMCE2 IN CELL CYCLE PROGRESSION AND GENOME MAINTENANCE

Lily Michelle Dixon BSc (Hons)
Medical Sciences MSc by Research
Supervised by Dr Elaine Taylor

Lancaster Medical School
Faculty of Health and Medicine
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I, Lily Dixon, confirm that the work presented in this thesis is my own and has not been submitted in substantially the same form for the award of a higher degree elsewhere. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

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Abstract

Preserving genome integrity is vital for protection against the development and progression of cancer. The SMC5/6 complex is an evolutionarily conserved protein complex thought to be involved in this process, specifically its SUMO ligase NSMCE2 subunit. Prior to this thesis, CRISPR-Cas-9 mutant NSMCE2 cell lines were produced; NSMCE2 knockout, NSMCE2 knockout with wildtype NSMCE2 re-expression and NSMCE2 knockout cells with ligase-dead NSMCE2 expression. The purpose of this research was to characterize these cell lines with the aim of suggesting possible roles for NSMCE2. Both the NSMCE2 knockout and NSMCE2 ligase-dead cell lines demonstrated proliferation defects and slowed S phase progression by FACS analysis, with the defect exacerbated in the ligase-dead cells. The remainder of this project investigated the cause of these defects, determining that it is not due to the activation of the replication checkpoint, although the ligase-dead cell lines did activate the S phase DNA damage checkpoint. Based on what is already known about NSMCE2 function, it is suggested that the most likely cause of the proliferation defects is un-sensed, unresolved replication stress characteristics. Whilst other proteins may compensate for the total loss of NSMCE2, the ligase-dead cell line is expected to be the result of a dominant-negative mutation, which may explain the more pronounced S phase defect. The NSMCE2 ligase-dead cell lines also demonstrate a >4N DNA content, so it is speculated that unresolved replication stress characteristics may also cause segregation difficulties in this cell line. However, immunofluorescence and decatenation inhibitor treatment has shown that NSMCE2 may instead or also be involved in activating the decatenation checkpoint to allow for efficient segregation. A bioSUMO method for isolation of specific SUMOylation targets has also been developed with the aim to analyse specific NSMCE2 modifications within the future.

Table of Contents

List of Figures

Fig 1 Diagram illustrating the hallmarks of cancer

Fig 2 Diagram illustrating the two main hypotheses for the involvement of genome instability in the initiation of cancer formation

Fig 3 Diagram illustrating the classical DNA damage pathway

Fig 4 Diagram demonstrating the recruitment of DNA-PK, ATM and ATR in the DNA damage response

Fig 5 Diagram illustrating the downstream signalling of p53

Fig 6 Diagram illustrating the inactive pre-replication complex

Fig 7 Diagram illustrating the bi-directional movement of replication forks and the overall process of eukaryotic replication throughout the cell cycle

Fig 8 Diagram illustrating some of the causes of replication stress

Fig 9 Diagram demonstrating the activation of ATR-ATRIP following replication stress

Fig 10 Diagram demonstrating the activation of Chk1 by ATR through interactions with And-1 and Claspin

Fig 43 bioSUMO strategy western blot of MRC5 cells transfected with eiter pbio-V or pbio-SUMO1 incubated with anti-biotin HRP antibody

Fig 44 bioSUMO strategy western blot of MRC5 cells transfected with eiter pbio-V or pbio-SUMO1 incubated with anti-RanGAP antibody

List of Tables

Abbreviations

AND-1	Acidic nucleoplasmic DNA-binding protein 1
ATM	Ataxia-telangiectasia mutated
ATMi	Ataxia-telangiectasia mutated inhibitor
ATR	Ataxia-telangiectasia and Rad3-related protein
ATRIP	ATR interacting protein
BRCA1	Breast cancer type 1 susceptibility protein
BER	Base excision repair
BLM	Bloom syndrome protein
Cas9	CRISPR associated protein 9
CDK	Cyclin dependent kinase
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CPT	Camptothecin
CRISPR	Clustered regularly interspaced short palindromic repeats
DAPI	4'6-diamidino-2-phenylindole
DDR	DNA Damage response
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dsDNA	Double strand DNA
RanGAP1	Ran GTPase-activating protein 1
ECL	Enhanced chemiluminescence
FACS	Fluorescence activated cell sorting
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Genome instability
H2AX	H2A (Histone family member X)
HR	Homologous recombination
HU	Hydroxyurea
kDa	kilodalton
LOH	Loss of heterozygosity
MCM	Minichromosome maintenance protein complex
MMR	Mismatch repair
RS	Replication stress
MRN	Mre11-Rad50-Nbs1 protein complex
NCS	Neocarzinostatin
NDRG1	N-Myc Downstream regulated 1
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NSMCE	Non-structural maintenance of chromosomes element
ORC	Origin of replication complex
p53	Phophoprotein p53
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PIAS	Protein inhibitor of activated STAT
RGS GAP	Regulator of G-protein signalling GTPase accelerating protein
Pre-IC	Pre-initiation complex
Pre-RC	Pre-replication complex
RAD50	DNA repair protein RAD50
RAD51	DNA repair protein RAD51 homolog 1
RAD52	DNA repair protein RAD52 homolog
RANBP2	RAN Binding protein 2
rDNA	Ribosomal DNA
REV1	DNA repair protein REV1
RNA	Ribonucleic acid
RPA	Replication protein A
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDSA	Synthesis dependent stand annealing
SENP	Sentrin-specific protease
Sgs1	Slow growth suppressor 1
SIM	SUMO interacting motif
SMARCAL1	SWI/SNP-related matrix associated actin-dependent regulator of chromatin subfamily A-like protein 1
SMC	Structural maintenance of chromosomes

SP-RING	Siz/PIAS RING
ssDNA	Single Stranded DNA
SUMO	Small ubiquitin-like modifier
TOPBP1	DNA topoisomerase 2-binding protein 1
TopoII	Human topoisomerase II
TRAX	Translin-associated protein X
TRF1	Telomeric repeat-binding factor 1
TRF2	Telomeric repeat-binding factor 2
WRN	Werner syndrome RecQ like helicase

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I would also like to thank my parents, boyfriend and close friends who have supported me throughout this entire process. This accomplishment would not have been possible without them.

Chapter 1: Introduction

1.1 Overview of Cancer

Cancer is a collection of over 200 different life-changing diseases, affecting different tissues and cell types, all characterized by dysregulated cell growth and division, and usually, their ability to spread around the body. Most people within the UK will be affected in some way throughout their lives, with 1 in every 2 people receiving a cancer diagnosis (Cancer Research UK, 2014). For this reason, it is important that every effort is made to learn more about cancer formation and progression in order to support the development of new methods for prevention and treatment.

Cancer cells all share a variety of characteristics which inherently define them as cancerous. Originally, six hallmarks of cancer were described; self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis (See Fig. 1A) (Hanahan and Weinberg, 2000). More recently this list has been expanded to include two further emerging hallmarks which are involved in the pathogenesis of cancer (see Fig.1B). These are: deregulating cellular energetics, by which cancer cells reprogram their glucose metabolism to rely on aerobic glycolysis; and avoiding immune destruction, by which solid tumours have evolved to avoid cell death by becoming undetectable to all arms of the immune system. As well as emerging hallmarks, the list has also been expanded to include two enabling characteristics; genome instability and mutation, which drives genotypic changes that underpin phenotypic alterations in cell behavior; and tumour promoting inflammation, that provides the perfect microenvironment for cancer development through the supply of growth factors and mutagenic molecules for example (Hanahan and Weinberg, 2011).
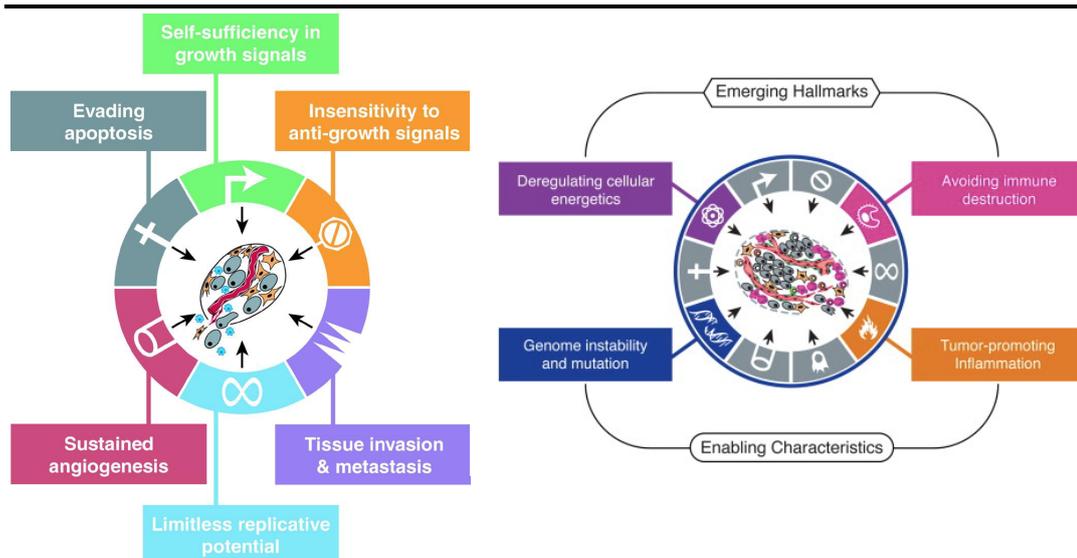
Figure 1 – A -The original six hallmarks of cancer. Cells acquire these capabilities to allow the formation of malignant tumours. Figure taken from (Hanahan and Weinberg, 2000). B- The further four hallmarks of cancer; two emerging hallmarks involved in the pathogenesis of cancer, and two enabling characteristics which allow and encourage the development of the other hallmarks. Figure taken from (Hanahan and Weinberg, 2011).

One of the key characteristics of a malignant tumour is its ability to proliferate with little to no control. This is highlighted by the fact that three of the six original cancer hallmarks describe the ways in which cells achieve this. During this dysregulated proliferation, tumour cells often experience high levels of replication stress (RS) (Gaillard et al., 2015). Therefore, cancer cells must be able to tolerate this RS in order to continue growing and dividing. Due to this reliance on RS tolerance mechanisms for cancer cell survival, there is significant interest in targeting these activities as a potential treatment strategy. For this reason, a key aim of this project is to characterize a poorly understood aspect of the RS response, with the goal of identifying new possibilities for therapeutic intervention.

1.2 Genome instability

Genome instability (GI), defined as an increased chance of genetic changes during cell growth and division, is a key feature of nearly all cancers. When cells are genetically unstable, they are more likely to acquire mutations which drive the development of additional cancer hallmarks, giving cancer cells a growth advantage and driving cancer progression (Yao and Dai, 2014).

There are two key hypotheses which associate GI with cancer formation; the mutator hypothesis and the oncogene-induced DNA replication stress model (OIRS) (see Fig. 2). The mutator hypothesis states that GI is an initiating feature of precancerous lesions, that causes an increase in spontaneous mutations, driving the development of the other hallmarks of cancer (Loeb, 1991). There is substantial evidence in support of this model in the case of hereditary cancers, where an inherited mutation, in a DNA repair gene for example, causes the initial genome instability allowing more mutations, and so, further cancer characteristics to arise (Loeb, 2016). Good examples of this include inherited mutations in nucleotide excision repair (NER) genes found in patients with Xeroderma pigmentosum (Cleaver, 1968), a genetic disease causing dyspigmentation of the skin and a predisposition to skin cancer. These patients develop skin cancer at much younger ages than the general population due to their mutated NER genes, which cause increased sensitivity to UV light, allowing further mutations to arise (Lambert and Lambert Muriel, 2014). The mutator hypothesis is also supported in the development of hereditary non-polyposis colorectal cancer, where affected patients have an inherited mutation in a single copy of a mismatch repair (MMR) gene. Somatic inactivation of the other MMR gene copy results in genetic instability, encouraging the development of colorectal (and other forms of) cancer (Peltomäki, 2001). Similarly, colorectal cancer can also be developed as a consequence of mutations in the proof reading catalytic subunits of DNA polymerases ε and δ, which also leads to the genome instability (Palles et al., 2012).

By contrast, the oncogene-induced DNA replication stress model claims that, in most cancer cases, it is a sporadic mutation within an oncogene or tumour suppressor which initiates and drives the cancer formation through increased cell proliferation. This uncontrolled and increased proliferation causes increased RS which in turn leads to increased GI (Yao and Dai, 2014). In precancerous lesions, this increased RS and associated damage triggers the DNA damage response (DDR), activating p53 and causing cell cycle arrest, senescence or apoptosis. p53 acts as a tumourigenesis barrier preventing cancer development. However, ongoing RS and GI provides a continual pressure for p53 mutation, which would lead to the evasion of cell death (see Fig. 2) and impair the ability of the cell to repair damage. This explains why p53 mutations

are so common in cancer cells, as a mutation allows cells to avoid cell death following RS and GI, and so continue proliferating and generating further cancer hallmarks (Halazonetis et al., 2008).
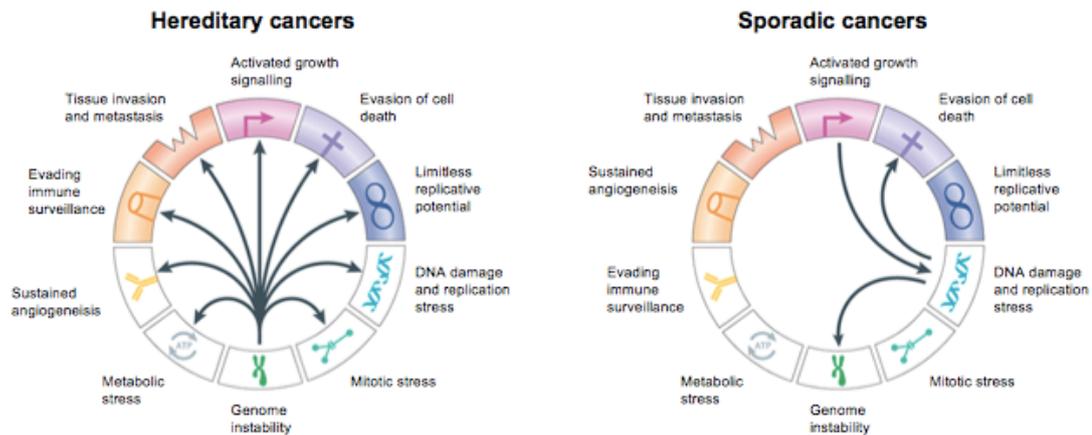
Figure 2 - Diagrammatic representation of the two main hypotheses describing how genome instability is involved in the initiation of cancer formation. Figure adapted from (Negrini et al., 2010) by Dr Elaine Taylor

1.3 The DNA damage response pathway

The ability to deal with DNA damage effectively is vital for avoiding the harmful effects of RS and GI which drive carcinogenesis (Pause et al., 2003). DNA is constantly subject to damage, not only as a consequence of RS but also from free radical species, which are produced as a by-product of normal metabolic processes within the cell (Valko et al., 2006) as well as from exposure to various exogenous sources of damage such as ultraviolet light (which causes damage both directly and by the production of genetically damaging reactive oxygen species) (Schuch et al., 2017), ionizing radiation (Ward, 1988) and chemical carcinogens (Reviewed in: (Jackson and Bartek, 2009)

The human body has evolved a complex network of pathways which it uses to deal with the various forms of DNA damage and alterations which result from these different damaging processes. In essence, the DNA damage response (DDR) is initiated by sensory molecules that each detect different types of DNA lesion and, through the action of specific transducer molecules, signal the presence of this DNA damage to a range of effector molecules (Fig. 3). These effectors trigger a number of cellular

responses to limit the consequences of this DNA damage. These responses include repair, cell cycle arrest, senescence or apoptosis (This pathway is reviewed in (Harper and Elledge, 2007)).
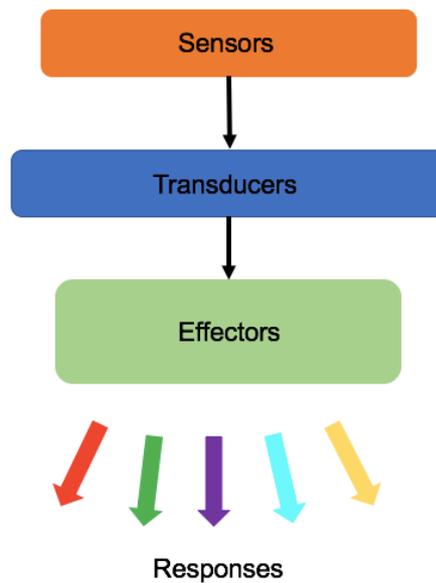
Figure 3 -The classical DNA damage response signalling pathway involving the 3 key molecules, sensors, transducers and effectors. Sensors sense the different forms of DNA damage within the cell before passing the information to transducers which can recruit many different effectors. The effectors can then carry out the response necessary to combat the damage formed (Reviewed in (Harper and Elledge, 2007).

Like many other pathways within the cell, the DDR is controlled by phosphorylation. Two key molecules which act early in these signaling pathways, as sensors, are the phosphatidyl-inositol 3-OH kinase-like kinases, ATM (Ataxia telangiectasia mutated) and ATR (ATM and Rad Related) (Savitsky et al., 1995; Bentley et al., 1996). These kinases must be closely controlled in order to prevent dysregulated activation which would cause untimely cell cycle effects. For this reason, both ATM and ATR need specific co-factors for their recruitment and activation at sites of damage. For ATM this is the MRN complex, made up of MRE11, RAD50 and NBS1, whereas ATR requires ATRIP (Falck et al., 2005). Although there is some overlap between the roles of ATM and ATR in the DDR, ATR is predominantly activated by ssDNA, coated with Replication Protein A (RPA), that is generated by many different types of DNA damage, especially

during DNA replication, whilst ATM is largely activated by the presence of double strand breaks (DSBs) (Zou and Elledge, 2003; Shiloh, 2003). Another key protein kinase involved in the early stages of the DNA damage response is the DNA-dependent Protein Kinase (DNA-PK). This kinase requires the co-factor Ku to become activated following DSBs (Dvir et al., 1992) (Gottlieb and Jackson, 1993). The recruitment and activation of these three important kinases is illustrated in Fig. 4.
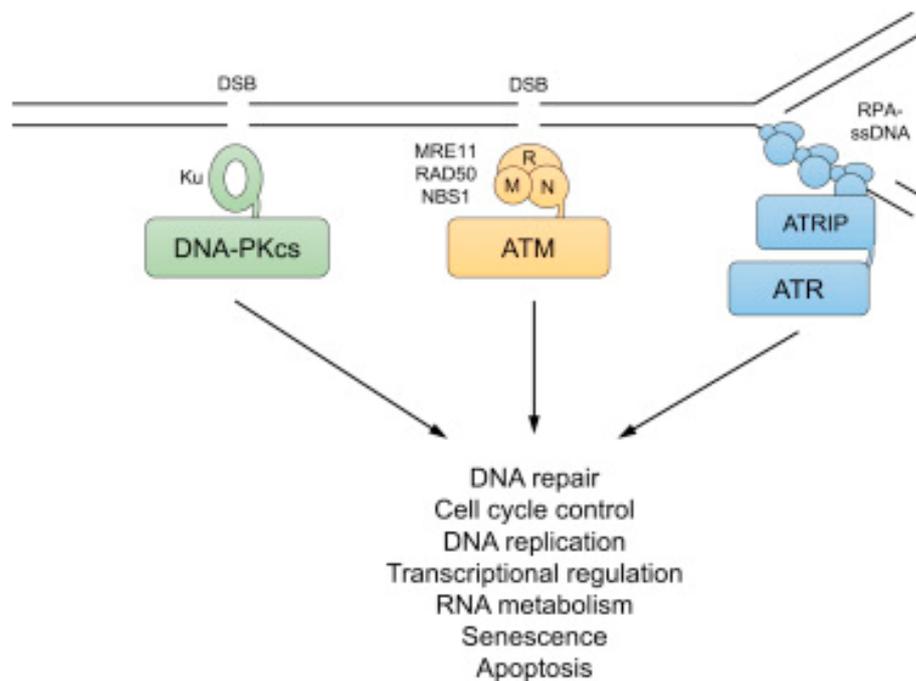
Figure 4 – Diagrammatic model of the recruitment and activation of the three key kinases in the early DNA damage response; ATR, ATM and DNA-dependent protein kinase (DNA-PK) Figure taken from (Blackford and Jackson, 2017).

Following the activation of these early acting kinases of the DDR, there are over 700 phosphorylated transducer and effector proteins that can, in turn, be activated (Matsuoka et al., 2007), allowing this network to become very complex and give a wide range of specific responses. Two of these transducer/effector proteins, which are found to work in close partnership with ATM and ATR, are the secondary kinases Chk1 and Chk2. ATM is normally seen to interact with Chk2, and ATR with Chk1, however crosstalk and some function crossover has been documented (Bartek and Lukas, 2003).

The activation of either Chk1 and Chk2 are essential to achieve cell cycle arrest. They both activate checkpoints within the cell cycle, allowing the cell extra time to deal with any issues faced during replication or following DNA damage. Chk1 is identified as the main activator of the S-phase replication checkpoint, through phosphorylation and inactivation of replication proteins, when cells have encountered problems during DNA replication. This halts replication until the issues have been dealt with, such as by preventing late origins from firing so that resources can be directed to the replication forks which need it, and ensuring that the cells do not enter G2 or mitosis until replication has been completed (González Besteiro and Gottifredi, 2015). Chk2, unlike Chk1, activates the G1/S checkpoint as well as the G2/M checkpoint, by phosphorylation of different targets in response to double strand breaks. For example, Chk2 can activate the G1/S checkpoint by phosphorylating p53 and activate the G2/M checkpoint by phosphorylating CDC25c (Chehab et al., 2000) (Matsuoka et al., 1998).

Following cell cycle arrest, if there is any damage to the DNA, DNA repair pathways are activated to restore genome integrity. There are several different types of repair pathway which can be undertaken in response to DNA damage signaling depending on the type of damage incurred, including; base excision repair (BER), nucleotide excision repair (NER), ICL repair, SSBR, and repair of DSBs by repair homologous recombination (HR) or non-homologous end joining (NHEJ) (reviewed in: (Giglia-Mari et al., 2011) and (Caldecott, 2008).

Ultimately, if the genetic damage cannot be repaired or the repair machinery becomes overwhelmed by the quantity of DNA damage, cells undergo cell death by activating a pathway such as apoptosis. Following damage, p53 is signaled to activate the transcription of both anti-apoptotic/repair genes (see Fig. 5) as well as pro-apoptotic genes. As the repair machinery cannot deal with the damage any longer, p53 allows for more pro-apoptotic gene transcription than repair gene and so causes the activation of the death receptor and consequently apoptosis (Roos and Kaina, 2013).
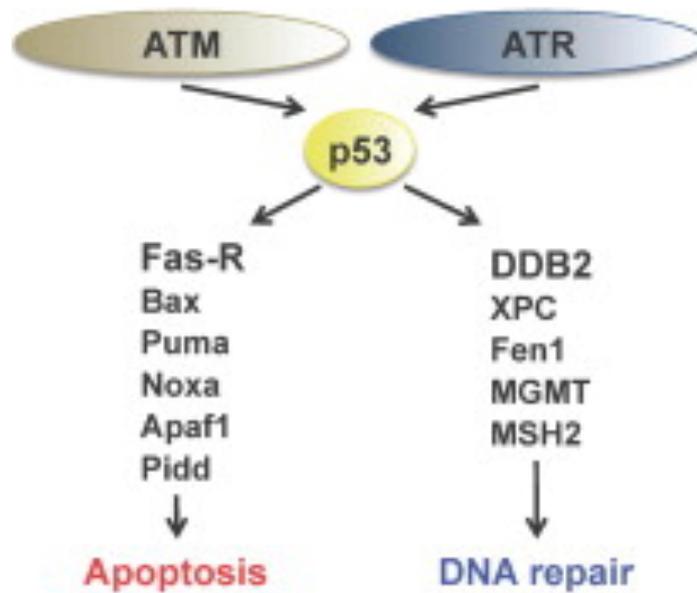
Figure 5 – The downstream signalling of DNA Damage to p53. p53 induces the transcription of both pro-apoptotic and DNA repair genes. Once the repair machinery becomes overwhelmed, the balance is shifted towards pro-apoptotic protein production causing cell death to occur. Figure taken from (Roos and Kaina, 2013).

1.4 Eukaryotic DNA replication

When cells divide, they must accurately copy millions of individual base pairs. This is a very complex process and as such, has the possibility to become faulty and malfunction. To prevent this, cells have developed an intricate mechanism to tightly coordinate DNA replication, to minimize errors and avoid genome instability.

In eukaryotes, replication of the entire genome occurs exactly once during S phase in each cell cycle in each cell. To ensure that replication of large eukaryotic genomes occurs quickly and efficiently, DNA replication occurs in parallel at many sites along the DNA, with replication forks proceeding in a bi-direction manner, copying the DNA until they reach another fork. The locations on eukaryotic DNA where replication begins are called origins. Activation of replication origins is tightly controlled and occurs as a two-step process, origin licensing and replication initiation. During G1, origin licensing takes place as the pre-replication complex is assembled at the origin. The pre-RC is made up of the Origin recognition complex (ORC), Cdc6, Cdt1 and the MCM2-7 replicative helicase (See Fig. 6). At this point, the pre-RC is in an inactive state and DNA replication will not begin unless it becomes activated through the initiation

step (Reviewed in: (Tanaka and Araki, 2010; Bell and Dutta, 2002)). Many more origins are licensed for replication than those that actually fire in S phase. The ones which do not fire are named dormant origin sites and these origins are reserved in case there are problems in S-phase and replication can then begin from a different site (Blow and Dutta, 2005).
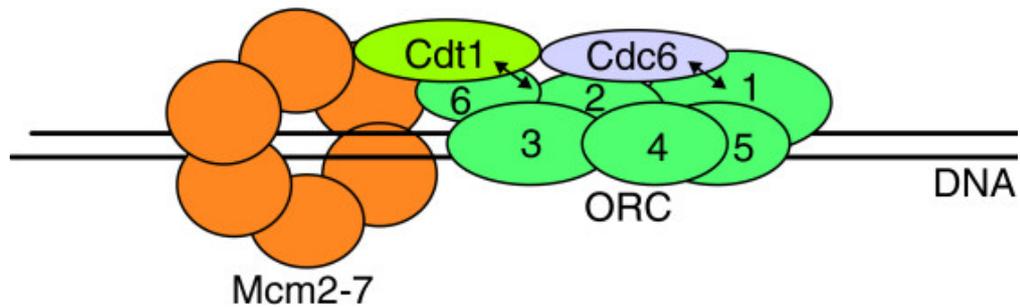
Figure 6 – The inactive pre-replication complex as found on eukaryotic dsDNA origins, made up of Mcm2-7, Origin recognition complex, cdc6 and cdt1. Figure taken from (Duncker et al., 2009).

Activation of the pre-RC to form the pre-IC begins in a cell cycle dependent manner which

ensures the binding of a growing list of proteins, including Cdc45, GINS, Sld3, Sld2 and Dpb11, to the replication origins. The pre-IC can then be activated, forming an active replication complex, following helicase activation and the recruitment of DNA polymerases, allowing the replication forks to begin moving, bi-directionally, away from the origins, with MCM2-7 slightly ahead to open the dsDNA (see Fig. 7A) (Remus and Diffley, 2009; Méchali, 2010; Fragkos et al., 2015). Once the replication forks have moved away from their origins, they continue replicating the genome until they reach another replication fork. This occurs until the entire genome has been replicated (see Fig. 7B) (Gaillard et al., 2015).
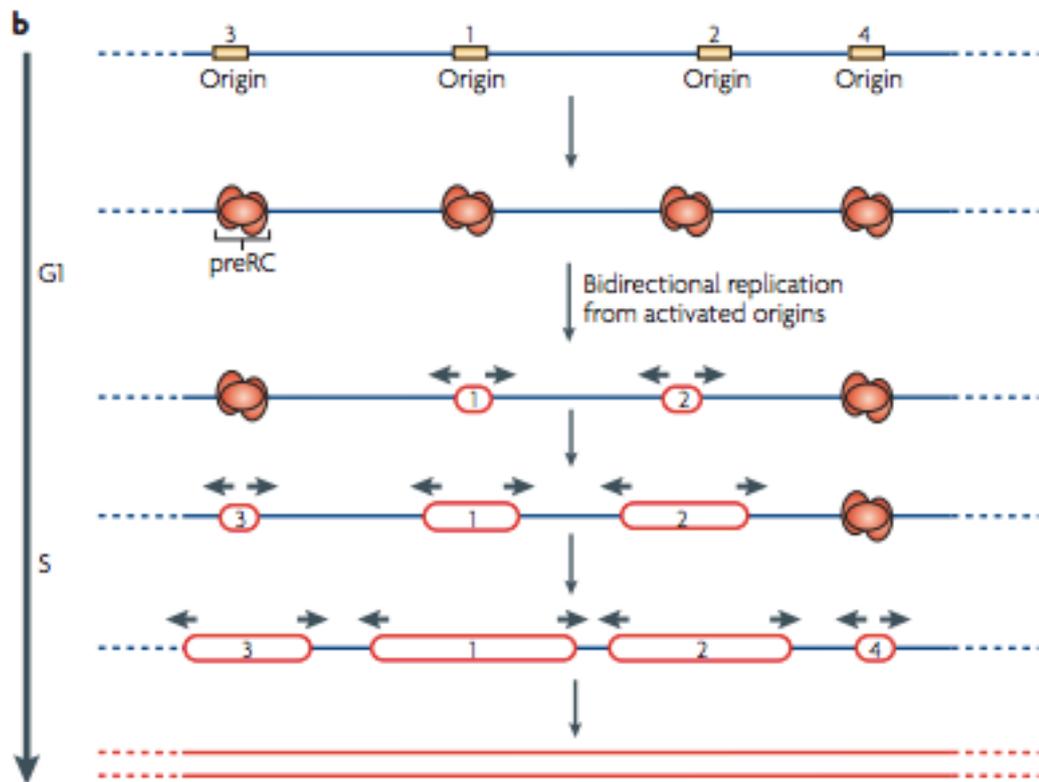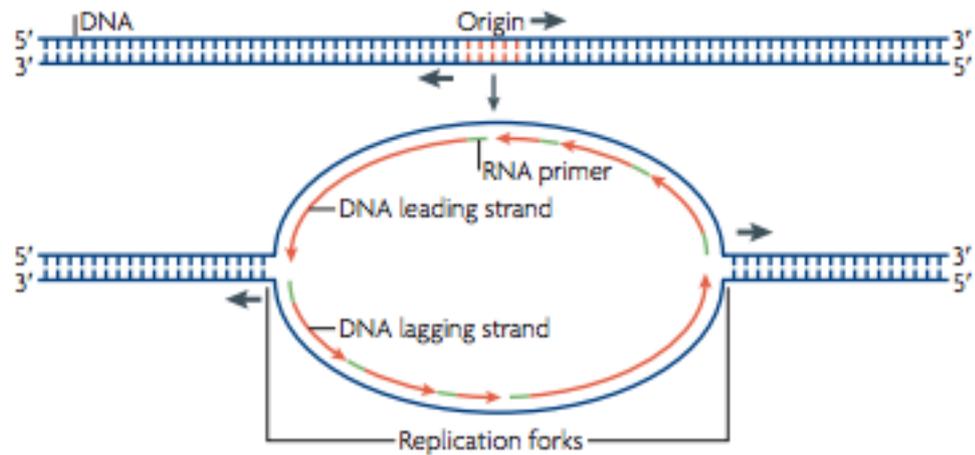
Figure 7 -A) Diagrammatic representation of two replication forks moving of bi-directionally on eukaryotic DNA at one replication origin. B) The overall process of eukaryotic DNA replication through the cell cycle, from replication origin licensing during G1 to firing and replication of the whole genome during S phase. Figure taken from (Méchali, 2010)

1.5 Replication Stress

The complex process of DNA replication is susceptible to problems such as the slowing, stalling or even collapse of replication forks, termed replication stress (Zeman and Cimprich, 2014). There are many replication challenges which cause RS to arise during

normal replication. These include physical blocks to the replication fork, lack of replication resources, collisions between the replication fork and transcriptional machinery, and dysregulated origin use (Mazouzi et al., 2014). Many of these impediments can be seen in Fig. 8.
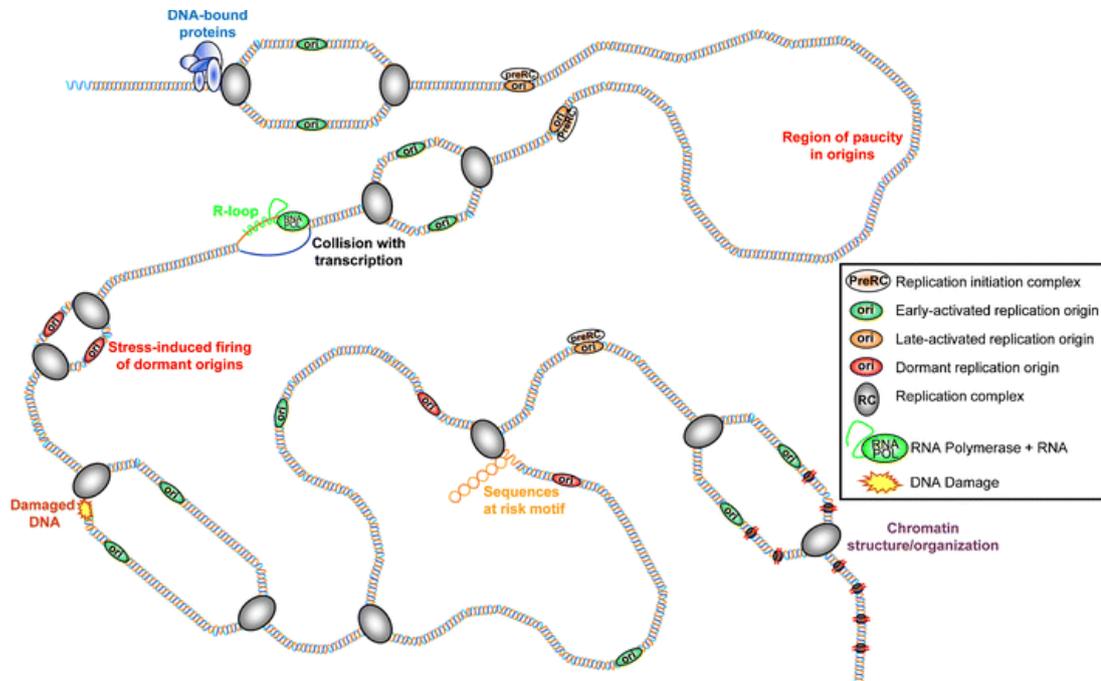
Figure 8 – Overview of some of the barriers faced by the replication forks during DNA replication which can cause replication stress. Figure taken from (Lambert and Carr, 2013).

A physical obstacle within the genome can be extremely detrimental for replication. There are many different types of physical blocks to the progress of a replication fork, a key one being DNA lesions resulting from exogenous or endogenous damage (Lambert and Carr, 2013). Another form of physical obstacle faced by the replication machinery is protein-bound DNA. The helicases found in the replication complex struggle to unwind the dsDNA when it is protein-bound, causing the stalling and possible dissociation of the replication complex (Brüning et al., 2014). Secondary structures, commonly found within DNA, are also difficult for the replication machinery to pass through. For this reason, common fragile sites (due to their high A/T content), trinucleotide repeats and GC-rich DNA are problematic due to their tendency to form structures such as hairpins and G-quadraplexes (Mazouzi et al., 2014; Zeman and Cimprich, 2014).

Similarly, RS can also be caused by a lack of replication resources such as nucleotides or components of the replication machinery. If this occurs, large exposed lengths of ssDNA will occur as the cells run out of the raw materials needed to continue replicating. The importance of this replication hindrance has been shown through the ability to rescue RS by exogenously supplying nucleosides (Poli et al., 2012; Bester et al., 2011).

Conflicts between replication and other DNA associated processes can also lead to RS. For example, both the replication machinery and the transcription machinery are competing for the same dsDNA template, both unwinding the DNA, and carrying out their individual roles. Although coordinated closely, they are likely to come into contact with each other on occasions leading to replication fork stalling. It is thought that the stalling could occur due to head on collisions between the machinery but it has been shown that even before the two collide, there is an increase in RS and fork stalling due to their topological effects, such as the positive supercoiling, that they both create (Bermejo et al., 2012). Collisions are particularly likely when transcribing long genes in human cells as transcription can lead into a second cell cycle, where replication has begun again. When the machinery collides and replication is halted, R-loops, interactions between DNA and RNA, can form (as shown in Fig. 8). This can lead to severe genetic damage such as DSB's as RNA displaces the template DNA strand, causing further RS and obstruction to the replication machinery (Helmrich et al., 2011)

Unregulated origin usage can also influence RS. To prevent re-replication, origins are usually unable to be licensed after the start of S-phase, even if a problem arises during replication. This means that there would be no replacement replication machinery if a fork were to collapse, and many sections of DNA would be left un-replicated. To avoid this situation, cells normally license more origins than they fire at the start of S phase, leaving many origins lying dormant unless they are required. However, this can lead to RS if not appropriately regulated, for example, if too many or too few origins are fired or if origins are able to re-fire (Alver et al., 2014). If, at the start of S phase, not enough origins are fired for replication, then isolated replication forks will arise. These forks would need to travel further, increasing the likelihood of stalling and possibly

collapsing. If not enough origins have been licensed either, this would be a genetic catastrophe as there would be fewer dormant origins to replace the stalled forks so the genome could not be entirely replicated. Further issues could arise following reduced origin firing as there would be an increased chance that the genome would only be part replicated before entering mitosis, even assuming no replication challenges occur. This would not only cause the loss of genetic material, but it would also mean that the sister chromatids will still be linked during cytokinesis so the DNA strands would break and not segregate equally (Hills and Diffley, 2014). If the opposite occurs and too many origins are fired, the replication machinery may be unable to continue replicating due to a shortage of replication substrates, such as nucleotides, as well as the increased likelihood of collisions between transcriptional and replication machinery, causing RS as described above (Beck et al., 2012; Jones et al., 2013). There is also the possibility that origins could re-fire. This dysregulated re-firing would lead to the detrimental replication consequences described if there were too many origins fired, as well as the possibility that the replication forks themselves could collide into each other as the forks would be found closer together (Taylor and Lindsay, 2015).

Oncogene-induced RS

By driving dysregulated cell proliferation, activation of oncogenes or inactivation of tumour suppressors can lead to RS by a variety of mechanisms. One key problem that arises from untimely and increased replication is a lack of nucleotides needed to keep up with this replication. This was demonstrated to be the case when the Cyclin E oncogene is activated, stimulating the Rb-E2F pathway which caused disturbed replication and RS. This RS phenotype could be reversed with the addition of exogenous nucleosides, establishing that it was the nucleosides which were the limiting factor and cause of the RS in this case (Bester et al., 2011). However, although this is one way by which oncogenes can cause RS, it is clear that this is not always the case. For example, Cyclin E overexpression can also cause RS by increasing origin firing. This can cause conflicts between the replication and transcription machinery as described above, leading to compromised fork progression (Jones et al., 2013). The MYC oncogene is one of the master regulators of the cell cycle, influencing the expression of many genes involved in proliferation such as cyclin dependent kinases

(CDKs). MYC overexpression is a common occurrence in many cancers and it has been shown to increase RS by increasing origin licensing density as well as altering the origin firing pattern such as by influencing replication timing and increasing the number of early-replicating origins (Srinivasan et al., 2013). Overexpression of the oncogenes RAS and MOS also produces RS by upregulating the expression of CDC6 and so causing an increase in active origins and replication forks, thereby resulting in the RS phenotypes described above (Di Micco et al., 2006; Bartkova et al., 2006). As well as resulting from oncogene activation, RS can also be a consequence of loss of the tumour suppressor gene p53. Where p53 is functional, even following over-expression of the replication initiation factors Cdt1 and cdc6, p53 can prevent re-replication from a single origin through the activation of p21. However, following p53 tumour suppressor inactivation, as found in many cancer cells, re-replication of the genome is permitted, and so RS occurs at a much higher rate (Vaziri et al., 2003).

1.6 The replication stress response

Replication fork stalling following RS causes the uncoupling of the MCM helicase from the DNA polymerase upon the replication fork. To continue replication, the replication complex therefore needs to reform and it does so upon the activation of the replication checkpoint (Mourón et al., 2013). The uncoupling of the replication machinery is a vital signal for the activation of this replication checkpoint as it causes the unwinding of dsDNA to ssDNA behind the helicase but ahead of the stalled replication machinery. This region of ssDNA becomes coated in Replication Protein A (RPA), stabilizing the ssDNA and serving as the site of localisation and activation of ATR along with its interacting partner ATRIP (Zou and Elledge, 2003). It is the junction of ssDNA with dsDNA at the fork stall that is the vital structure required for the binding and activation of ATR-ATRIP, as ATRIP interacts with RPA. As well as the recruitment of ATR/ATRIP to the stalled forks, for complete activation of the replication checkpoint, both the RAD17-RFC and Rad9-Rad1-Hus1 (9-1-1) complexes need to be recruited to the site, with the RAD17-RCF clamp loader complex acting as a loader for the 9-1-1 complex onto stalled fork site (Ellison and Stillman, 2003). The loaded 9-1-1 complex is then vital for the activation of a further replication checkpoint protein, the

DNA topoisomerase II binding protein 1 (TOPBP1), which is initially recruited through the presence of ATRIP and the MRN complex (Duursma et al., 2013; Choi et al., 2010). It is currently thought that it is the N-terminus of TOPBP1 which stabilizes the entire checkpoint complex and stimulates the full kinase activity of ATR, allowing it to interact with its downstream effectors (Choi et al., 2010). The mechanism of ATR-ATRIP activation can be seen in Fig. 9, demonstrating the interdependency each of the checkpoint activation proteins has upon each other.
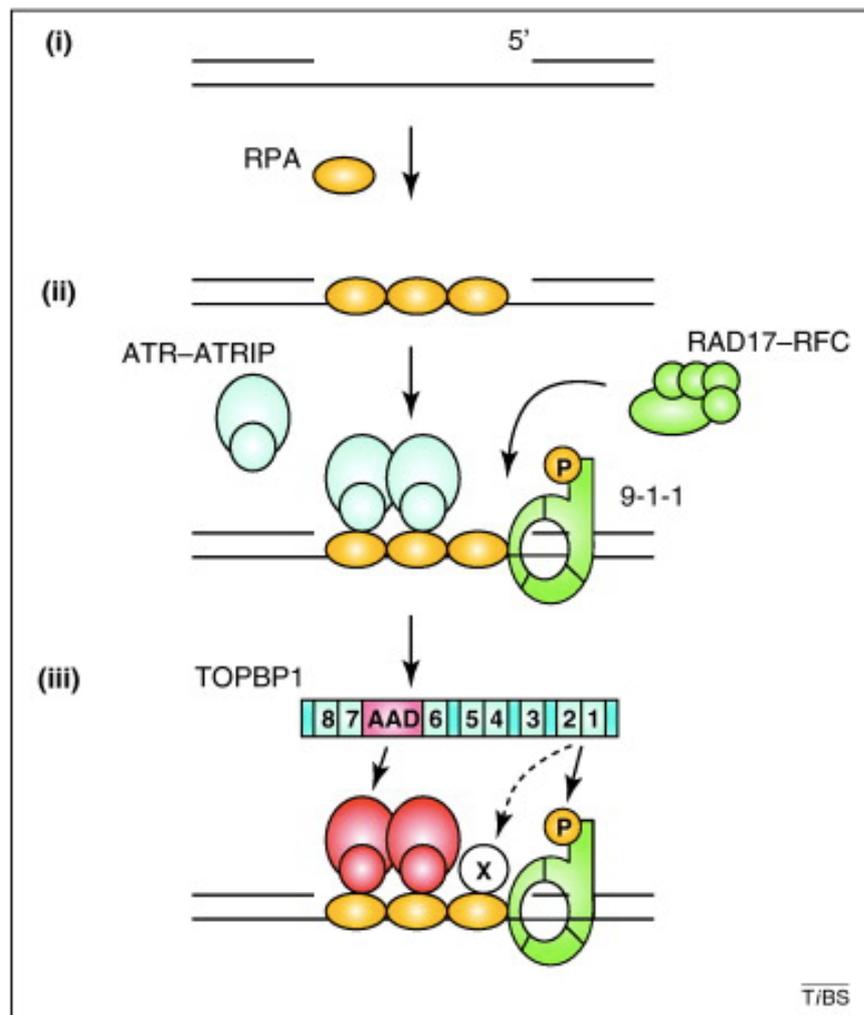
Figure 9 - The activation of ATR-ATRIP following replication stress. The activation of ATR requires all of the molecules shown in this diagram; Replication protein A (RPA, RAD17-RCF, The 9-1-1 complex, TOPBP1 and a possible unknown protein 'X'. Figure taken from (Flynn and Zou, 2011).

As discussed earlier, the activation of the downstream kinase Chk1 by ATR is an important element of the replication checkpoint. As with most biological processes, it is not as simple as ATR phosphorylating Chk1 alone, there are other vital proteins

required for this activation to occur. In this case, it has been found that the mammalian proteins, Timeless and Tipin, promote the phosphorylation of Chk1 by ATR through interactions with RPA on ssDNA and by promoting Claspin (Mrc1) protein activation which then binds to Chk1 (Kemp et al., 2010). Recently, it has been demonstrated that the And-1 protein is also involved in promoting Chk1 activation (see Fig. 10). At the site of RS, there is an increase in And-1 which becomes phosphorylated by ATR, allowing And-1 to interact with Claspin (Mrc1). This association is required for Chk1 to bind to Claspin, and so be phosphorylated by ATR. These interactions allow for efficient activation of Chk1 and so give rise to all of the replication checkpoint effects to be described below (Hao et al., 2015).
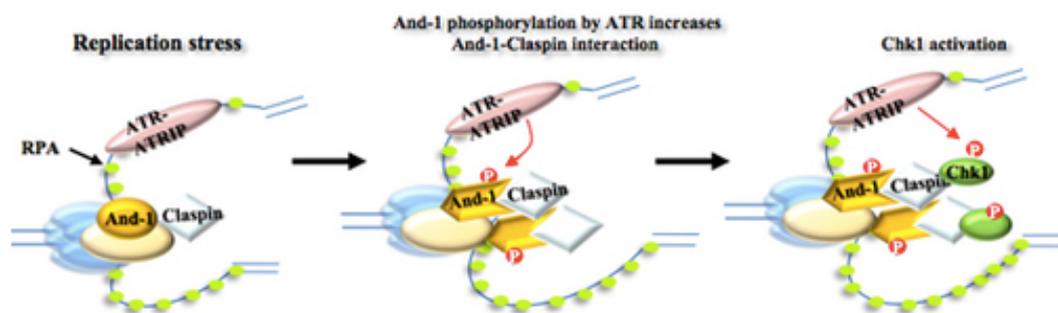
Figure 10 - Activation of Chk1 by ATR through interactions with And-1 and Claspin. Figure taken from (Hao et al., 2015)

Upon phosphorylation of Chk1, the S phase replication checkpoint can then be effected in full through phosphorylation of proteins which produce cell cycle arrest, reduced origin firing, replication fork stabilization, removal of the source of RS, and replication fork restart.

Importantly, to allow for cell cycle arrest and to inhibit origin firing, ATR/Chk1 quickly induces the degradation of Cdc25A, through phosphorylation, which causes the inhibition CDK2 which is required for entry into G2 (Sørensen et al., 2003). To ensure adequate time is given to allow recovery, ATR/Chk1 kinase activity also reduces the number of new active origins following RS through the inhibition of proteins involved in origin licensing, although some dormant origins near the site of RS are permitted to

fire to aid the DNA recovery (Ge and Blow, 2010). As well as allowing for cell cycle arrest, ATR must also act quickly to stabilize and promote recovery of the stalled fork, to reduce GI and ensure the fork does not collapse before recovery takes place. This is achieved by ATR-dependent phosphorylation of various recovery proteins including WRN, SMARCAL1 as well as the AND-1 protein discussed above, which prevent aberrant fork structures from arising (Ammazzalorso et al., 2010; Couch et al., 2013; Hao et al., 2015; Syljuåsen et al., 2005).

As the cell cycle becomes arrested and stalled forks become stabilized, fork recovery, removal of the RS source and repair of the replication stress feature can then begin. One of the key causes of RS can be the lack of nucleosides. The kinase activity of Chk1 activates E2F6 which leads to the activation of genes involved in nucleoside synthesis, removing the stress and so allowing replication to continue (Bertoli et al., 2013). RS is also often caused by physical blocks to the genome and so ATR/Chk1 activation provides the ability to activate the DNA damage response, to remove these blockages and restart the replication fork. These processes mainly occur through HR-dependent mechanisms which involve the DNA becoming reprimed, degraded or rearranged (fork reversal) in order to form a functioning replication fork once more. These techniques, shown in Fig. 11, are different depending on whether the replication challenge is found on the leading or lagging strand. Many of the HR mechanisms used to reform the replication fork on the leading strand (Figure 11A) do not actually repair any damage but simply tolerate and move around it. This is the case when the fork is reprimed and repaired (Figure 11A - 2b, 3c, 4c and 4b) and when fork reversal is carried out causing chicken foot RS intermediates (Figure 11A - 2b, 3d, 4d and 5d) (Li and Heyer, 2008). The involvement of ATR/Chk1 in the process of fork restart is vital, as a critical HR protein, Rad51, is directly activated by Chk1, whilst BRCA1 is activated by ATR (Sørensen et al., 2005; Tibbetts et al., 2000). Low-fidelity translesion synthesis polymerases such as POLH, POLI, and REV1, can also be used to tolerate the challenge rather than repair it, by moving through the lesion (Figure 11A - 3b) and replicating the DNA despite its complications (Sale et al., 2012). The most effective pathway allows for the repair of the initial replication block (Figure 11A - 1, 2a, 3a, 4a, 5a) through the cleavage of the stalled fork to form a double strand break. This break can

then also be repaired through HR (Li and Heyer, 2008), again stimulated through ATR and Chk1.

The pathways are marginally different when the blockage is found on the lagging-strand (Figure 11B), with all pathways beginning with re-initiation of synthesis downstream leaving a gap around the blockage (Figure 11B - 1) and ending with tolerance of the replication challenge (Figure 11B- 4). These mechanisms involve the use of a paranemic joint (Figure 11B - 3a), D-loop (Figure 11B- 2b and 3b) or translesion synthesis polymerases (Figure 11 - 2c) (Li and Heyer, 2008).
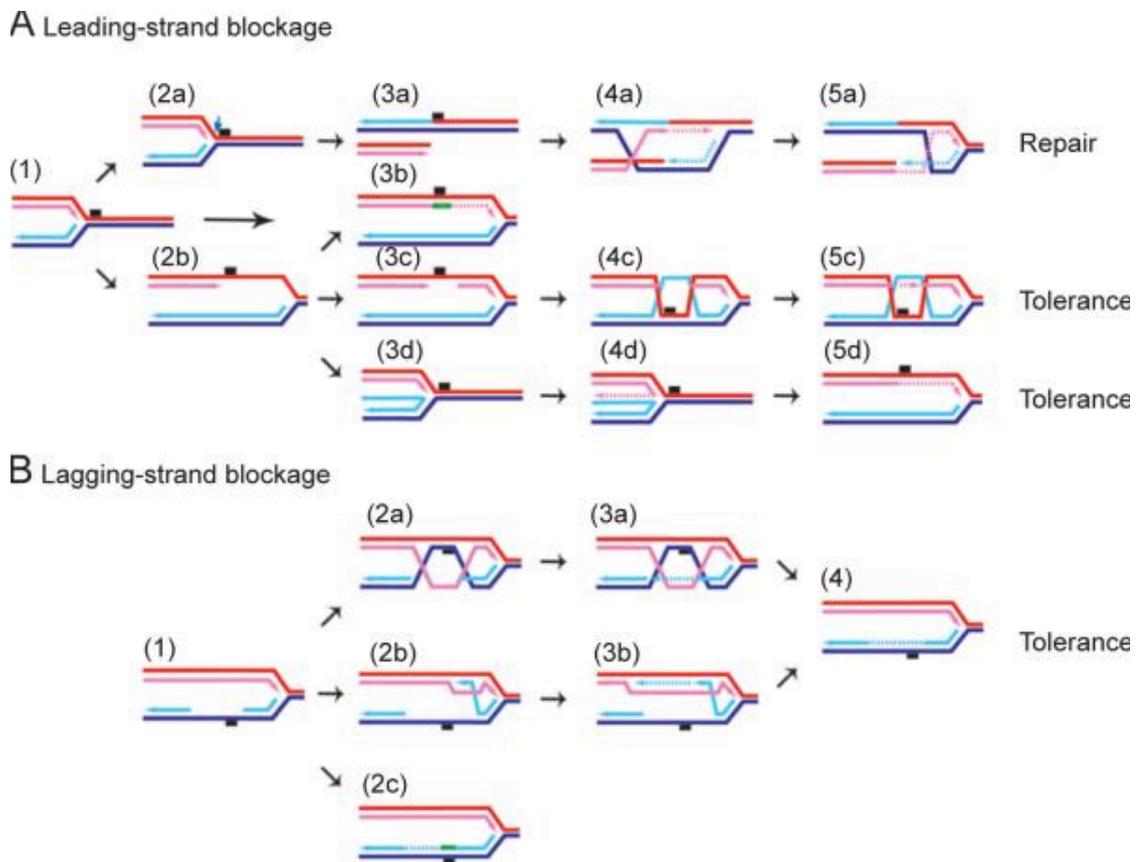
Figure 11 - Mechanisms of re-starting stalled or broken replication forks using homologous recombination pathways. This diagram shows the different pathways undertaken depending if the lesion is found within the leading or lagging strand during replication. Most of the pathways do not repair the damage found within the replication fork but simply tolerate it using homologous recombination mechanisms, through either repriming and repairing, fork reversal or the action of translesion synthesis polymerases (11A - 2b, 3c, 3d, 4c, 4d, 5c and 5d, 11B), however the replication blockage can be repaired through fork cleavage and homologous repair (11A 2a, 3a, 4a and 5a). Figure taken from (Li and Heyer, 2008).

As well as the fork reversal and re-priming mechanisms described above, an alternative mechanism using nucleases to degrade the replication challenge has a role in restarting stalled forks after genotoxic stress. DNA2 nuclease has been shown to degrade the reversed replication fork DNA and restart the replication machinery meaning no intermediates are formed (Thangavel et al., 2015).

Once the fork has been reformed through any of the mechanisms described, DNA replication can continue until the entire genome has been replicated.

If the stalled forks cannot be restarted, the replication fork will eventually collapse due to their unstable nature. If forks do collapse then double strand breaks frequently occur which can cause serious damage to the genetic material and possible cell death (Marians, 2000).

The three-stage checkpoint activated HR pathway is often used to repair these double strand breaks, as well as to remove byproducts and intermediates of RS such as intrastrand crosslinks and cruciform structures. The general process of HR when repairing a double strand break is shown in Fig. 12. As this figure shows, the process of repairing double strand breaks through HR can occur through three different forms; synthesis dependent strand annealing (SDSA), Break induced repair (BIR) or double strand break repair (DSBR). The majority of these repair mechanisms cause large sections of loss of heterozygosity (LOH) as a full replication fork is formed, but some DSBR products do demonstrate crossover products after Holliday junction formation (Li and Heyer, 2008). DSBs which occur following fork collapse are mainly repaired through BIR as they only possess one free DNA end, losing genetic material through LOH. However, if two collapses occur close together, DSBR mechanisms can be used to avoid these long LOH tracts (Llorente et al., 2008).
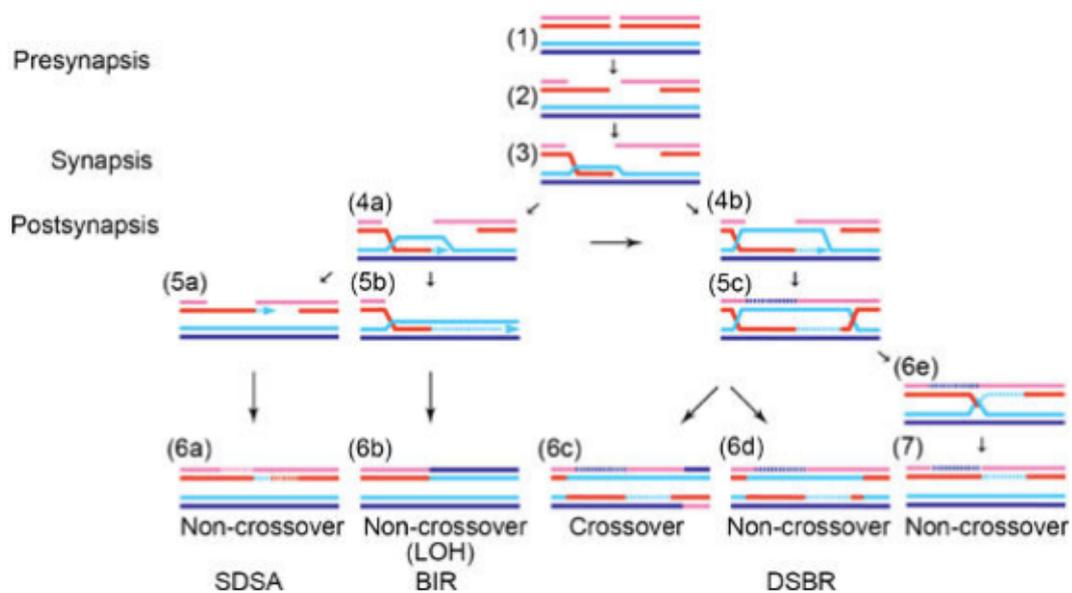
Figure 12 - Homologous recombination pathway following the occurrence of a double strand break formed following fork collapse. The pathway involves 3 key stages; presynapsis, synapsis and post synapsis, where the mechanism could take forms of homologous recombination; synthesis dependent strand annealing, break induced repair and double strand break repair. Figure taken from(Li and Heyer, 2008).

1.7 SMC proteins and the SMC5/6 complex

Structural maintenance of chromosomes (SMC) proteins are found in all forms of life from bacteria to humans. These essential proteins act as molecular DNA linkers, which organize the structure and function of chromosomes throughout the cell cycle, and are vital for the survival of many forms of life (Losada and Hirano, 2005).

SMC proteins are large polypeptides of up to 1300 amino acids in length, which share a characteristic domain structure. This comprises two nucleotide binding domains, one found at each the N and C terminal domains, separated by two extensive coiled coil domains and a flexible hinge region at the centre (Hirano, 2006). Each SMC protein folds at the central hinge region such that the coiled region forms antiparallel dimers and the Walker A and Walker B ATP binding and hydrolysis motifs within the terminal domains are brought together to form the ATP binding 'head' domain. The individual SMC proteins then come together to form dimers, interacting through the hinge

regions, allowing the formation of the well known V-shape, shown in Fig. 13 (Melby et al., 1998). In prokaryotes, these complexes are homodimers but in eukaroyotes, six SMC proteins interact in specific heterodimer combinations, in association with other complex components, to undertake vital chromosome organizational roles. These are cohesin, containing SMC1 and SMC3, condensin, containing SMC2 and SMC4 and the SMC5/6 complex, based on a heterodimer of SMC5 and SMC6 (Hirano, 2006). Both cohesin and condensin have the classic architectural V- shape (see Fig. 14). The primary function of cohesin is well established as the cohesion protein that links sister chromatids, and is therefore important for chromosome segregation in mitosis. It is also crucial for efficient DNA repair and controlling gene expression throughout the cell cycle (Peters et al., 2008). Condensins, of which there are two in eukaryotes, play a role in the overall shaping of eukaryotic chromosomes, organising the structure in chromosome assembly as well as in segregation throughout the stages of mitosis (Hirano, 2016).
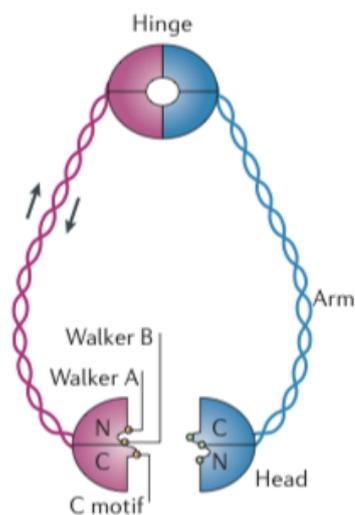
Figure 13 – Diagrammatic representation of the basic structure of an SMC dimer. The two monomers interact at the hinge region forming the well-known 'V shape' with antiparallel regions (indicated by the arrows) leading to the nucleotide binding head regions. Figure taken from (Hirano, 2006).
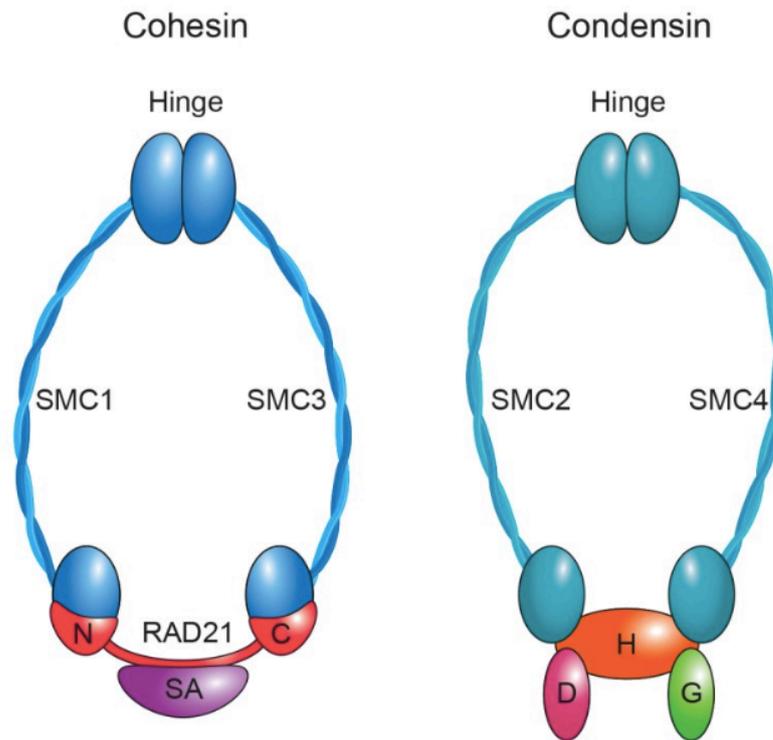
Figure 14 - Diagrammatic representation of the structure of the SMC protein complexes, Cohesin and Condensin. Both have the classic SMC dimer V-shape with the two SMC proteins interacting at the hinge region, as well as non-SMC subunits, such as the kleisin components, RAD21 in Cohesin and CAPD3 (H) in Condensin, which interact with the SMC proteins at the head domains to connect the two heads. Figure taken from (Yuen and Gerton, 2018)

The remaining SMC complex, SMC5/6, has a much less well known role in chromosome organization but is thought to be involved in RS recovery and repair. The first component of the SMC5/6 complex to be identified was the product of the *Schizosaccharomyces pombe (S. pombe) rad18* gene (Lehmann et al., 1995), later renamed *smc6* as sequence analysis revealed the Rad18/Smc6 protein to be a new member of the SMC family. *S. pombe* Smc6 was subsequently shown to be part of a larger complex comprising its heterodimeric SMC partner, Spr18/SMC5 along with six non-SMC element proteins (Fousteri and Lehmann, 2000) (McDonald et al., 2003). Of these, Nse1-4 were initially identified and were found to be highly conserved between both budding and fission yeast. Upon their discovery, it was first indicated that the complex may play an important role in DNA repair through sensitivity of yeast in SMC5/6 mutants to DNA damaging agents (Fujioka et al., 2002) (McDonald et al., 2003) (Pebernard et al., 2004) (Hu et al., 2005). The two further non-SMC complex components were then identified, Nse5 and Nse6, which were found to be essential in *S. pombe* but non-essential in *Saccharomyces cerevisiae (S. cerevisiae)* (Pebernard

et al., 2006) (Duan et al., 2009). The structure of the SMC5/6 complex in both yeast strains is shown in Fig. 15.
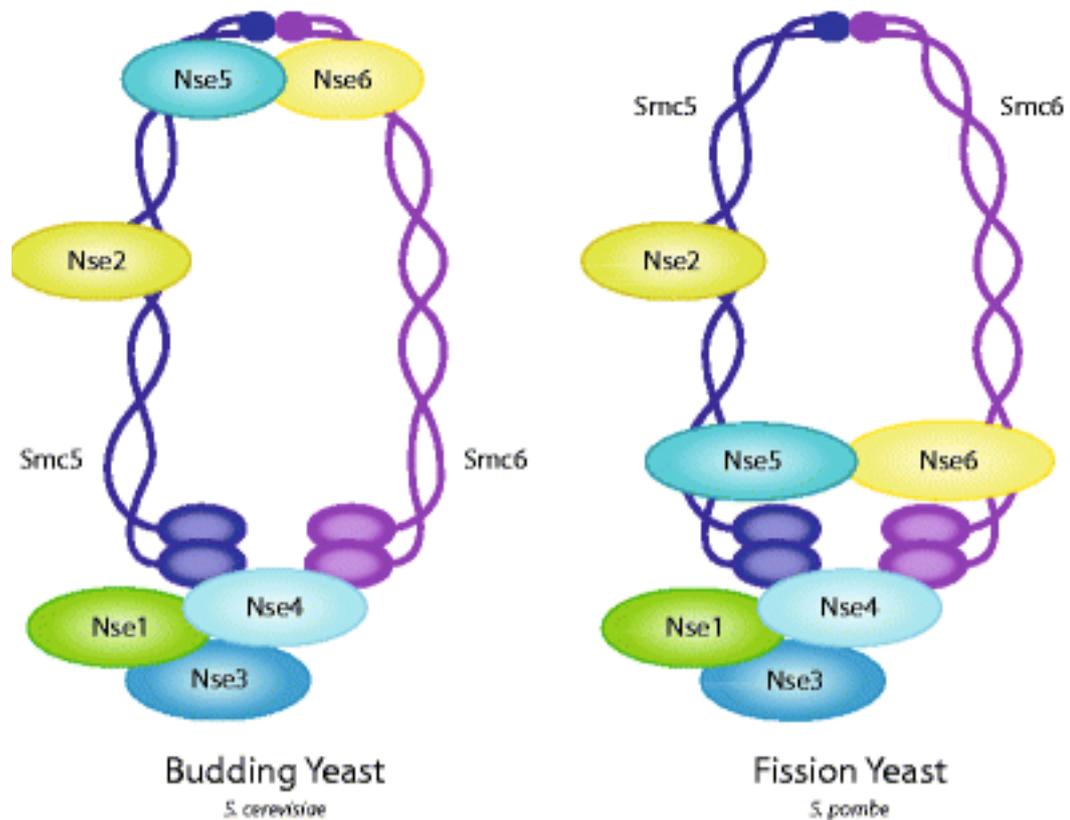
Figure 15 -Diagrammatic representation of the architecture of the yeast strains of the SMC5/6 complex. Figure taken from (Verver et al., 2016a)

As shown in Fig. 15, the yeast SMC5/6 complexes also have the characteristic V-shape expected from an SMC protein dimer, with Nse4 in a highly-conserved location as it bridges the gap between the head domains. The C-terminal domain of Nse4 directly interacts with the head of SMC5, much like the kleisin components, RAD21 and CAPD3 of Cohesin and Condensin (Palecek et al., 2006). Nse4 also directly binds to both Nse1 and Nse3 (Palecek et al., 2006; Pebernard et al., 2008). Nse3 contains a MAGE (melanoma-associated antigen gene) domain and it has been shown to enhance the activity of its binding partner Nse1, a ubiquitin ligase protein (Pebernard et al., 2004; Doyle et al., 2010). As well as the Nse1 ubiquitin ligase, the SMC5/6 complex has

another ligase function in Nse2, an E3 SUMO ligase. These ligase proteins are not found in other SMC complexes but are conserved in the SMC5/6 complex in all known organisms (McDonald et al., 2003). The positioning of NSMCE2 upon the coiled section of SMC5 is markedly different compared to the other non-SMC components of the other SMC complexes. The Nse5 and Nse6 components share little sequence homology between the two yeast species and were also found to be associated with different positions within the complex in the two yeasts, as shown in Fig. 15 (Pebernard et al., 2006) (Duan et al., 2009).

Following the discovery of each of the SMC5/6 components in the two yeast strains, human orthologs of each SMC5/6 complex component were also identified. The SMC6 and SMC5 genes and their protein products were identified relatively quickly (Taylor et al., 2001), followed by NSMCE1-4, the orthologs of Nse1-4 (Harvey et al., 2004; Potts and Yu, 2005; Taylor et al., 2008). Recently, the Nse5 and Nse6 equivalents, SLF1 and SLF2, were also discovered in human cells. These proteins have little homology with either of the yeast Nse5 and Nse6 proteins and they are not part of the core complex (Räschle et al., 2015). However, the ligase activities of both the NSMCE1 and NSMCE2 components have been confirmed in the human SMC5/6 complex, further indicating that they are evolutionarily and functionally important (Potts and Yu, 2005; Doyle et al., 2010). The likely structure of the SMC5/6 complex including its non-SMC proteins within humans can be seen in Fig. 16.
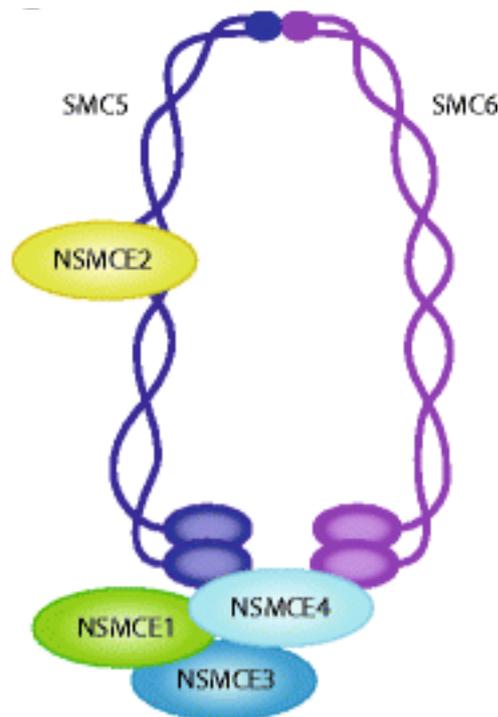
Figure 16 -Diagrammatic representation of the architecture of the SMC5/6 complex in mammals. Figure taken from (Verver et al., 2016a)

1.8 Functional roles of the SMC5/6 complex

The SMC5/6 complex has been associated with maintaining genome stability since the very beginning of its discovery. The initial functional work surrounding the complex was undertaken in yeast (*S. pombe and S. cerevisiae*) before being extended to mammalian cells and, more recently, to human patients.

Since the first discovery of Smc6 through complementation of the DNA damage sensitivity of a yeast mutant, studies of the SMC5/6 complex have tended to focus on its role in DNA repair (Lehmann et al., 1995). Initially, yeast mutants of SMC5/6 complex components were shown to be sensitive to a wide range of DNA damaging agents (Andrews et al., 2005; Verkade et al., 1999; Fujioka et al., 2002; McDonald et al., 2003; Pebernard et al., 2004; Hu et al., 2005) with genetic analysis in *S. pombe* suggesting a role in HR-dependent double strand break repair, along with RAD51 (McDonald et al., 2003; Harvey et al., 2004; Pebernard et al., 2004). Following this, studies in *S. cerevisiae* demonstrated that Myc-tagged SMC6 associated 5-7 fold more with the DNA regions flanking DSBs than when no breaks were present, further

supporting that the complex was involved in DSB repair (De Piccoli et al., 2006). Again, genetic analysis indicated that this involvement related to HR-dependent repair of dsbreaks (De Piccoli et al., 2006). This data was supported in NSMCE2 siRNA knockdown studies in human cells, where their ability to carry out HR was decreased but there was no effect upon NHEJ (Potts et al., 2006). Consistent with these results, analysis of mutant SMC5/6 complex strains of A. thaliana and DT40 chicken cells each confirmed that the SMC5/6 complex facilitates HR between sister chromatids (Watanabe et al., 2009; Stephan et al., 2011a).

Further analysis in yeast has revealed that the SMC5/6 complex plays a vital role when cells are subject to RS. This was initially discovered in *S. pombe* where analysis of *smc6* mutants indicated that the complex was vital in the resolution of DNA intermediate structures formed following replication fork collapse (Ampatzidou et al., 2006). It was suggested that the SMC5/6 complex may have two roles in RS recovery, both an early and late stage function. It is thought that once the stalled replication forks are stabilized, the early function of the complex is to restart the replication fork through the loading of Rad52 and RPA onto the chromatin, maintaining an appropriate configuration. The late stage function involves the resolution of collapsed fork intermediates and fork resetting through HR before replication can restart (Irmisch et al., 2009). These two functions, early and late, can be seen in Fig. 17. The late stage function was supported by studies in *S. cerevisiae Mms21/nse2* mutants, which demonstrated that the complex was involved with the resolution of recombination intermediates such as cruciform structures, often the result of collapsed replication forks, as part of the RS recovery (Branzei et al., 2006). Additionally, the early RS recovery function of the SMC5/6 initially found *in S. pombe* was later corroborated in *S. cerevisiae* (Bustard et al., 2012), where the complex was shown to be vital in preventing replication fork collapse after stalling and directly ensuring efficient restart.

Early function

Stalled replication fork (stable)

S phase check point Cds1

Late function

Collapsed fork

Replication Complex dissociates

Smc5/6 keeps stalled fork primed for restart

Strand invasion

Smc5/6 required for HJ resolution, fork resetting

Replication restart
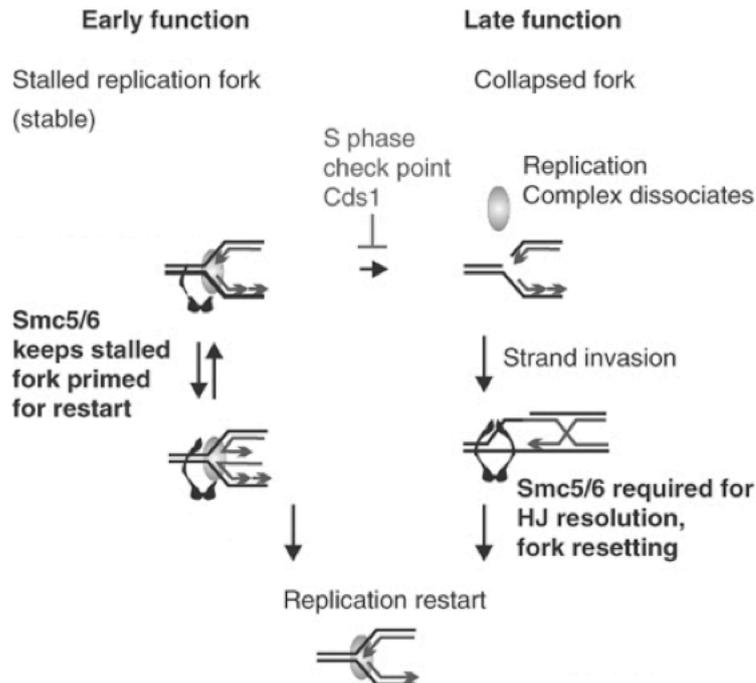
Figure 17 - Diagrammatic representation of the two functions of the SMC5/6 complex in homologous recombination following replication stress. The early function requires the SMC5/6 complex to restart replication in a stalled fork by recruiting Replication Protein A and rad52. The late function of SMC5/6 is to move intermediate structures from collapsed replication forks in order to restart normal replication. Figure adapted from (Irmisch et al., 2009).

Several studies have analysed the role the SMC5/6 complex plays in the replication and stable maintenance of ribosomal DNA (rDNA). It was shown in *S. pombe* and *S. cerevisiae* that the complex is enriched at the ribosomal gene array (Torres-Rosell et al., 2005; Ampatzidou et al., 2006), indicating that the SMC5/6 complex may have a specific role in this area of DNA. This was supported in *S. cerevisiae* where rDNA regions were associated with increased collapsed fork RS intermediates as well segregation defects in SMC5/6 mutants (Torres-Rosell et al., 2005). rDNA is replicated unidirectionally, which means that replication forks cannot converge to continue replication when one stalls. This means that stalled or collapsed forks must be recovered, in order to complete the replication. Because of this, it has been suggested that the effects seen within mutant SMC5/6 cells are just an exaggerated version of those seen in the rest of the genome as the RS recovery functions are even more important in rDNA regions. This is supported further as rDNA is made up of repeat units which are inherently susceptible to RS (Murray and Carr, 2008). Moreover, it has

recently been found in *S. cerevisiae* that the SMC5/6 complex plays a vital role in allowing replication through natural pausing sites as well as processing DNA structures formed during DNA damage tolerance pathways following RS. These features are frequently found, although not exclusively, in rDNA (Menolfi et al., 2015; Peng et al., 2018). This study gives further support that the genome maintenance and repair role of SMC5/6 occurs genome wide but is more significant in rDNA due to its susceptibility to fork stalling and the reduced back-up capabilities.

Extensive research supports a role for the SMC5/6 complex in HR, both within DNA damage repair and RS recovery, however some questions were also thrown up. In particular, yeast SMC5/6 components are encoded by essential genes, yet classical HR genes are not essential in yeast, suggesting a role for SMC5/6 beyond HR (Murray and Carr, 2008) This gave big clues that the function of the SMC5/6 complex did not solely like in HR, but it must also have roles outside of this as well.

There have been various studies undertaken which indicate that one of the key functions of the SMC5/6 complex lies within maintaining chromosome architecture. The initial evidence to suggest this comes from budding yeast where the association of the SMC5/6 complex to DNA is chromosome length dependent, i.e. the longer the chromosomes, the higher the interaction rates (Betts Lindroos et al., 2006; Kegel et al., 2011). This suggested that the complex must be able to 'sense' the length of the chromosomes, possibly due to topological stress. Topological stress is the stress within dsDNA that occurs as the two strands separate in order to allow replication. The DNA ahead of the fork becomes supercoiled, and these supercoils need to be removed by topoisomerases in order to allow complete replication (Keszthelyi et al., 2016). If the SMC5/6 complex was to be involved with the removal of topological stress, then it may interact with topoisomerases. This was supported in *S. pombe,* where overexpression of Smc6 led to nuclear changes similar to those following the loss of topoisomerases I and II, and in *S. cerevisiae* where the SMC5/6 association with longer chromosomes was topoisomerase dependent (Harvey et al., 2004; Kegel et al., 2011). A study in *S. cerevisiae* later found that the SMC5/6 complex is loaded onto sites of topological stress by cohesin, demonstrating an interplay between these SMC complexes

(Jeppsson et al., 2014). This was supported in human cells where the SMC5/6 complex was shown to associate with chromatin throughout the cell cycle in the same pattern as cohesin. This study also found that the SMC5/6 complex may interact with condensins as well as TopoII directly in order to organize and coordinate chromosome architecture, indicating further interplay between the SMC complexes for chromosome maintenance (Gallego-Paez et al., 2014).

It is thought that one of the main purposes for interactions between cohesin and the SMC5/6 complex is for ensuring accurate chromosome segregation. Studies in *S. pombe* demonstrated a failure to complete mitosis in some SMC5/6 mutants due to the inability of these cells to remove cohesin from the chromosome arms (Outwin et al., 2009). Early studies have often suggested a role in chromosome segregation for the SMC5/6 complex. Temperature sensitive *smc6 S. pombe* mutants demonstrated that without the complete complex, cells exhibit incomplete segregation of chromosomes, with the chromatids often found to be stretched along the division plane (Harvey et al., 2004; Sergeant et al., 2005). Moreover, a role for the SMC5/6 complex in chromosome segregation was also indicated as it was demonstrated that the SMC5/6 complex is necessary for the segregation of repetitive regions of DNA (Torres-Rosell et al., 2005). However, there are two main types of connections found between sister chromatids which need to be resolved before segregation; cohesin protein connections as well as DNA mediated linkages. DNA mediated linkages can be further divided into catenations which are formed as sister chromatids pair up during normal replication, resolved by condensin and Topoisomerases, as well as other DNA mediated linkages such as unresolved RS characteristics and unfinished DNA replication. It was shown by Bermúdez-López et al., (2010) that in *S. cerevisiae* the main connection removed for segregation by the SMC5/6 complex was the DNA mediated linkages caused by unfinished replication structures, not the removal of cohesin as previously believed. This was also shown by Branzei et al., 2006, in mutant *S. cerevisiae* cells which expressed non-functional SMC5/6. Furthermore, mutant SMC5/6 cells were also shown to undergo catastrophic meiotic divisions as DNA linkages between the chromosomes remained as inappropriate meiotic segregation was attempted (Farmer et al., 2011). On the other hand, in SMC5 knockout mouse

embryonic stem cells, it has also been revealed that the loss of SMC5/6 causes reduced chromosome segregation possibly due to abnormal condensin localization, implicating the complex in removal of catenations (Pryzhkova and Jordan, 2016). A study within human cells has also shown that depletion of Smc5 and Smc6 caused segregation defects, possibly causally related to an abnormal distribution of condensins and topoisomerase II (Gallego-Paez et al., 2014). This indicates that the pathway is not as clear as first expected and the complex may aid segregation through several different pathways along with being cell type and/or species specific.

In recent years, as well as cellular research to attempt to identify the function of the SMC5/6 complex, several patient studies have been undertaken. Four clinical patients have been identified carrying NSMCE3 mutations which resulted in early childhood death after development of pulmonary disease and viral pneumonia. As the NSMCE3 subunit is an integral part of the SMC5/6 complex architecture, loss of NSMCE3 destabilises the entire complex so the effects seen are thought to be the result of the loss of the entire complex, not solely NSMCE3. The patients presented with T and B cell immunodeficiency, indicating that the SMC5/6 complex plays a role in human lymphocyte development, as well as cells from these patients displaying defective HR repair, increased chromosome breakage, increased sensitivity to DNA damaging agents, increased numbers of micronuclei, and RS sensitivity at a molecular level (van der Crabben et al., 2016). These phenotypes support much of the previous research associating the SMC5/6 complex with HR repair and recovery from RS, indicating that the complex functions similarly in a whole organism as previously shown on a cellular level. Patients presenting with NSMCE2 mutations have also been investigated. These patients displayed primordial dwarfism, gonadal failure and insulin resistance as well as increased numbers of micronuclei and nucleoplasmic bridges at a molecular level. The cells from these patients demonstrated a clear reduction in RS recovery which the study indicated was due to loss of the NSMCE2 SUMO ligase function (Payne et al., 2014). These findings were supported by an NSMCE2 study in mice where knockouts demonstrated an increase in cancer and aging as well as presenting with Blooms syndrome hallmarks such as difficulty segregating their DNA, increased recombination as well as micronuclei. The similarity between these characteristics and Blooms

syndrome further suggest that it is most likely resolving DNA mediated linkages which the SMC5/6 complex undertakes before chromosome segregation. However, many of these molecular features were surprisingly shown to be independent of the NSMCE2 SUMO ligase function in mice, suggesting that the function of the NSMCE2 subunit may not be as simple as first anticipated (Jacome et al., 2015).

It is clear from the literature that the SMC5/6 complex may play a wide range of roles in maintaining genome stability during DNA replication and segregation. It is possible that the complex may be acting as an all-round control unit for these processes, possibly through the NSMCE2 SUMO ligase subunit which could potentially interact with a wide range of substrates.

1.9 Mechanism of SUMOylation

SUMOylation is an essential post-translational protein modification in eukaryotic cells which can alter protein interactions and localization within cells. It has been linked to the development of diseases such as neurodegeneration (Droescher et al., 2013) and heart failure (Kho et al., 2011) and plays a role in a wide variety of biological processes. The mechanism of SUMOylation is a very similar concept to ubiquitination, involving the covalent attachment of SUMO (small ubiquitin-like modifier) molecules to lysine residues within target proteins via an enzyme cascade (Wilkinson and Henley, 2010).

There are four paralogues of SUMO proteins found within mammals, SUMO-1-4. SUMO-1 was discovered first, having roles in DNA strand exchange, binding to RAD51/52 (Shen et al., 1996), as well as binding to the tumour suppressor PML protein (Boddy et al., 1996). The discovery of SUMO-2 protein closely followed (Mannen et al., 1996) and the SUMO-2 and SUMO-3 human genes were then confirmed. Research showed that these genes shared 87% identity, whilst 47% identity was shared between SUMO-1 and SUMO-3 (Lapenta et al., 1997), verifying these proteins as the human SUMO gene family. Following this, a relatively unknown SUMO was also identified, SUMO-4. This SUMO molecule is intron-less and found mainly in the kidney (Bohren et al., 2004). It has been questioned whether SUMO-4 can in fact SUMOylate targets

due to an inhibitory proline residue, but it is thought to have other non-covalent roles (Owerbach et al., 2005).

SUMOylation occurs through an enzymatic cascade resulting in the SUMOylation of target proteins at lysine residues. It starts with the transformation of immature SUMO molecules into mature proteins presenting a C-terminal diglycine motif which is necessary for adenylation by the SUMO E1 activating enzyme. The E1 enzyme can then form an E1-SUMO thioester bond before the SUMO is transferred onto the E2 conjugation enzyme, forming a E2-SUMO thioester bond. Some E2 enzymes can then directly transfer the SUMO molecule onto target lysine residues, but usually E3 ligases enhance this process and increase the specificity through two mechanisms. They either form a complex with the E2-SUMO, forming a scaffold allowing transfer of the SUMO residue to the target protein or the E3 ligase can stimulate E2 to release the SUMO directly onto a substrate, acting as a sort of catalyst (Gareau and Lima, 2010). This general mechanism can be seen in Fig. 18. SUMOylation is a reversible process and the SUMO modifications can be removed through SENPS (sentrin-specific proteases). These proteases cleave at the C-terminus of SUMO and therefore reverse any effects the SUMO had on the protein (Johnson, 2004).
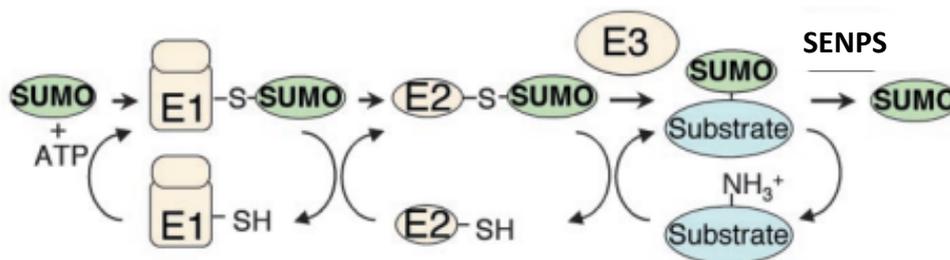
Figure 18 – The enzymatic cascade resulting in the SUMOylation of target proteins at lysine residues. Figure adapted from (Johnson, 2004)

Within mammalian cells, there is only one of each E1 and E2 enzymes which activate and conjugate all three of the key SUMO molecules (SUMO1-3), UBA2 and Ubc9 respectively (Schulman and Harper, 2009; Saitoh et al., 1998). However, there are at least two different groups of E3 ligases in mammalian cells, with several distinct enzymes in each category. The first category are the SP-RING E3 ligases (similar to ubiquitin RING E3 ligases) (Hochstrasser, 2001) which act as the framework to allow

the transfer of SUMO from E2 onto the substrate. These SP-RING motif ligases can be further sub-divided into PIAS family proteins or 'others'. The PIAS proteins, for example PIAS1, have a shared 400 amino acid sequence at their N terminal as well as the shared SP-RING (Kahyo et al., 2001) (Geiss-Friedlander and Melchior, 2007), whilst the 'other' category ligases, such as NSMCE2, do not share this N terminal domain (Geiss-Friedlander and Melchior, 2007). The second main type of E3 ligases have no similarity with the ubiquitin ligase pathway and share little sequence similarities, but instead act as the catalyst for SUMO release from the E2 enzyme onto the substrate. An example of this subset is RANBP2, a protein involved in controlling nuclear transport (Pichler et al., 2004). As mentioned previously, SUMO modifications can be removed from substrates through the action of SENPS. There are six SUMO-specific SENPS encoded within the human genome; SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7, which share a 200 amino acid catalytic domain. Each SENP can carry out deconjugation and processing of a range of proteins, but often in a specific biological process or point in development. For example, SENP2 acts early in embryonic development (Nayak and Müller, 2014).

As well as the addition of one SUMO molecule to a protein substrate, mono-SUMOylation, poly-SUMO chains made up of mainly SUMO-2 and SUMO-3 can form on target proteins (Ulrich, 2008). SUMO polymerization occurs through interactions between SUMO molecules and the surface of E2 enzymes which allow the chains to form (shown in Fig. 19) (Capili and Lima, 2007). The effects of mono and poly SUMOylation are different depending on the protein and it widens the range of cellular effects SUMOylation has upon a cell.
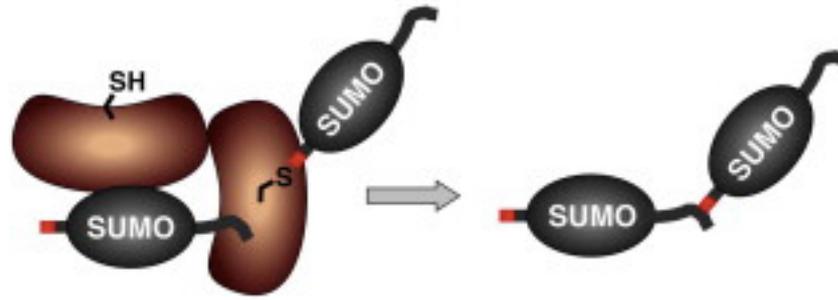
Figure 19 - Diagrammatic representation of the formation of SUMO chains on target proteins using the conjugating Ubc9 (E2) enzyme shown in brown. Figure taken from (Ulrich, 2008)

The importance of SUMOylation has been demonstrated many times, right from embryo development through to cell death following trauma (Nacerddine et al., 2005; Zhang et al., 2016). One of the main effects of SUMO modifications is to alter the activity of the specific protein, either positively or negatively. For example, when the protein product of the RGSZ2 gene, a regulator of G protein signaling, RGS GAP, becomes covalently SUMOylated, it removes its GTPase-activating protein capabilities, regulating the activity of the protein (Garzón et al., 2011). However, it is often not the covalent binding of the SUMO residue itself which causes the effects of SUMO modification seen in many biological processes. SUMO interacting motifs (SIMs) are protein regions which non-covalently interact with SUMO molecules/chains upon covalently SUMOylated proteins, causing an effect to take place (Kerscher, 2007). The effects of SIM binding can also be demonstrated through the RGS GAP protein, as when a SUMO residue covalently bound to another protein, non-covalently binds to the RGS GAP RH domain SIM, RGS GAP becomes unable to interact with other G protein coupled receptor signaling subunits and carry out its signaling function (Garzón et al., 2011). This is an example of how SUMO modifications can affect the functional interactions of a protein, but it is also possible that SUMO modifications can affect interactions which alter the localization of the proteins within the cell. For example, RanGAP1 can become covalently SUMOylated with SUMO-1, which allows it to move to and associate with RanBP2 at the cytoplasmic periphery of the nuclear pore complex, through its SIM (Mahajan et al., 1997). It is also thought that the Polo-like kinase 1 (Plk1)- interacting checkpoint helicase (PICH) locates at the centromere to allow for correct chromosome segregation through interactions between its SIM-3 and

an unknown covalently modified SUMO protein (Sridharan and Azuma, 2016). SUMO modification can also have an impact upon protein stability, as demonstrated by NDRG1, the metastasis suppressor protein. Upon SUMOylation by SUMO-2, the protein becomes destabilized, affecting the protein function and its concentration within the cell (Lee and Kim., 2015). Another SUMO stability mechanism occurs with the promyelocytic leukaemia protein (PML), as when it becomes poly-SUMOylated it is signaled for its degradation through the ubiquitin pathway (Tatham et al., 2008).

It has been demonstrated that the differing effects of poly-SUMO chains compared to individual SUMO modifications may occur as proteins with multiple SIMs within their amino acid sequence can bind more securely to the SUMO chains than mono-SUMO residues. This may explain how the two types of modification are distinguished and how the differences in effects between poly and mono SUMOylated proteins occur, giving rise to the wide range of effects noted (Xu et al., 2014).

SUMO modifications play a significant role in maintaining the stability of the genome. They are vital for correct chromosome segregation, removal of cruciform structures following replication fork collapse, cell cycle progression as well as the important checkpoint activation, amongst many other cellular processes (Nacerddine et al., 2005; Branzei et al., 2006; Seufert et al., 1995). One of the most important genome stability roles SUMO modifications have is within DNA damage repair, although how it does so is still not well understood. SUMO-1, 2 and 3 all accumulate at the sites of double strand breaks in mammalian cells indicating that SUMOylation may play a role in regulating the processes which occur following the damage (Galanty et al., 2009). It has been found that the central HR protein Rad52 is directly regulated by SUMOylation (Altmannova et al., 2010; Sacher et al., 2006), along with other well-known DNA repair proteins PCNA, Sgs1 helicase, BLM and WRN (Kawabe et al., 2000; Eladad et al., 2005; Hoege et al., 2002; Bermúdez-López et al., 2016). This provides strong evidence that SUMO modifications may hold the key to how DNA repair and genome stability processes are all coordinated and controlled. SUMO-targeted E3 ubiquitin ligases (STUbLs) are also thought to play an important role in genome maintenance. These ligases use SIMs to recognize their targets and regulate the levels of SUMOylated

proteins within each cell by targeting these proteins for ubiquitin-dependent proteasomal degradation (Perry et al., 2008). Limited targets have been found making it difficult to make specific judgements but the human STUbL, RNF4, has been shown to interact with the human E2 and E3 ligases, indicating that it downregulates SUMO signaling as part of its role to maintain the genome (Kumar et al., 2017).

1.10 The SUMO ligase function of the SMC5/6 complex

Two components of the SMC5/6 complex have E3 ligase roles; NSMCE1 as an E3 ubiquitin ligase and NSMCE2 as an E3 SUMO ligase. These catalytic roles were initially proposed following sequence analysis of these subunits by (McDonald et al., 2003), where NSMCE1 featured a RING finger motif, suggesting its role as a possible E3 factor in ubiquitinylation and NSMCE2 featured a zinc finger similar to the DNA binding protein Miz1, indicating its role in SUMOylation. The ubiquitin ligase function of NSMCE1 was then confirmed in vitro, through the study of MAGE proteins such as MAGEG1 (Nse3 in yeast), where it was demonstrated that whilst NSMCE1 has its own weak ligase activity, NSMCE3 promotes and enhances this activity as part of the SMC5/6 complex (Doyle et al., 2010). The SUMOylation function of Nse2 was first confirmed in *S. pombe*, before this activity was then replicated in human cells (Andrews et al., 2005; Potts and Yu, 2005).

The roles of these ligase subunits have been studied intensely over the past 20 years, with their targets and functions still largely unidentified. However, quickly after the proteins discovery it was found that the Nse2/NSMCE2 SUMO ligase, in both yeast and human cells, had an important function in DNA damage repair and in the avoidance of DNA damage during normal mitotic cell division (Potts and Yu, 2005; Andrews et al., 2005). This led to the suggestion the NSMCE2 SUMO ligase may play a wide role in chromosome maintenance and RS recovery. Subsequently much of the research has focused upon this area.

In support of this suggestion, mutant Mms21/Nse2 *S. cerevisiae* cells were shown to spontaneously incur DNA damage as well as being very sensitive to RS, which resulted

in many cell cycle progression defects and chromosome breakage, possibly due to the non-functioning Nse2 SUMO ligase (Rai et al., 2011). Furthermore, several other yeast studies have also demonstrated a role for the Nse2 SUMO ligase in RS recovery, resolving recombination intermediates such as cruciform structures, produced following collapsed replication forks (Chavez et al., 2010; Branzei et al., 2006). As well as studies using yeast, there has also been support for the NSMCE2 DNA repair function in DT40 chicken cells, where the SUMO ligase ability was shown to be vital for DNA repair as well as recovery from RS following exogenous damage, with a strong suggestion for a role within HR (Kliszczak et al., 2012).

Primordial dwarfism, has now been determined to be the result of many DNA damage response/repair- associated genetic defects (Alcantara and O'Driscoll, 2014). Human patients with NSMCE2 mutations, have been described which present with primordial dwarfism, along with other defects such as gonadal failure and insulin resistance, indicating that the SUMO ligase function of NSMCE2 may be vital for DNA damage repair on a whole mammalian system level. The cells from these patients displayed increased numbers of micronuclei and nucleoplasmic bridges as well as reduced recovery from RS during DNA synthesis. These abnormalities could be reversed through the addition of wildtype NSMCE2 but not through the addition of ligase-dead NSMCE2, supporting the previous yeast studies that it is specifically the ligase function of NSMCE2 which carries out these RS recovery and repair roles. The same research group have also found that NSMCE2 knockdowns in Zebrafish produce the same dwarfism phenotype seen within the human patients. This again could be reversed through re-expression of wildtype NSMCE2 but not the ligase-dead form, further indicating that it may be the reduced tolerance to RS due to the removal of the SUMO ligase which causes the dwarfism (Payne et al., 2014).

Although there is now fairly strong evidence in support of a genome stability role for the NSMCE2 SUMO ligase, to date, only a handful of NSMCE2 SUMOylation targets have been identified. Initial studies in *S. pombe* revealed that Nse2 SUMOylates both itself, SMC6 and Nse3, whilst in vitro assays using human NSMCE2 have similarly confirmed NSMCE2 autoSUMOylation and modification of SMC6 (Andrews et al.,

2005) (Potts and Yu, 2005). It has been reported that SMC5 may also be SUMOylated, in both yeast and in human cells, but it is not yet clear whether this modification is solely NSMCE2 dependent (Bustard et al., 2012; Zhao and Blobel, 2005).

As well as components of the SMC5/6 complex itself, other reported targets of NSMCE2 SUMOylation include TRAX, the Sgs-1 -Top3-Rmi1 complex, KU70, Scc1, TRF1 and TRF2. These targets often support the case that the NSMCE2 SUMO ligase function lies within chromosome maintenance and RS repair. For example, the Sgs-1-Top3-Rmi1 (STR) complex is employed by the cell to remove and repair RS intermediates, and its ability to do so is provided by SUMOylation by NSMCE2. Upon SUMOylation, the STR complex accumulates at repair sites and is able to form inter-subunit interactions, promoting its activity, aiding RS tolerance (Bonner et al., 2016). The SMC5/6 complex has also been shown to SUMOylate Scc1, a human subunit of cohesin. The SUMOylation of Scc1 by NSMCE2 causes antagonisation of Wap1, allowing HR to be carried out within human cells, removing damage and providing protection from RS (Wu et al., 2012). As well as this, NSMCE2 is also known to SUMOylate the telomere binding proteins, TRF1 and TRF2. These proteins allow telomere elongation through HR to occur in a process named alternative lengthening of telomeres, often used by malignant cells (Potts and Yu, 2007).

As the range of NSMCE2 targets has only just beginning to be revealed, it is possible that nearly all of the genome stability and repair functions of the SMC5/6 may be explained through the NSMCE2 SUMO ligase ability. It is possible that the complex may act as a caretaker for the whole system, overseeing and directing the process to ensure genome stability throughout replication.

1.11 This Thesis

The introduction has outlined the important roles RS and GI play in the development and progression of cancer. For this reason, mechanisms for repairing genetic damage and resolving replication stress are vitally important, acting as a surveillance system to preserve genome integrity. A key protein complex thought to be involved in this

process is the evolutionarily conserved SMC5/6 structural maintenance of chromosomes complex, however its specific role remains unclear. Previous studies have indicated that the ligase activity of the NSMCE2 subunit of SMC5/6 is critically important for the function of the complex, therefore this study aimed to characterize human cell lines mutated for NSMCE2 to explore this further.

Prior to the commencement of this study, two NSMCE2 knockout cell lines (N2A KO and N2B KO) were created through CRISPR-Cas9 gene targeting using LifeTech GeneArt CRISPR gene editing kit. The mutations were directed against two different sequences within exon 1 of the NSMCE2 open reading frame. For the N2A KO cell line, the targeted sequence (tgtatcaactctggtatggac) was at 97-117bp, whilst for N2B KO, the targeted sequence (ggacgttccagttcaaattc) was at 7-26bp. To complement these cell lines, NSMCE2 knockout cell lines with wildtype NSMCE2 re-expression (N2 WT) along with NSMCE2 knockout cells expressing ligase-dead NSMCE2 (N2 R) were also created. These rescue clones were derived from both respective knockout cell lines by stable transfection with wildtype NSMCE2 or NSMCE2 RING mutant in pCl-neo mammalian expression vector.

Following their creation, proliferation assays were then carried out, as shown for the N2A cell lines in Fig. 20. These data demonstrate that loss of the NSMCE2 subunit causes a moderate proliferation defect that can be rescued by re-expression of wild type NSMCE2. Surprisingly, expression of catalytically inactivated NSMCE2 exacerbates this proliferation defect. These data suggest that the observed proliferation defect is due to the specific loss of NSMCE2 or its catalytic activity only.
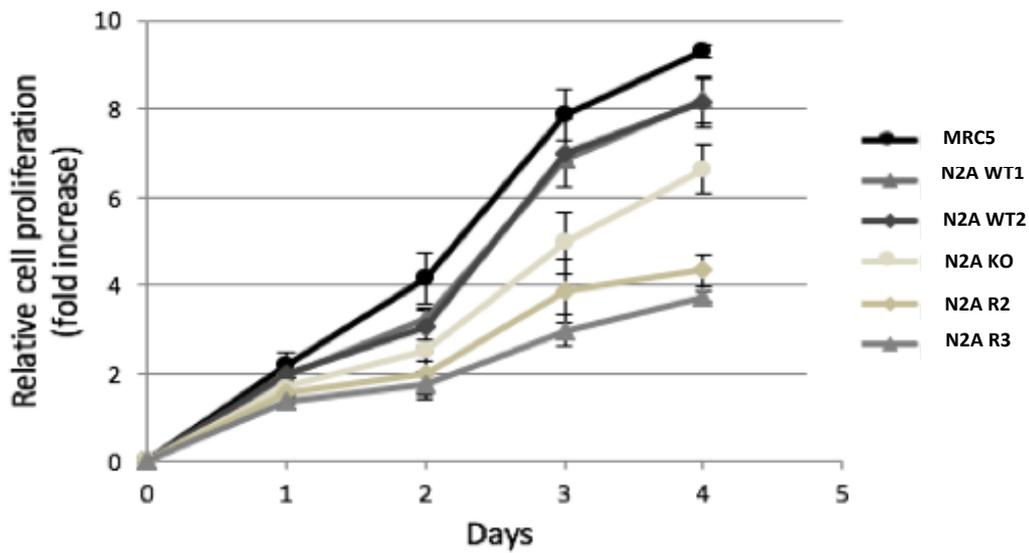
Figure 20 - Cell line proliferation assay for each the CRISPR-Cas9 genome edited cell lines; NSMCE2 knock out (N2A KO), NSMCE2 knockout with wildtype NSMCE2 re-expression (N2A WT1 and N2A WT2) and NSMCE2 knockout cells with ligase-dead NSMCE2 re-expression (N2A R2 and N2A R3), created prior to the commencement of this study.

The aim of this project was to further characterize these NSMCE2 cell lines with the intention of determining the genome maintenance and repair functions of NSMCE2 and its SUMO ligase. Experiments were first carried out to validate the cell lines; assessing the expression level of each of the SMC5/6 components and the association of those components before further analysis of their ability to progress through the cell cycle, focusing on DNA replication and chromosome segregation. This enabled us to ascertain whether NSMCE2 and its SUMO ligase play a role within these processes. Additionally, a protocol was developed with the aim of identifying specific SUMOylation targets of NSMCE2, of which few are currently known.

Chapter 2: Materials and Methods

Composition of solutions used

Solution	Composition
Resuspension Buffer (for total cell extracts)	50mM Tris pH7.5, 150mM NaCl, 1mM MgCl$_2$
Phosphatase Inhibitor Resuspension Buffer (for total cell extracts)	50mM Tris pH7.5, 150mM NaCl, 1mM MgCl$_2$, 20mM NaF, 10mM β-glycerophosphate, 2mM Na$_a$VO$_3$
Lysis Buffer (for routine total cell extracts and Immunoprecipitation)	50mM Tris pH7.5, 150mM NaCl, 1mM MgCl$_2$, 0.1% SDS, 1µl/ml Base muncher (Expedeon®), Protease inhibitor cocktail (Expedeon®) at 1/100. For immunoprecipitation, 0.1% NP40 was used instead of 0.1% SDS.
5x Loading Buffer for SDS-PAGE electrophoresis	0.25% bromophenol blue 0.5M dithiothreitol, 50% glycerol, 10% SDS
SDS-PAGE running buffer	24.8mM Tris-base, 192mM Glycine, 0.1% SDS
Transfer Buffer (for semi-dry transfer)	48mM Tris-base, 39mM Glycine, 0.038% SDS, 20% Methanol
PBS	8mM Na$_2$HPO$_4$, 15mM KH$_2$PO$_4$, 3mM KCl, 137mM NaCl, pH 7.3
PBS-T	PBS plus 0.02%, 0.1% or 0.5% Tween
Blocking Buffer 1	5% milk powder in PBS-T (0.1 or 0.5%)
Blocking Buffer 2	3% BSA in PBS-T (0.1%)
Blocking Buffer 3	1x Casein blocking buffer (Sigma Aldrich ®)
ECL Reagent	20ml of 100mM Tris pH 8.0 with 6µl Hydrogen Peroxide (30% solution), 50µl

	90mM p-coumaric acid, 100μl 250mM luminol
Stripping Buffer	200mM glycine pH2, 0.1% SDS
Immunofluorescence Blocking Buffer	2% BSA in 1X PBS
Immunofluorescence Permeabilisation Buffer	PBS with 0.1% TritonX-100
Immunofluorescence Antibody Buffer	0.5% BSA in PBS
Biotin Stock Solution	1mM Biotin dissolved in Culture Medium
SUMO Lysis Buffer	8M Urea, 1% SDS, 60mM N-ethylmaleimide, Protease Inhibitor Cocktail (Expedeon®) at 1/100 in 1xPBS
SUMO Binding Buffer	3M Urea, 1M NaCl, 0.25% SDS in 1xPBS
SUMO Wash Buffer 1 (WB1)	8M Urea, 0.25% SDS in 1xPBS
SUMO Wash Buffer 2 (WB2)	6M Guanidine Hydrochloride in 1xPBS
SUMO Wash Buffer 3 (WB3)	6.4M Urea, 1M NaCl, 0.2% SDS in 1xPBS
SUMO Wash Buffer 4 (WB4)	4M Urea, 1M NaCl, 10% Isopropanol, 10% Ethanol, 0.2% SDS in 1xPBS
SUMO Wash Buffer 5 (WB5)	8M Urea, 1% SDS in 1xPBS
SUMO Wash Buffer 6 (WB6)	2% SDS in 1xPBS
SUMO Elution Buffer	4x Loading Buffer for SDS-PAGE electrophoresis, 200mM DTT
SUMO Elution Buffer 2	2% SDS, 10mM Biotin, 8M Urea in 1xPBS
SUMO Elution Buffer 3	10mM EDTA, 10mM Biotin in 1xPBS

Primary antibodies used

Antibody	Routine Incubation Conditions	Origin (Secondary Antibody)	Concentration	Supplier	Buffer
ATM (pS1981)	Overnight at 4°C	Mouse	1:1000	Cell Signaling Technology®	Blocking Buffer 2
ATR (pS428)	Overnight at 4°C	Rabbit	1:1000	Cell Signaling Technology®	Blocking Buffer 2
Chk1	Overnight at 4°C	Mouse	1:1000	Cell Signaling Technology®	Blocking Buffer 1 (0.1%)
Chk1 (pS345)	Overnight at 4°C	Rabbit	1:1000	Cell Signaling Technology®	Blocking Buffer 2
Chk2	Overnight at 4°C	Mouse	1:1000	Cell Signaling Technology®	Blocking Buffer 1 (0.1%)
Chk2 (pT68)	Overnight at 4°C	Rabbit	1:1000	Cell Signaling Technology®	Blocking Buffer 2
GAPDH	60 minutes at RT	Mouse	1:4000	Abcam	Blocking Buffer 1 (0.1%)
g-H2AX	Overnight at 4°C	Mouse	1:1000	Upstate Biotechnology	Blocking Buffer 1 (0.1%)

Histone H3	60 minutes at RT	Rabbit	1:2000	Abcam	Blocking Buffer 1 (0.1%)
NSMCE1	Overnight at 4°C	Rabbit	1:000	Raised in-house	Blocking Buffer 1 (0.1%)
NSMCE2	Overnight at 4°C	Rabbit	1:1000	Raised in-house	Blocking Buffer 1 (0.1%)
NSMCE3	Overnight at 4°C	Rabbit	1:1000	Raised in-house	Blocking Buffer 1 (0.1%)
NSMCE4	Overnight at 4°C	Rabbit	1:1000	Raised in-house	Blocking Buffer 1 (0.1%)
p53	Overnight at 4°C	Mouse	1:2000	Cell Signaling Technology®	Blocking Buffer 1 (0.1%)
p53 (pS15)	Overnight at 4°C	Mouse	1:1000	Cell Signaling Technology®	Blocking Buffer 1 (0.1%)
RanGAP1	Overnight at 4°C	Rabbit	1:1000	Cell Signaling Technology®	Blocking Buffer 1 (0.1%)

SMC5	Overnight at 4°C	Rabbit	1:1000	Raised in-house	Blocking Buffer 1 (0.1%)
SMC6	Overnight at 4°C	Rabbit	1:1000	Raised in-house	Blocking Buffer 1 (0.1%)
Biotin HRP	Overnight at 4°C	N/A	1:2000	Cell Signaling Technology®	Blocking Buffer 3
Streptravidin HRP	Overnight at 4°C	N/A	1:2000	Cell Signaling Technology®	Blocking Buffer 1 (0.5%)
Tubulin	Overnight at 4°C	Rabbit	1:40	Cell Signaling Technology®	Immunofluorescence antibody buffer

Secondary antibodies used

Secondary Antibody	Host Species	Supplier
Anti-Mouse HRP	Rabbit	Dako
Anti-Rabbit HRP	Goat	Dako
Alexa Fluor 633	Goat	Invitrogen

2.1 Molecular Biology Techniques

2.1.1 Transformation of Plasmid into DH5α competent cells.

For transformation, one 50µl aliquot of DH5a cells was thawed on ice, before 1µl plasmid DNA was added and incubated on ice for 30min. The cells were heat shocked

for 20-30 secs at 42°C before being incubated for a further 2 min on ice. Transformed cells were plated and incubated overnight on selective agar plates with Ampicillin (100 μg/ml) at 37°C.

2.1.2 Growth of E. coli in liquid cultures

To expand the quantity of plasmid DNA before harvesting, *E. coli* colonies grown following transformation, were inoculated into 100ml LB media containing 100μg/ml carbenicillin before incubation at 37°C overnight.

2.1.3 EndoFree MidiPrep

To harvest and purify plasmid DNA from liquid bacterial cultures, midipreps were carried out using Qiagen-tip 100 materials according to the manufacturer's instructions, with a few minor adjustments. Following isolation of DNA-containing supernatant after addition of P3, DNA was precipitated by addition of 10ml isopropanol and centrifugation (5000rpm, 30min). To remove any endotoxins, the DNA was re-suspended in 0.5ml TE with 4.5ml QBT containing 10% TritonX-100 and incubated at 4°C for 30min before being applied to the Qiagen column. The final plasmid DNA pellet was re-suspended in 400μl Buffer TE.

2.1.4 Determining DNA Concentration

To quantify the concentration of DNA, a spectrophotometer, reading dsDNA settings, was used to measure absorbance (A_{260}). For each sample, 2μl of DNA was added to 58μl dH_2O, mixed, and compared to a negative control.

2.2 Protein Techniques

2.2.1 Total cell protein extract preparation

To analyse the protein content of various cell samples, total cell protein extraction took place. Two methods were routinely used for protein extraction; standard total cell extract lysis or direct cell lysis.

Standard total cell extract lysis – For general total protein analysis by SDS-PAGE, standard cell lysis was carried out. Each cell sample was spun down (4k, 1min) and washed in 1ml PBS. Following a further 4k spin, pellets were lysed in 100μl lysis buffer (per 5x10^5 cells) for 10min at RT (or 4°C for immunoprecipitation sample preparation). Extracts were clarified through centrifugation (13k, 5min), Bradford assay sample taken and the remaining supernatant boiled at 95°C, 3min following addition of 25μl 5x SDS-PAGE loading buffer.

Direct cell lysis – When it was necessary to minimize any alteration to the cell samples during extract preparation, eg. loss of protein phosphorylation, direct cell lysis was carried out. Cells were counted and an equal number collected. Each sample was spun down (4k, 1min) and washed in 1ml cold PBS before being re-suspended in 100μl of either cold Resuspension Buffer or Phosphatase Inhibitor Resuspension Buffer (per 5x10^5 cells). 25μl 5x SDS-PAGE loading buffer was immediately added and the samples boiled (3min, 95°C). Base Muncher (Expedeon®)(1μl per sample, diluted 1 in 5 in resuspension buffer) was then added to each sample and incubated for 5-10min (RT) before heating for a further 3min (95°C).

2.2.2 Bradford Assay Method

To determine the overall protein concentration of each sample, Bradford assays were undertaken. Cell extract samples (1-2μl) were added to 600μl of Bradford Ultra reagent (Expedeon) in a cuvette, mixed and analysed (colour change relative to a negative control) using a spectrophotometer reading at OD600.

2.2.3 Acetone Precipitation

To concentrate protein for SDS-PAGE gel loading and western blot detection, acetone precipitation was carried out. Four volumes of cold acetone (-20°C) were added to protein samples and incubated at -20°C (>1h) before centrifugation (13k, 30min.) The

protein pellet was washed in 50% acetone before being re-suspended in an appropriate volume of buffer for loading.

2.2.4 SDS PAGE Gel Electrophoresis

To separate proteins based on molecular weight, SDS-PAGE gel electrophoresis was carried out using Biometra™ mini-gel electrophoresis equipment. The resolving gel was prepared according to Table 1 and 5.5ml poured between the assembled gel plates, before being overlaid with 100µl of butan-2-ol. Once the gel had set (30-40 minutes), the remaining butan-2-ol was poured off, the stacking gel (Table 2) added and the comb inserted. The gel was allowed to set for 20 minutes before being transferred to a Biometra™ mini-gel electrophoresis tank in SDS-PAGE running buffer. The protein extracts (pre-prepared with the addition of 5x SDS-PAGE gel loading buffer) were loaded onto the gel and run at a constant voltage of 120V through the stacking gel and 160V through the resolving gel until the dye front had run off.

Table 1- Recipes for 10ml SDS-PAGE Resolving gels to be used in SDS-PAGE gel electrophoresis.

	8% Gel Volume (ml)	10% Volume (ml)	12% Volume (ml)	14% Volume (ml)
ddH2O	4.6	4.0	3.3	2.6
30% Acrylamide mix	2.7	3.3	4.0	4.7
1.5M Tris (pH 8.8)	2.5	2.5	2.5	2.5
10% SDS	0.1	0.1	0.1	0.1
10% APS	0.1	0.1	0.1	0.1
TEMED	0.006	0.004	0.004	0.004

Table 2- Recipe for 3ml SDS-PAGE Stacking gel to be used in SDS-PAGE gel electrophoresis.

	Volume (ml)
ddH2O	2.1
30% Acrylamide Mix	0.5
1M Tris (pH 6.8)	0.38
10% SDS	0.03
10% APS	0.03
TEMED	0.003

2.2.5 Western Blotting – Semi-Dry Transfer

For western blot detection, proteins were transferred from SDS-PAGE gels to nitrocellulose membranes by semi-dry transfer. The western blot materials (3x Filter paper and 1x Nitrocellulose membrane) were soaked in transfer buffer before being assembled on semi-dry transfer equipment (Bio-Rad™). The layers were constructed accordingly; two sheets of filter paper, nitrocellulose membrane, SDS-PAGE gel and a final filter paper with air bubbles rolled out between each layer during assembly. The proteins were transferred at 20V for 50 minutes.

2.2.6 Antibody probing of the Nitrocellulose membrane

To allow detection of specific proteins of interest, nitrocellulose membranes were probed with specific antibodies. The membranes were first incubated in blocking buffer specific for the antibody (see Table 4) for 1h at RT, before incubation in primary antibody in the same buffer (for conditions see Table 2). Following this, membranes were washed in PBS-T (4x 5min) (tween concentration the same as blocking buffer) and incubated in the appropriate secondary HRP-conjugated antibody (see Table 3) diluted in Blocking Buffer 1 (1h, RT.) before further washes in the appropriate PBS-T (4x 5min). For western blots of immunoprecipitation samples, protein A/G conjugate was used instead of secondary antibody to allow for native antibody visualization only.

2.2.7 Developing western blots by enhanced chemiluminescence (ECL)

To allow visualisation of antibody-bound proteins, western blots were developed through enhanced chemiluminscence (ECL). After washing the nitrocellulose membrane in appropriate PBS-T and blotting dry, freshly made ECL reagent was applied for 1min. The membrane was again blotted dry and visualized using the Bio-rad ChemiDoc™ on Chemi Blot settings.

2.2.8 Stripping of Western blot

To allow re-probing of the nitrocellulose membranes, previously bound antibody was removed through membrane stripping. The membranes were washed in 10ml of stripping buffer (2x 5min) before being washed in PBS-T (0.1%, 3x 5mins). The membranes could then be re-probed with desired antibodies.

2.2.9.1 Preparation of antibody-coupled magnetic beads for immunoprecipitation

To isolate proteins of interest by immunoprecipitation, antibodies were first crosslinked to magnetic beads. Protein A-Dynabeads (Invitrogen ™)(50µl) were incubated with 10µg SMC6 or control IgG antibody for 2h. at 4°C after a PBS-T (0.02%) wash. To facilitate the washes, the beads were immobilized with a magnet. To crosslink, beads were washed in 1M borate, incubated with 10mg DMP crosslinking agent for 1h, before a final wash in Tris 1.5M pH 8.8. The beads were stored in PBS-T (0.02%) (4°C) before use.

2.2.9.2 Immunoprecipitation

Cell extract for immunoprecipitation was prepared through standard total cell extract lysis with NP40 detergent (see 2.2.1). Protein concentrations of individual cell extracts were determined and 150µg of protein for each cell extract was mixed with each set (IgG and SMC6) of prepared Dynabeads (4°C, 2h). After binding, beads were washed in PBS-T (0.02%) and the protein eluted in 50µl 0.2M glycine pH2 then immediately neutralized with 8µl Tris pH8.8.

2.2.10 Detection of SUMOylated proteins through a bioSUMO Strategy

To analyse and isolate SUMOylated proteins within cells, a bioSUMO approach was used, adapted from (Pirone et al., 2016). This protocol specifically isolates SUMOylated proteins utilizing the strong interaction between biotin and Streptavidin beads. Cells to be analysed are initially transfected with a bio-tagged SUMO conjugate expression plasmid. As the bio-tagged SUMO becomes expressed, it is rapidly biotinylated by the co-transfected *E.coli* BirA enzyme, due to the bio tag. These SUMO molecules are then quickly used by the endogenous SUMOylation system within cells and the biotinylated SUMO molecules become conjugated to SUMOylation targets within the cell. This then allows for streptavidin-based affinity chromatography to take place due to the biotin tags, and the specific isolation of SUMOylated proteins.

Cells to be analysed through this bioSUMO strategy were grown xon 10cm plates to 70-80% confluency. The cells were then transfected with the appropriate vector (see Table 3) (using method 2.3.2) or treated as an untransfected control. 1mM Biotin stock was added to each plate to a concentration of 50µM (immediately or up to 24h post-transfection) and the cells harvested between 24-72h later.

Table 3- Vectors used within the development of the bioSUMO protocol, for the detection of SUMOylated human proteins. Vectors were received from (Pirone et al., 2016)

Short name	Construct	Prom otor	Module1	T2A	Module 2	T2A	Modu le 3
pbio-V	BirA-puro	CAG	empty	empt y	BirA[opt] -V5	GSG - T2A	puro (1)
pbio-SUMO1	bioSUMO1A_BirAV5_puro	CAG	BioSUMO1 (human)	GSG-T2A	BirA[opt] -V5	GSG - T2A	puro (1)
pbio-SUMO2	bioSUMO2A_BirAV5_puro	CAG	BioSUMO 2 (human)	GSG-T2A	BirA[opt] -V5	GSG - T2A	puro (1)
pbio-SUMO3	bioSUMO3A_BirAV5_puro	CAG	BioSUMO3 (human)	GSG-T2A	BirA[opt] -V5	GSG - T2A	puro (1)

To harvest the cell samples, each plate was washed with cold PBS (2x) and the cells scraped into a microcentrifuge tube on ice. The cells were pelleted (500g, 5min.) before being re-suspended in 0.75ml SUMO lysis buffer per plate. Each sample was sonicated to remove viscosity (routinely 4x15s, 18µm) and centrifuged for 20 min (13k). The supernatant was collected and added to 3 volumes SUMO binding buffer before an extract sample was taken (40µl added to 10µl 5x SDS-PAGE loading buffer). Following this, equilibrated (1xPBS and 2x SUMO bind buffer) high capacity Neutravidin® agarose resin beads (ThermoFisher Scientific™) were added (13.3µl per plate used) to each sample and incubated overnight (RT).

After incubation, the Neutravidin® beads were pelleted through centrifugation (100g, 5min) and a flow through sample taken (40µl added to 10µl 5x SDS-PAGE loading buffer). The beads were then re-suspended and washed in 300µl (per 10cm plate) of the following buffers: 2xWB1, 3xWB2, 1xWB3, 3xWB4, 1xWB1, 1xWB5 and 3xWB6. To change wash, the beads were either spun at 2k, 30-45secs through a spin column or the wash was aspirated off. As the last wash was removed, 50µl SUMO elution buffer was added to the beads, vortexed and boiled (99°C, 8mins). The elution mixture was vortexed again, spun briefly and boiled for a second time (99°C, 5mins) before the elution was collected through a 13k spin for 5 mins.

2.3 Cell Culture Techniques

2.3.1 Cell Culture Conditions

MRC5-V1 and all other cell lines used within this project were cultured in defined medium (see Table 4) at 37°C in 5% CO_2. The media within growth vessels was changed every 2-3 days when necessary.

Table 4 -Culture medium used for culturing MCR5-V1 and all other cell lines used in this project. This media was stored at 4°C in the dark

	Volume (ml)
DMEM/4.5g Glucose with L-glutamine	500
Foetal Bovine Serum	50
Penicillin/Streptomycin (Gibco®)	5

.

Routinely, cells were grown in 75cm^2 flasks however, for many experiments they were grown in different sized vessels. The size of each vessel and volume of media required can be seen in Table 4. When reaching confluence, cells were split and sub-cultured allowing for further growth. To do this, the media was aspirated from the cells before washing in PBS and incubation in 0.5% trypsin solution (5% trypsin in PBS) (Gibco®) (see Table 5) at 37°C until the cells were in suspension. The cells were then spun through an equal volume of fresh media (500rpm) to remove the trypsin and re-suspended in an appropriate volume of medium. Cells could then be put back into the growth vessels at an appropriate concentration to allow growth. Where desired, cells were counted using a haemocytometer.

Table 5– Description of each growth vessel used to grow MRC5-V1 as well as the other cell lines within this project.

Size of vessel	Volume medium required (ml)	Volume of Trypsin required (ml)	Approx. Cell Yield
Medium Flask (75cm^2)	15	5	7×10^7
Small Flask (25cm^2)	5-7	3-4	2.5×10^6
10cm Dish	10-14	4	5.5×10^6
6cm Dish	4-5	2-3	2×10^6
6 well plate	4	1-2	9.5×10^5
12 well plate	2	1	3.8×10^5
24 well plate	1	0.2-0.5	1.9×10^5

Cells were frozen (-80°C or in liquid nitrogen for longer term storage) in cryovials, at approximately 1-2 $\times10^6$/ml, in medium containing 10% DMSO. Once removed from the freezer or liquid nitrogen, cells were quickly thawed within a 37°C waterbath and spun through 5ml medium (500rpm, 5min) before being transferred into an appropriate vessel to allow growth.

2.3.2 Transfection

In order to allow expression of specific proteins, expression plasmids were transfected into mammalian cells. Cells to be transfected were routinely grown on a 10cm plate, in antibiotic-free medium, to 70-80% confluency and were transfected using FuGENE® HD Transfection Reagent. For each plate, 18µg plasmid DNA was mixed with 1ml Serum-free media before the addition of 50µl FuGENE®. This was mixed thoroughly and incubated at RT for 15-20min. Following incubation, this transfection mix was added drop-wise to each plate and gently rocked to enable even distribution.

2.3.3 Fluorescent Activated Cell Sorting (FACS)

Cells to be analysed by FACS were harvested, counted and a roughly equivalent number collected from each sample ($\approx 1 \times 10^6$). The cells were then washed in cold PBS, centrifuged (200G, 10min) and re-suspended in 0.5ml PBS, before being added drop-wise to 4.5ml 70% ethanol (4°C) whilst being vortexed. Fixed cells were then stored at 4°C for at least 1h before use.

To hydrate the cells in preparation for FACS analysis, fixed cells were spun (200G, 10min) and re-suspended in 5ml cold PBS. The hydrating cells were incubated at RT for 20 min before being re-suspended in an appropriate volume of PBS ($1\text{ml}/1 \times 10^6$ cells) containing 5µg/ml Propidium Iodide and 100µg/ml RNase. After 30 min incubation at 4°C, FACS analysis was then undertaken using a Beckman Coulter CytoFLEX™ Flow Cytometer with CytExpert™ Software. The number of cells analysed was dependent on each specific experiment. These are shown in each results figure.

2.3.4 Immunofluorescence

For antibody staining and immunofluorescence analysis, cells were grown on glass coverslips within a 24 well plate until 80% confluency. The coverslip-adhered cells were then washed with warm PBS, fixed in 4% formaldehyde in PBS (15min, RT) and washed twice more with PBS (5min, RT). The cells were permeabilised through incubation with the immunofluorescence permeabilisation buffer (5min, RT), washed

in PBS (2x5min, RT), then incubated in immunofluorescence blocking buffer for 1h, RT within a humid light-tight box. The coverslips were then incubated in anti-tubulin antibody (Cell Signalling Technology®) diluted in Immunofluorescence antibody buffer overnight at 4°C. Following incubation, the coverslips were washed in PBS (3x5min, RT), incubated with the secondary antibody (Alexa Fluor 633 anti-rabbit IgG) (1h, RT, in the dark), followed by three more PBS washes (3x5min, RT). The coverslips were mounted onto glass microscope slides using DAPI mounting solution (Vectashield®) and then stored at 4°C in the dark until required. The cells were visualised and analysed using Zeiss ZEN software a confocal microscope. To analyse cell size, the largest linear distance across each cell was measured.

Chapter 3: Results

3.1 Validating the NSMCE2 cell lines

Prior to the start of this project, several NSMCE2 mutant cell lines were generated in this laboratory. These cell lines comprise NSMCE2 knockouts (N2AKO and N2B KO), NSMCE2 KO with wild type NSMCE2 expression restored (N2A WT1, N2A WT6 and N2B WT3), and NSMCE2 KO cells with stably expressed ligase-dead NSMCE2 (N2A R2, N2A R3 and N2B R2). Very little previous work had been undertaken in order to validate these NSMCE2 cell lines, other than the proliferation assays shown in section 1.11. Before any further analysis could be undertaken, it was vital to confirm the appropriate expression of NSMCE2 in each cell line and determine the effects of these NSMCE2 mutations on the rest of the SMC5/6 complex.

3.1.1 Analysis of SMC5/6 component expression within the NSMCE2 cell lines

In order to determine whether the expression of each of the SMC5/6 components was as expected within each of the NSMCE2 mutant cell lines, expression analysis was carried out. Previous work has shown that the human SMC5/6 complex contains six key components; SMC5, SMC6, NSMCE1, NSMCE2, NSMCE3 and NSMCE4 (Taylor et al., 2001; Taylor et al., 2008; Harvey et al., 2004; Potts and Yu, 2005), so it was vital that expression of these components was evaluated within each of the NSMCE2 cell lines, with only the knockout cell lines expected to not express NSMCE2.

To investigate this, total cell extracts were prepared, as described in section 2.2.1, from each of the NSMCE2 mutant cell lines, along with the parental MRC5-V1 cells, and analysed by western blotting. Antibodies were used to detect each of the SMC5/6 components along with GAPDH which served as the loading control. The results, shown in Fig. 21, demonstrate that in both the N2A KO and N2B KO cell lines, the NSMCE2 knockout has been successful, with NSMCE2 expression apparently abolished. In both cases, expression of the other SMC5/6 components was unaffected. In addition, the results show that expression of NSMCE2 has been sucessfully restored in the wild type NSMCE2 corrected cells (N2A WT1, N2A WT6, N2B WT3), although

marginally overexpressed compared to the MRC5 control cells. Ligase-dead NSMCE2 is expressed at comparable levels in N2A R2 and N2A R3, and at a slightly reduced level in N2B R2. As expected, the expression of the other SMC5/6 components within the corrected cell lines (ligase dead or wild type NSMCE2) is also unaffected. This data confirms the validity of the NSMCE2 cell lines in terms of SMC5/6 component expression. From this point forward, the experiments presented focus on the NSMCE2A cell lines, hereafter referred to as the NSMCE2 cell lines.
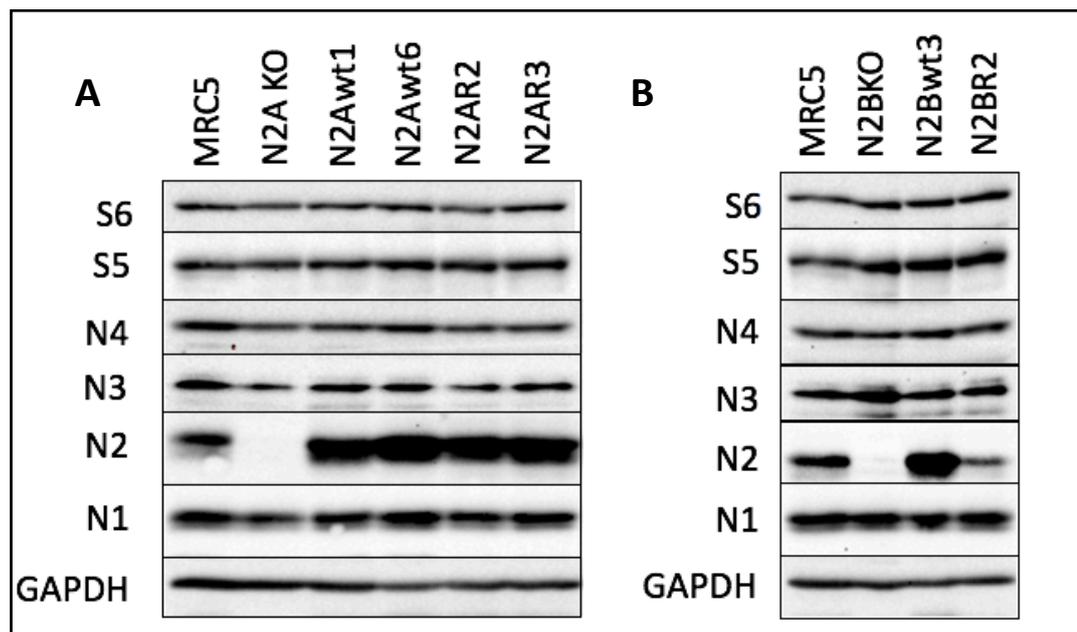
Figure 21- Western blot showing expression of SMC5/6 components in A) wild type (MRC5) and NSMCE2 knockout (N2AKO) cells as well as N2A KO cells following re-expression of wild type NSMCE2 (N2Awt1, N2Awt6) or ligase-dead NSMCE2 (N2AR2, N2AR3) and in B) wild type (MRC5), NSMCE2 knockout (N2BKO), and N2B KO cells following re-expression of wild type NSMCE2 (N2Bwt3) or ligase-dead NSMCE2 (N2BR2). GAPDH is used as the loading control. This experiment was performed several times before the commencement of this project, ensuring the validity of this data.

3.1.2 Analysis of SMC5/6 complex formation in the NSMCE2 mutant cell lines

Since the expression of the SMC5/6 components within the NSMCE2 cell lines was as expected, it was important to discover whether loss of the NSMCE2 protein in its entirety, or the loss of only its SUMO ligase activity, affected the structure of the complex. In yeast, it has been found that the NSMCE2 component only interacted with SMC5 within the SMC5/6 complex (Duan et al., 2009; Andrews et al., 2005; Sergeant et al., 2005). Therefore, it was expected that the loss of this protein component would not make a significant difference to the overall integrity of the SMC5/6 complex, with the remaining components still able to associate. To confirm this,

immunoprecipitation assays (see method 2.2.9.2) were carried out. The SMC5/6 complex was immunoprecipitated from cell extracts prepared from MRC5, N2A KO and N2A KO cells expressing wild type NSMCE2 (N2A WT1) or ligase dead NSMCE2 (N2A R2), using anti-SMC6 antibodies (or non-specific IgG as a negative control). The input extract samples and the immunoprecipitated proteins were then analysed by western blotting (Fig. 22).
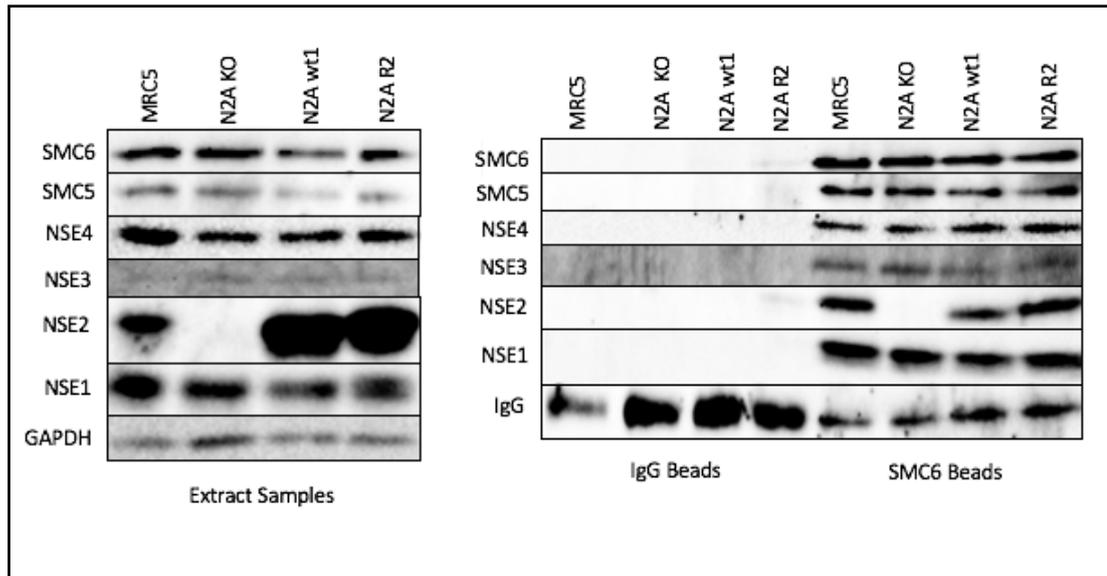
Figure 22 - Western Blot showing results of an Immunoprecipitation assay on the NSMCE2A cell lines; Wild type (MRC5), NSMCE2 knockout (N2AKO) as well as N2A KO cells following re-expression of the wild type NSMCE2 (N2A wt1) or ligase-dead NSMCE2 (N2AR2). 75µg of cell protein extract was used in each immunoprecipitation assay and 1/5th used as the extract sample. IgG concentration is used as the antibody control and GAPDH as the loading control for the extract samples.

The data presented in Fig. 22 (right panel) demonstrate that immunoprecipitation of SMC6 results in co-immunoprecipitation of the entire complex when either wild type or ligase-dead NSMCE2 is present. Moreover, even in the absence of NSMCE2 expression, the remaining components of the complex are immunoprecipitated along with SMC6, confirming that the expression of the NSMCE2 protein is not required for association of the remaining SMC5/6 complex components. No complex components were immunoprecipitated on the IgG-bound beads confirming that immunoprecipitation of the SMC5/6 components is specific to their association with SMC6 and the SMC6 antibody. These results indicate that all further data collected using the NSMCE2 mutant cell lines will be specific to the loss of the NSMCE2 component or is activity and not due to loss of the integrity of the entire SMC5/6 complex.

It was previously identified that both the NSMCE2 knockout and ligase-dead cells proliferated at a slower rate (see proliferation data in section 1.11). To determine whether this reduced proliferation rate related to slowed progression through the cell cycle, cell cycle analysis was performed through Fluorescent activated cell sorting (FACS). This would aid determination of whether these slow proliferating cell lines were having problems progressing through a specific cell cycle phase, through the entire cell cycle (such as due to a metabolic defect) or due to extensive cell death. Cells of each NSMCE2 cell line were fixed and the cellular DNA stained with propidium iodide before cell cycle analysis was undertaken using Beckman Coulter CytoFLEX™ Flow Cytometer with CytExpert™ Software as described in 2.3.3. The results of this analysis are presented in Fig. 23.
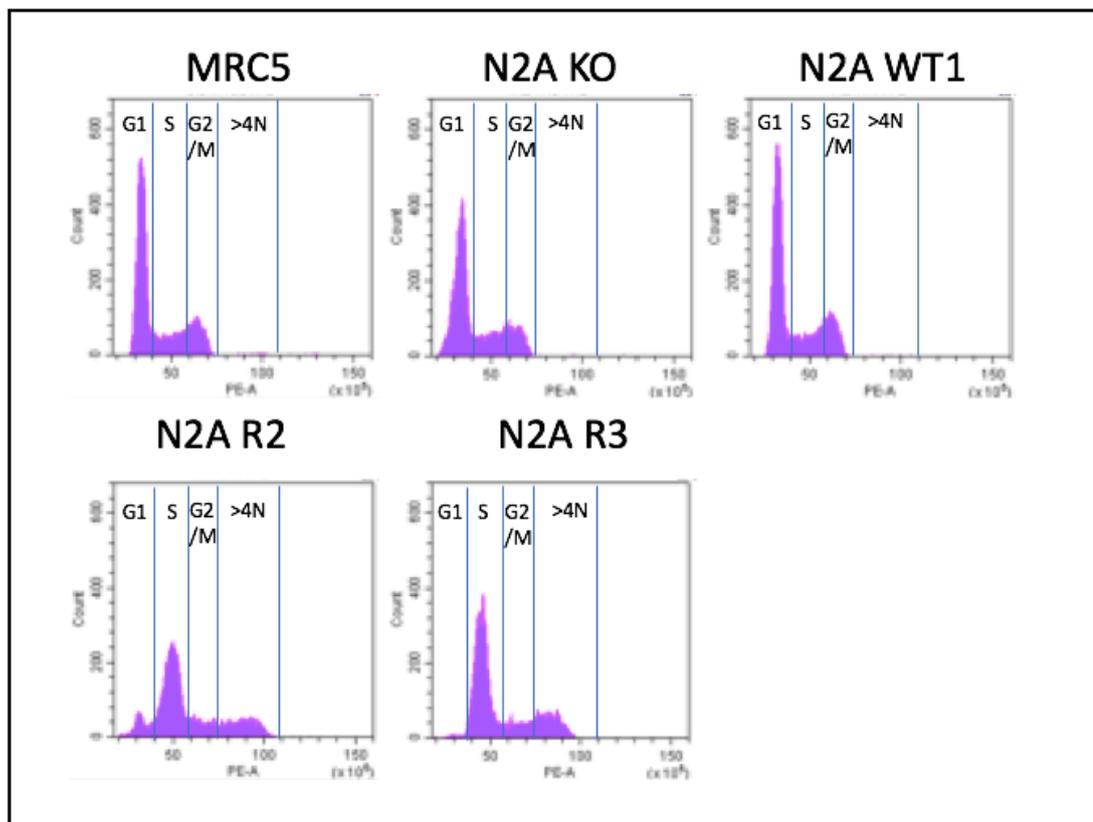
Figure 23 - *FACS analysis showing the cell cycle proportions at a specific time point for each of the NSMCE2A cell lines; wild type (MRC5), NSMCE2 knockout (N2AKO) and N2A KO cells following re-expression of wild type NSMCE2 (N2A WT) or ligase dead NSMCE2 (N2A R2 and N2A R3). The cells were stained with Propidium Iodide to visualise the DNA content. 10,000 cells analysed per cell line. The marked cell cycle stages are to be used as guidelines only. This experiment was repeated frequently throughout the project in order to ensure the reliability of each cell line. This figure demonstrates a representative example.*

The data presented in Fig. 23 illustrate that the slowed proliferation rate of the NSMCE2 knockout cells may be due to a difficulty moving through S phase of the cell cycle, with a noticeably reduced proportion of cells within G1 and an increased S/G2 population. Moreover, the re-expression of wild type NSMCE2 in the corrected cells (N2A WT1) restores the FACS profile to trace the one attained by MRC5-V1 cells almost completely, as in the proliferation assay, indicating that the NSMCE2 knockout cell cycle defect is specific to NSMCE2 component loss, rather than an off-target effect of the CRISPR Cas9 modification.

The FACS profile of both NSMCE2 ligase-dead cell lines reveals that their ability to move through the cell cycle is significantly more affected than the NSMCE2 knockout cells. The population of cells are predominantly accumulated in mid-late S phase, suggesting that these cells are having difficulties in completing DNA replication and proceeding to G2/M. As well as this, there is a significant population of cells with >4N DNA content. This suggests that when these cells are able to finish replicating their DNA, they are then unable to effectively separate the replicated chromosomes into two new daughter cells, possibly due to issues arising during S phase, but instead begin to re-replicate their DNA without effective chromosome segregation, giving a >4N population of cells. Although a proportion of the N2A R2 and R3 cell populations expressing ligase-dead NSMCE2, must eventually replicate and segregate their DNA to continue proliferation, both of these processes appear compromised, perhaps explaining their extremely slowed proliferation rate. It is possible that the S phase delay in both the NSMCE2 knockout and ligase-dead cell lines is caused by the activation of the DNA damage or replication checkpoints, as this would halt the cells moving through S phase. Nevertheless activation of these checkpoints would not explain the >4N ligase-dead population, suggesting another process may be involved.

What is most interesting about this data, is that the loss of the entire NSMCE2 protein results in a cell cycle profile more closely related to wildtype MRC5 than that following re-expression of a catalytically inactive version of this protein. This trend is corroborated by the proliferation assay data. This indicates that the catalytically-inactivated NSMCE2 protein may exert a dominant-negative effect, where the ligase-dead protein acts antagonistically, possibly interacting with the normal proteins within

the cell and blocking their functions. This will be important to consider when interpreting all further data.

3.2 The activation of the Damage and Replication checkpoints

3.2.1 Validation of Damage and Replication checkpoint antibodies

The reduced proliferation seen within the knockout and ligase dead NSMCE2 cell lines has been associated with slow progression through S phase, especially in N2A R2 and N2A R3, as shown in Fig. 23. This suggests that NSMCE2 ligase activity is important for S phase progression. There are two checkpoints used by cells which can also halt progression through S phase; the DNA damage checkpoint and the replication checkpoint (González Besteiro and Gottifredi, 2015; Chehab et al., 2000). Since the SMC5/6 complex has been strongly linked to repair of damage and recovery from RS since its discovery (Lehmann et al., 1995; Nasim and Smith, 1975; Andrews et al., 2005; Zhao and Blobel, 2005), it was important to discover whether the altered progression through S-phase observed in our NSMCE2 mutant cell lines was at all associated with the activation of either of these checkpoint mechanisms. For these reasons, we wanted to test the NSMCE2 cell lines for activation of both the DNA damage and replication checkpoints, during normal replication and following exogenous stress.

Key molecules which are phosphorylated and act early in the DNA damage checkpoint are ATM and Chk2 (Chaturvedi et al., 1999), whilst ATR and Chk1 act mainly in response to RS and the single strand DNA which forms (Zou and Elledge, 2003). Phosphorylation of these important kinases denotes the activation of the two checkpoints respectively, and so we tested various antibodies against the phosphorylated forms of these checkpoint proteins (α-ATMpS1981, α-Chk2pT68, α-ATRpS428, α-Chk1pS345) for use in our subsequent analysis of NSMCE2 mutant cells. In order to test the detection ability of these phospho-antibodies, MRC5 cells were treated with a variety of genotoxic agents (5µM Etoposide (16h), 200 or 10µM H_2O_2 (1h) or 25, 10 or 2.5nM Neocarzinostatin (NCS) (1h) before total cell extracts were

prepared using the phospho-protein direct cell lysis method (see method 2.2.1). 50μg of total protein extract

was resolved through SDS-PAGE gel electrophoresis and western blotted (see methods 2.2.4 and 2.2.5) with each of the four antibodies. The result are shown in Fig. 24.

Treatment	Untreated	Etoposide	H₂O₂	H₂O₂	NCS	NCS	NCS
Concentration		5μM	200μM	10μM	25nM	10nM	2.5nM
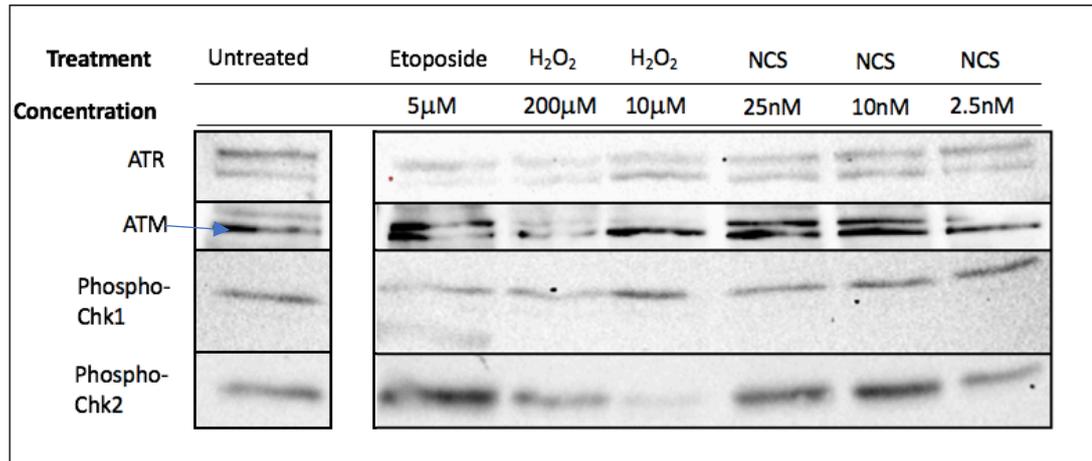
Figure 24 - Western blot to test the detection ability of ATM, ATR, Phospho-Chk1 and Phospho-Chk2 following damage treatment of wild type MRC5 cells. Cells were treated with either 5μM Etoposide (Etop), 200 or 10μM H₂O₂ or 25, 10 or 2.5nM Neocarzinostatin (NCS). 50μg of total protein cell extract was resolved through SDS-PAGE gel electrophoresis for each treatment.

Although the α-ATRpS428 antibody detected two bands in both untreated and treated cell extracts, it was not clear if either band represented phosphorylated ATR since there was no apparent increase in signal following treatment with agents that cause RS such as etoposide. For this reason, it was subsequently discounted from further testing and experiments. Moreover, the signal created by the α-Chk1pS345 antibody also does not appear to be quantitatively different following treatment with RS agents compared to the control extract. However, it was hoped that with the resolution of more protein, it may possibly generate a stronger more quantifiable α-Chk1pS345 signal. Conversely, the α-ATMpS1981 antibody showed more promise, with a relative increase in phosphorylated ATM (upper band indicated by arrow) within the damage treated samples compared to the controls. The upper band is more intense following etoposide treatment or treatment with increased amounts of NCS. The expected pattern of stronger kinase activation with greater amounts of genotoxic agent treatment was also demonstrated following use of the α-Chk2pT68 antibody,

mirroring the pattern created with ATM as expected. Additional experiments were undertaken to further test and validate the detection abilities of these antibodies.

To further assess the detection of phosphorylated checkpoint proteins using these phospho-specific antibodies, MRC5 were treated with either 2.5nM camptothecin (CPT) (16h), 2mM hydroxyurea (HU) (16h), or 5μM etoposide (16h). Total cell extracts were prepared and 75μg was resolved through SDS-PAGE gel electrophoresis before western blotting with α-ATMpS1981, α-Chk1pS345, α-Chk2pT68 and α-p53pS15 antibodies. It has been known for a long time that p53 is a key player in the DNA damage response, which becomes phosphorylated by ATM following the production of damage (Kastan et al., 1992). In these experiments, the phosphorylation of p53 was utilised to determine the presence of DNA damage in each of the NSMCE2 cell lines. The results from each antibody incubation can be seen in Fig. 25.
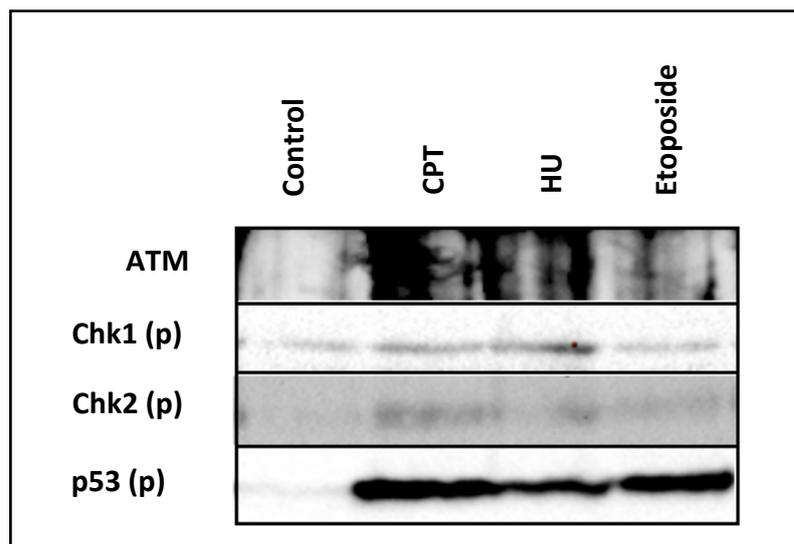
Figure 25 - Western blot to test the detection ability of ATM, phospho-Chk1, phospho-Chk2 and phospho-p53 following damage treatment of wild type MRC5 cells. Cells were treated with either 2.5nM Camptothecin (CPT), 2mM HydroxyUrea (HU), or 5μM Etoposide before 75μg of total protein extract was resolved through SDS-page gel electrophoresis.

As clearly shown in Fig. 25, the phospho-p53 antibody clearly detected the phosphorylated form of p53 in each of the damage treated cell extracts, relative to the untreated control, and so could be used to detect levels of DNA damage in future experiments. However, results for the other antibodies were less clear cut. As shown in Fig. 25, the α- ATMpS1981 antibody was found to be unusable due to the extensive

background signal generated and so it was discounted from use in further experiments. The signal generated by both the α-Chk1pS345 and α-Chk2pT68 antibodies following CPT and HU treatment was greater than in the control sample and so, despite some variability noted in subsequent experiments, it was determined that the antibodies were functional, and could be used to determine the activation state of the damage and replication checkpoints in each of the NSMCE2 cell lines.

3.2.2 Assessment of Damage Accumulation within the NSMCE2 cell lines

The slowed cell cycle progression noted in the NSMCE2 mutant cells could be a consequence of DNA damage arising in the absence of NSMCE2 ligase activity. DNA damage can arise as a consequence of RS and endogenously arising DNA damage could, in turn, hinder DNA replication, by interfering with fork progression. DNA damage can also prevent cell cycle progression by activating the DNA damage checkpoint, allowing cells to repair the damaged DNA (Reviewed in: (Zannini et al., 2014)). Therefore, we wanted to examine the possibility that DNA damage was accumulating within the NSMCE2 mutant cell lines and so hindering cell cycle progression. To do so, we analysed two markers of DNA damage within each of our NSMCE2 cell lines, the phosphorylated forms of H2AX (γ-H2AX) and of p53 (α-p53pS15). H2AX is a variant of the histone H2A protein found within the nucleosome core and its phosphorylation is a commonly used marker for DNA damage. Following replication stress and genetic damage, particularly dsbreaks, H2AX is phosphorylated at Ser139 by ATM and ATR (Rogakou et al., 1998; Burma et al., 2001; Ward and Chen, 2001). The phosphorylated form of p53, as discussed earlier, is also activated following DNA damage by ATM (Kastan et al., 1992) to allow for a wide range of DNA repair responses, or apoptosis when repair cannot occur (Williams and Schumacher, 2016).

To determine the level of these DNA damage markers within each of the N2A cell lines, equal amounts of total cell extract (see method 2.2.1) from each cell line (MRC5, MRC5 (treated with 5μM etoposide (1h), N2A KO, N2A KO with wild type NSMCE2 (N2A WT1) and N2A KO with ligase dead NSMCE2 (N2A R2 and N2A R3)) were analysed by western blotting using antibodies against both γ-H2AX and α-p53pS15, along with relevant

controls (α- histone H3, α-p53 and α-GAPDH). These experiments were repeated three times and a representative blot can be seen in Fig. 26.
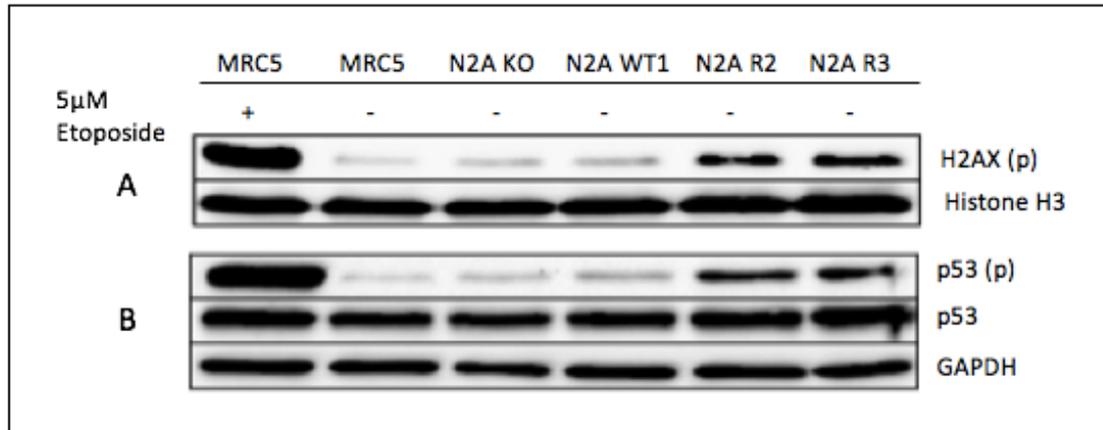
Figure 26 - Western showing the expression of A) phospho-H2AX and Histone H3 and B) phospho-p53, p53 and GAPDH in 5µM etoposide (1h) treated MRC5 wild type, untreated MRC5 wild type and untreated NSMCE2 knockout (N2AKO) cells as well as N2A KO cells following re-expression of wild type NSMCE2 (N2A wt1) or ligase dead NSMCE2 (N2ALD1 and N2A LD2). Loading controls are A) Histone H3 and B) GAPDH. The etoposide treated MRC5 cells act as a positive control for the DNA damage response. 7.5x10⁴ cells per sample. Representative of 3 independent repeats.

As shown in Fig. 26, there is no evidence to suggest that there are increased levels of DNA damage in the NSMCE2 knockout cell line over the background levels of damage noted within both the wild type MRC5 cells and the NSMCE2 KO with wild type NSMCE2 re-expression. This indicates that the slightly reduced S/G2 phase progression and reduced proliferation rate noted in the NSMCE2 knockout cells is not due to increased levels of DNA damage. However, it is clear that expressing the ligase-dead form of NSMCE2 does cause increased endogenous DNA damage compared to the wild type cell lines, as shown by increased expression of the phosphorylated forms of both H2AX and p53. This suggests that increased DNA damage may contribute to the reduced S phase progression seen in cells with catalytically inactivated NSMCE2.

3.2.3 Activation of the Damage checkpoint by the NSMCE2 cell lines

Since it is clear that there is an increase in DNA damage within the NSMCE2 ligase-dead cell lines, activation of the DNA damage checkpoint could be contributing to slowed S phase progression in these cells. To determine whether this could be the case, we decided to analyse Chk2 phosphorylation as a marker for activation of the ATM-dependent DNA damage checkpoint response (Chaturvedi et al., 1999).

As in the previous experiment, equal amounts of total cell extract (method 2.2.1) from each of the NSMCE2 cell lines; MRC5, N2A KO, N2AWt1, N2AR2 and N2A R3 as well as MRC5 cells treated with 5µM etoposide, were analysed by western blotting with antibodies against phosphorylated (α-Chk2pT68) and un-phoshorylated forms of Chk2 as well as GAPDH as the loading control. The results of the western blot can be seen in Fig. 27.
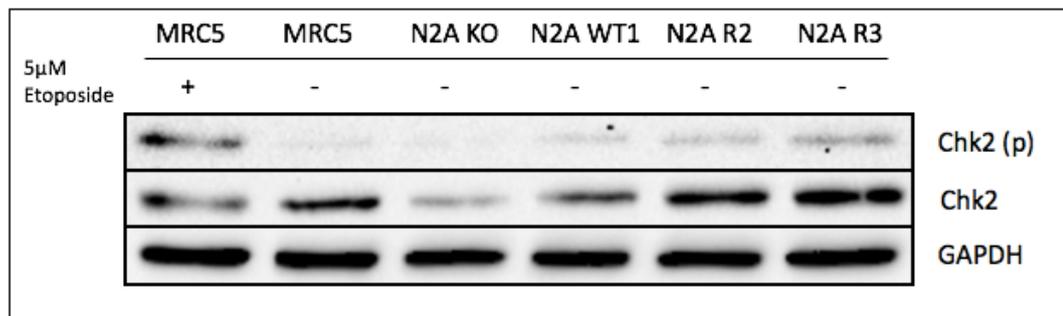
Figure 27 -Western blot showing the expression of phospho-chk2, chk2 and GAPDH in 5µM etoposide (1h) treated MRC5 wild type, MRC5 wild type and NSMCE2 knockout (N2AKO) cells as well as N2A KO cells following re-expression of wild type NSMCE2 (N2A wt1) or ligase dead NSMCE2 (N2ALD1 and N2A LD2). GAPDH is used as the loading control. $7.5x10^4$ cells per sample. This experiment was independently repeated three times. This figure demonstrates a representative example.

As expected, Fig. 27 illustrates that there is no increased activation of the DNA damage checkpoint within the NSMCE2 knockout cell line compared to both the control MRC5 and NSMCE2 knockout cells with wild type NSMCE2 re-expression, as shown by the level of phosphorylated Chk2. This supports the previous data which showed no phosphorylation of the DNA damage markers, γ-H2AX and p53pS15, indicating that it is unlikely that increased DNA damage or activation of the damage checkpoint is the cause of the reduced proliferation rate seen within the NSMCE2 knockout cell lines. Moreover, unexpectedly, the NSMCE2 knockout cell line has a decreased level of unphosphorylated Chk2 compared to the other cell lines. This may explain why there is no increase in phosphorylated Chk2 as there is less overall Chk2 to become phosphorylated. This indicates that NSMCE2 may be involved with the activation of the DNA damage checkpoint at an early stage, and this cell line may have difficulty activating their DNA damage checkpoint at all.

Conversely, both cell lines expressing the ligase-dead NSMCE2 do exhibit some Chk2 phosphorylation, so indicating activation of the DNA damage checkpoint in the presence of the DNA damage noted in Fig. 26. The activation of Chk2 seems to correlate with the level of damage accrued, as although the α-Chk2pT68 band is not as intense within the ligase-dead cell lines compared to the etoposide treated sample, this correlates with the damage accumulation shown in Fig. 26. This seems to validate that it may be DNA damage and activation of the DNA damage checkpoint which is, at least partly, causing the build-up of NSMCE2 ligase-dead cells within S phase. On the other hand, there is an increased level of un-phosphorylated Chk2 within both of the ligase-dead cell lines, suggesting that although there is an increase in phosphorylated Chk2 compared to the wild type cell line, there is the same proportion of unphosphorylated and phosphorylated Chk2. These data may indicate that the ligase-dead cell lines may not be phosphorylating Chk2 and activating the checkpoint any more than the wild type cell line.

3.2.3 Activation of the Replication Checkpoint by the NSMCE2 cell lines

The second checkpoint which could be activated, causing the slowed progression through S phase of the NSMCE2 knockout and NSMCE2 ligase-dead cells, is the replication checkpoint. This checkpoint involves the activation and binding of ATR to ssDNA following replication stress, which subsequently causes the phosphorylation of Chk1 and halting of the cell cycle through the involvement of many other proteins (as discussed in the introduction). To determine if this checkpoint activation is the cause, we decided to analyse the phosphorylation of Chk1 at Ser345 as a marker for activation of the ATR-dependent replication checkpoint. To do so, as in the previous experiment, equal amounts of total cell extract (method 2.2.1) from each of the NSMCE2 cell lines; MRC5, N2A KO, N2AWt1, N2AR2 and N2A R3 as well as MRC5 cells treated with 5μM etoposide, were analysed by western blotting with antibodies against phosphorylated (α-Chk1pS345) and un-phoshorylated forms of Chk1 as well as GAPDH as the loading control. This experiment was repeated three times and a representative blot can be seen in Fig. 28.
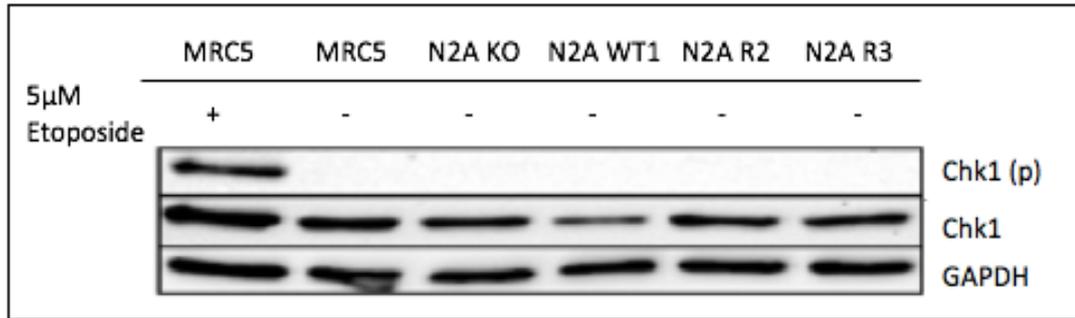
Figure 28 - Western blot showing the expression of phospho-chk1, chk1 and GAPDH in 5µM etoposide (1h) treated MRC5 wild type, MRC5 wild type and NSMCE2 knockout (N2AKO) cells as well as N2A KO cells following re-expression of wild type NSMCE2 (N2A wt1) or ligase dead NSMCE2 (N2ALD1 and N2A LD2). GAPDH is used as the loading control. 7.5x10⁴ cells per sample. This experiment was independently repeated three times. This figure demonstrates a representative example.

Fig. 28. indicates that, somewhat surprisingly, none of the NSMCE2 cell lines activate the replication checkpoint, with no phosphorylation of Chk1 detected in these cell lines, as compared with the etoposide-treated MRC5 cells used as positive control. This indicates that the slow progression of N2KO and NSMCE2 ligase-dead cells is not due to the activation of the replication checkpoint. It is possible that NSMCE2 mutant cells do activate the replication checkpoint to an extent that is below the level that we can detect, but there is no evidence of substantial checkpoint activation in this assay. These findings raised a question as to whether the problems incurred in the NSMCE2 ligase dead mutants, slowing S phase progression, are not sensed or responded to by the replication checkpoint, or whether these NSMCE2 mutant cells are unable to activate the replication checkpoint at all.

In order to determine the ability of each of the NSMCE2 cell lines to activate the replication checkpoint, each cell line was subjected to replication stress treatment with 2mM HU (16h), which should normally activate the replication checkpoint if cells have the capacity to do so (Koç et al., 2004). The cells were then analysed by flow cytometry (see method 2.3.3) to determine whether the cell cycle was halted at the replication checkpoint. The results of this experiment can be seen in Fig. 29.
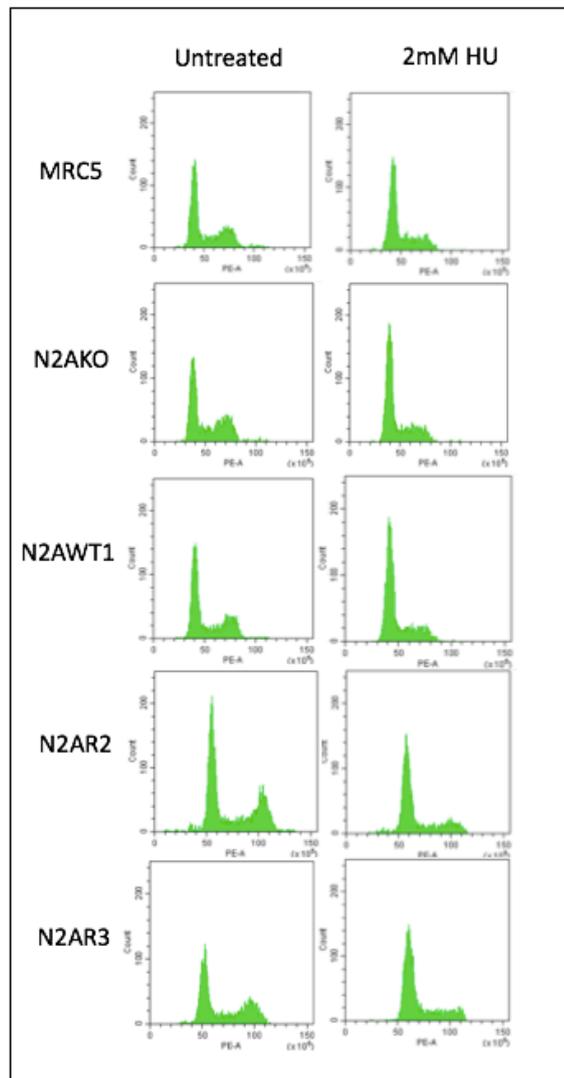
Figure 29 - Flow cytometry analysis showing the cell cycle proportions of untreated Wild type MRC5 cells, NSMCE2 Knockout (N2AKO) and N2AKO following re-expression of wild type NSMCE2 (N2A WT1) and re-expression of ligase dead NSMCE2 (N2AR2 and N2AR3) as well as 2mM Hydoxy urea treated cells (16h) from each cell line. Cells are strained with propidium iodide to visualise the DNA content. 4000 cells analysed. HydroxyUrea treatment carried out for 16h.

Upon analysis, 2mM HU treatment clearly does cause each NSMCE2 cell line to halt the cell cycle progression into G2, shown in Fig. 29. As expected, the cell cycle profile of the treated MRC5 cells displays a loss of the G2 peak, suggesting that there is an accumulation of cells at the G1/S border and in S phase. A similar result was also attained in both the N2A KO as well as the N2A KO with wild type NSMCE2 re-expression. Even in the NSMCE2 ligase-dead cell lines, where most of the population was in mid S phase even in the absence of replication stress treatment, it is clear that HU treatment has reduced the proportion of cells within the >4N population by

preventing re-replication. These data suggest that the NSMCE2 ligase activity of the SMC5/6 complex is likely not involved in the activation of the replication checkpoint.

However, in order to confirm this theory further, we analysed Chk1 phosphorylation following 2mM HU treatment (16h) of each NSMCE2 cell line. This was undertaken by western blot with antibodies against phosphorylated (α-Chk1pS345) and un-phoshorylated forms of Chk1. The results of this experiment can be seen in Fig. 30.
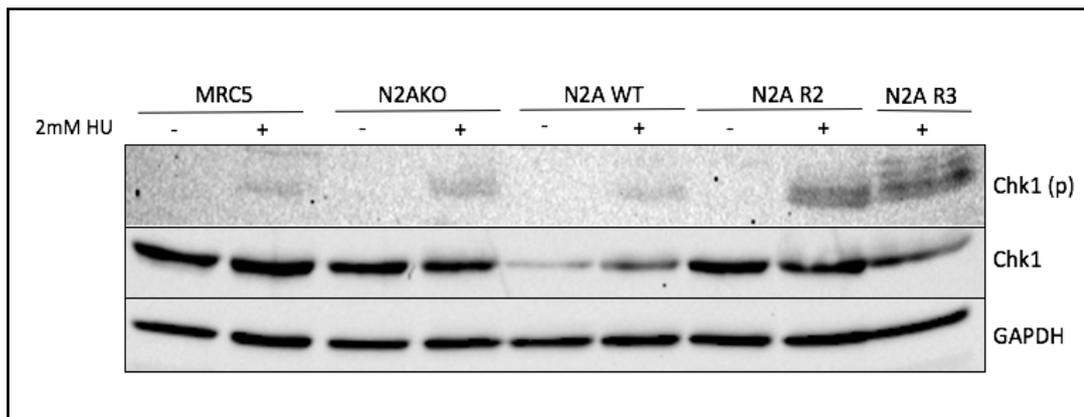
Figure 30 - Western blot showing the expression of phosphor-Chk1, Chk1 and GAPDH in both untreated and 2mM Hydroxy Urea (HU) treated (16h) Wild type MRC5, NSMCE2 knockout (N2AKO) as well as NSMCE2 knockout following re-expression of wild type NSMCE2 (N2A WT) and re-expression of ligase-dead NSMCE2 (N2A R2 and N2A R3) cell lines. GAPDH is used as the loading control.

Fig. 30 confirms that each of the NSMCE2 cell lines is able to activate the replication checkpoint and phosphorylate Chk1 when treated with HU, including the ligase-dead mutants. These data indicate that the replication checkpoint response is intact in both the N2A KO and ligase dead mutant cells. Indeed, the response is even more robust in the NSMCE2 ligase-dead mutants. However, at the top of the panel of the 2mM HU treated MRC5 cell sample, there is a faint band, possibly indicating another form of phosphorylated Chk1 which is present when the checkpoint is fully activated. This is also present within the 2mM HU treated N2A R3 cell line sample, but none of the other mutant cell lines. It is then possible that although these cell lines seem to be able to phosphorylate Chk1 and so activate the replication checkpoint, they may not be able to trigger the complete checkpoint response, implying NSMCE2 may be involved in this process.

Nonetheless, this data indicates that, although the ligase-dead mutants clearly struggle with replication, this replication challenge is either not enough to activate the checkpoint (although we question this following the cell cycle and proliferation data), or the specific replication challenge caused by mutation of the NSMCE2 ligase is not sensed or responded to by the replication checkpoint.

Unexpectedly, there is a decrease in the level of Chk1 expression within the N2A WT cell line. This suggests that although the re-expressed wild type NSMCE2 is sufficiently expressed and forms the full SMC5/6 complex, it may not be interacting with cellular pathways in exactly the same way as the wild type cell line and therefore is not producing the same effects. This is important to consider for future studies using these cell lines.

3.3 Assessment of the ability of the NSMCE2 cell lines to complete replication

3.3.1 Analysis of the NSMCE2 cell lines ability to activate the decatenation checkpoint

The slow proliferation seen by the NSMCE2 knockout cells as well as the NSMCE2 ligase-dead cells may be explained by their difficulty moving through S phase, as shown in the previous FACS data. We have determined that this is not due to the activation of the replication checkpoint although activation of the DNA damage checkpoint may contribute to the slowed S phase completion. The fact that the NSMCE2 ligase dead cells accumulate significant >4N DNA content suggests that these cells also may have a problem completing replication, particularly in efficiently segregating their DNA as they grow and divide. Moreover, several other SMC5/6 studies have implicated the complex in removing connections between chromosomes, which is vital to allow correct DNA segregation and cytokinesis (Gallego-Paez et al., 2014; Bermúdez-López et al., 2010; Torres-Rosell et al., 2007; Torres-Rosell et al., 2005).

To investigate the ability of the NSMCE2 cell lines to efficiently segregate their DNA, NSMCE2 mutant cells were treated with ICRF-193 (10µM) an inhibitor of DNA

topoisomerase II (Topo II). This enzyme is required for the separation of a specific type of connection between intertwined sister chromatids, catenations. Therefore, providing the cells are able to, inhibiting this enzyme should activate the decatenation checkpoint, which blocks the cell cycle within G2/M to allow for these chromatid connections to be removed, prior to mitosis (Holm, 1994; Charbin et al., 2014). However, if they are unable to activate the decatenation checkpoint, then inhibiting this process should not largely effect their ability to move through the cell cycle, but connections between sister chromatids will not be removed as TopoII is inhibited. To investigate this possibility, cell cycle analysis was performed by flow cytometry for each NSMCE2 cell line (see method 2.3.3). The result of this analysis can be seen in Fig. 31.
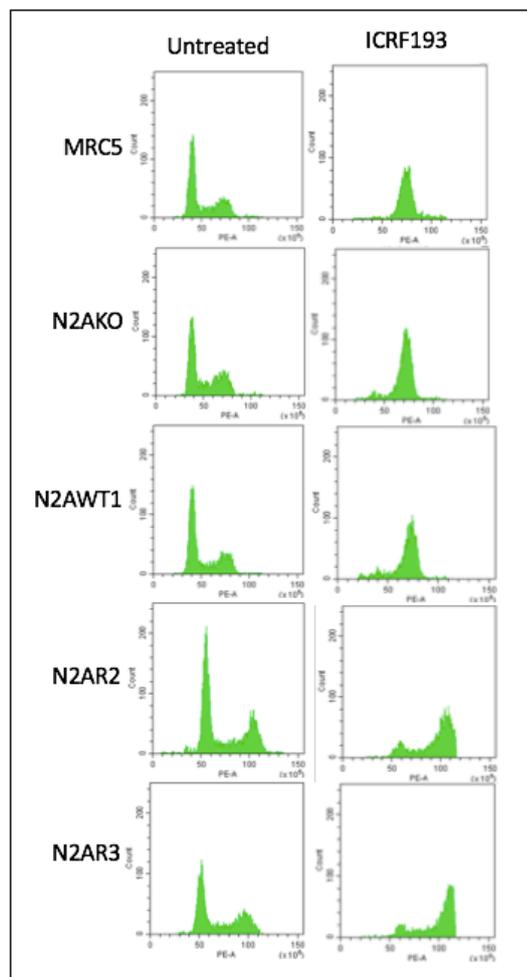
Figure 31 - Flow cytometry analysis showing the cell cycle proportions of untreated wild type MRC5 cells, NSMCE2 Knockout (N2AKO) and N2AKO following re-expression of wild type NSMCE2 (N2A WT1) and re-expression of ligase dead NSMCE2 (N2AR2 and N2AR3) as well as 10μM ICRF-193 treated cells from each cell line. Cells are strained with propidium iodide to visualise the DNA content. 4000 cells analysed. Treatment carried out for 16h.

As shown in Fig. 31, it is clear that within the MRC5, NSMCE2 knockout and NSMCE2 knockout cells with wild type NSMCE2 re-expression, ICRF-193 treatment is able to effectively activate the decatenation checkpoint. This suggests that these cell lines all have a functional decatenation checkpoint which halts the cells at the G2/M border successfully. However, within both the NSMCE2 ligase dead cell lines, following ICRF-193 treatment, the cells do not accumulate within G2, but instead continue to re-replicate their genome, producing a >4N population of cells. This suggests that the decatenation checkpoint is not operating to prevent missegregation when expressing the catalytically inactivated version of NSMCE2, implicating the NSMCE2 ligase activity within the activation or maintenance of the decatenation checkpoint and in the proper segregation of chromosomes.

3.3.2 Immunofluorescence analysis of the NSMCE2 cell lines

To further investigate the possibility that NSMCE2 may be involved in chromosome segregation, given the accumulation of >4N DNA content and an inability to block at the decatenation checkpoint in the NSMCE2 ligase-dead mutants, immunofluorescence was used to analyse cell division in each NSMCE2 cell lines. Two key parameters were monitored. Firstly, since the ligase-dead mutant cells accumulate a >4N DNA content and have problems segregating their DNA, we speculated that cell division may be compromised alongside chromosome segregation, and that this may result in increased cell size as cells continue to grow but fail to divide. Therefore, we measured overall cell size in each NSMCE2 cell line. Secondly, it is currently thought that some mutant cells that fail to accurately segregate their DNA, may do so because they enter mitosis before replication of the entire genome is complete. These cells would enter mitosis with unresolved connections between sister chromatids, such as unreplicated regions or collapsed fork intermediates, which then cause difficulty for successful DNA segregation. Cells which do this form mitotic DNA structures, such as micronuclei and anaphase bridges (Mankouri et al., 2013). Micronuclei are tiny extra-nuclear bodies of DNA. They are often caused by extensive DNA damage directly due to RS or due to defects in chromosome segregation often caused by RS (Fenech and Morley, 1985; Mateuca et al., 2006). Micronuclei are frequently noted as a footprint

for chromosome missegregation. As the SMC5/6 complex is often associated with RS resolution, it is possible that the chromosome segregation deficiency causing the >4N population within the ligase-dead cells could be due to an inability of the cells to remove replication intermediates characteristic of RS. Therefore, we determined the numbers of micronuclei within the NSMCE2 mutant cell lines to give further insight as to whether the NSMCE2 ligase activity may be involved in S phase completion before entry into mitosis, directly or indirectly.

In order to analyse the NSMCE2 cell lines by immunofluorescence, cells from each cell line; wild type MRC5, N2A KO, N2A KO with wild type NSMCE2 re-expression (N2AWt1) and N2AKO with NSMCE2 ligase-dead expression (N2AR2 and N2A R3), were grown on coverslips, then fixed and stained using method 2.3.4. Individual cells were then visualised by staining with α-tubulin antibody, detected using fluorophore 633-conjugated secondary antibody, while cellular DNA was stained with DAPI. The slides were then visualised using a confocal microscope and the average cell sizes and quantity of micronuclei present were determined for each cell line.

To investigate cell size, at least 200 cells of each of the NSMCE2 cell lines were measured and the result of this examination can be seen in Fig. 32. There is a significant increase in cell size between the MRC5 and each of the N2A KO, N2A R2 and N2A R3 cell lines (P<0.001). The average (mean) size of the MRC5 cells was 36.9μM and this increased to 43.3μM for the NSMCE2 knockout cells and to 51.9μM and 49.5μM for N2A R2 and N2A R3 respectively. The NSMCE2 knockout cells are therefore 17% bigger than the MRC5 cells. It is likely that this increase in size is due to continued growth throughout the extended cell cycle. However, there is a larger, approximately 35%, increase in cell size within both ligase-dead NSMCE2 cell lines compared to the MRC5 cells. These cells, which proliferate very slowly, were unable to activate the decatenation checkpoint and displayed a >4N DNA content under normal growth conditions, so it is likely then that these cells are increasing their size alongside an increased DNA content, and may have problems completing cell division. Moreover, these cell sizes indicate that it is possible the knockout cells are experiencing a mild version of the issues faced by the ligase-dead cells, although this is not possible to

explicitly determine from this data alone. A representative example of the sizes of each
of the NSMCE2 cell lines can be seen in Fig. 33.
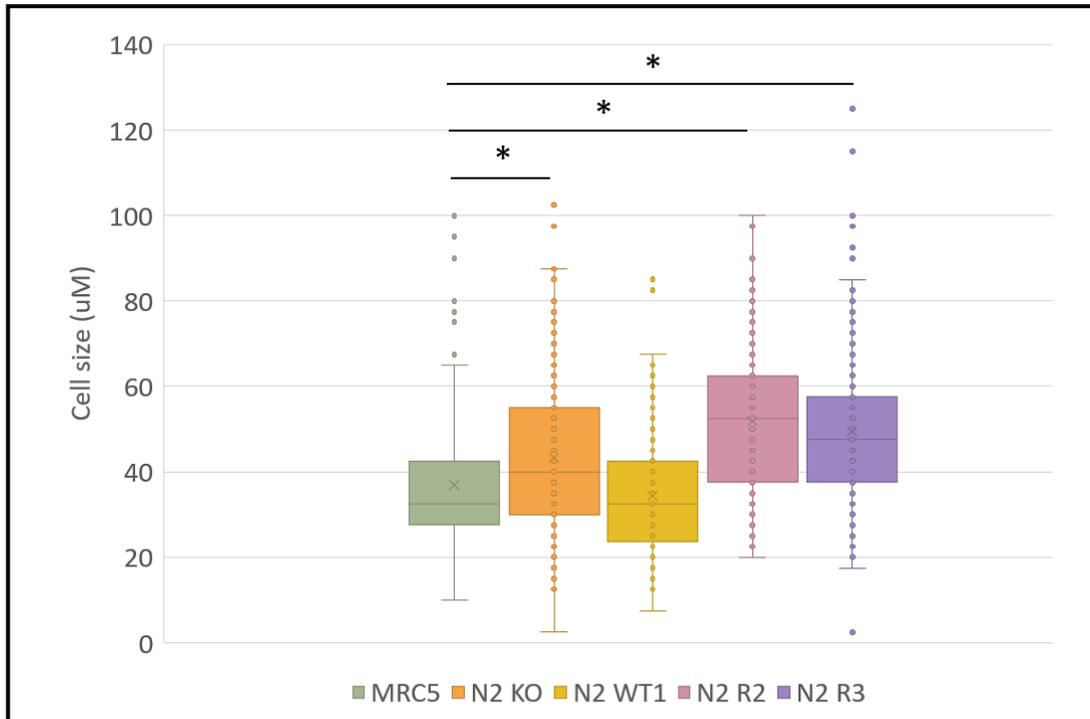
Figure 32 - Box plot to describe the cell sizes of each of the NSMCE2 cell lines; wild type MRC5, NSMCE2 knock out (N2A KO), NSMCE2 knockout with re-expression of wild type NSMCE2 (N2AWt1) and NSMCE2 knockout with re-expression of ligase-dead NSMCE2 (N2AR2 and N2A R3). Cells were visualised through confocal microscopy immunofluorescence using ZEN software and cell size was calculated as the largest measurable linear distance across each cell. Cells were mounted using Vectasheild which contained DAPI to stain the DNA. 200 cells analysed per cell line. Statistical analysis was carried out using an 2-way, unequal variances T test. *=p<0.001.
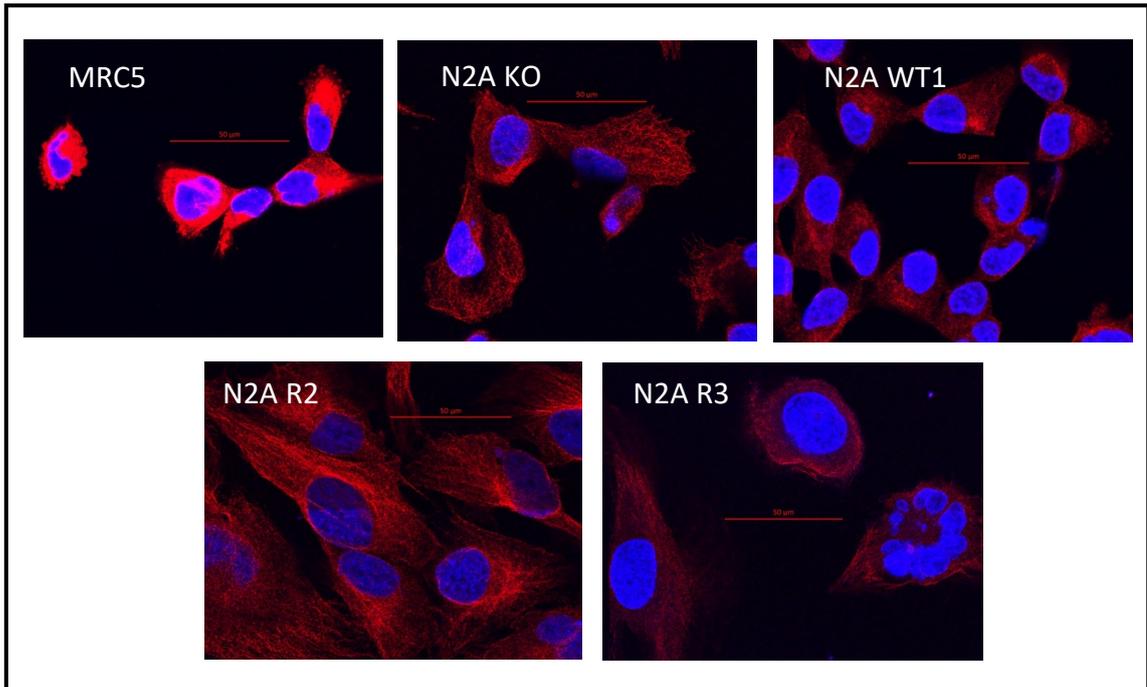
Figure 33 - Immunofluorescence pictures taken using a confocal microscope and visualised in ZEN software to analyse cell sizes of each of the NSMCE2 cell lines; wild type (MRC5) and NSMCE2 knockout (N2AKO) cells as well as N2A KO cells following re-expression of wild type NSMCE2 (N2Awt1) or ligase-dead NSMCE2 (N2AR2, N2AR3). *Cells were mounted using Vectasheild, which contained DAPI to stain the DNA blue.*

As well as studying the size of each of the NSMCE2 cell lines to examine the possibility the NMCE2 protein is involved in chromosome segregation, we also analysed the number of micronuclei, a product of faulty replication and increased DNA damage, produced by each of them. Examples of micronuclei can be seen in Fig 34. At least 200 cells were analysed for each cell line and the increase in the quantity of micronuclei compared to wild type MRC5 control cells is presented in Figure 35.
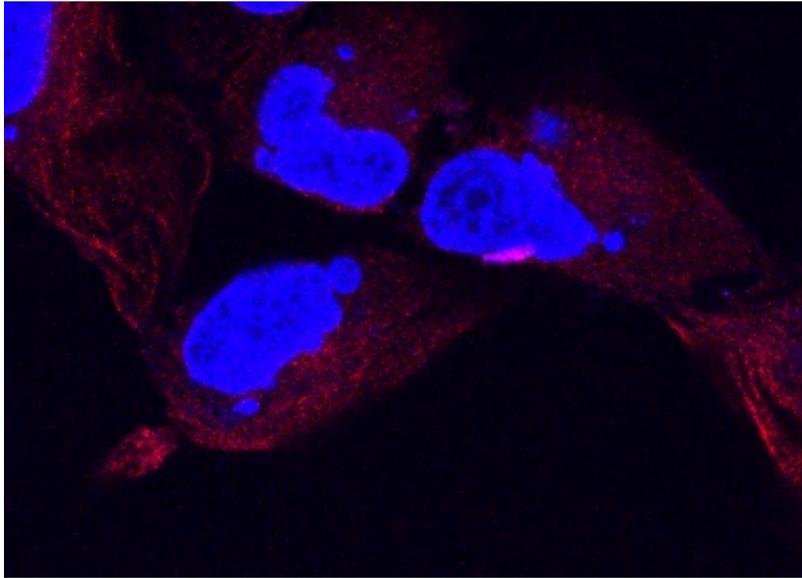
Figure 34 - Immunofluorescence pictures taken using a confocal microscope and visualised in ZEN software to analyse micronuclei within the NSMCE2 knockout cell line with ligase-dead NSMCE2 restoration (N2A R3). Cells were mounted using Vectashield, which contained DAPI stain the DNA blue.
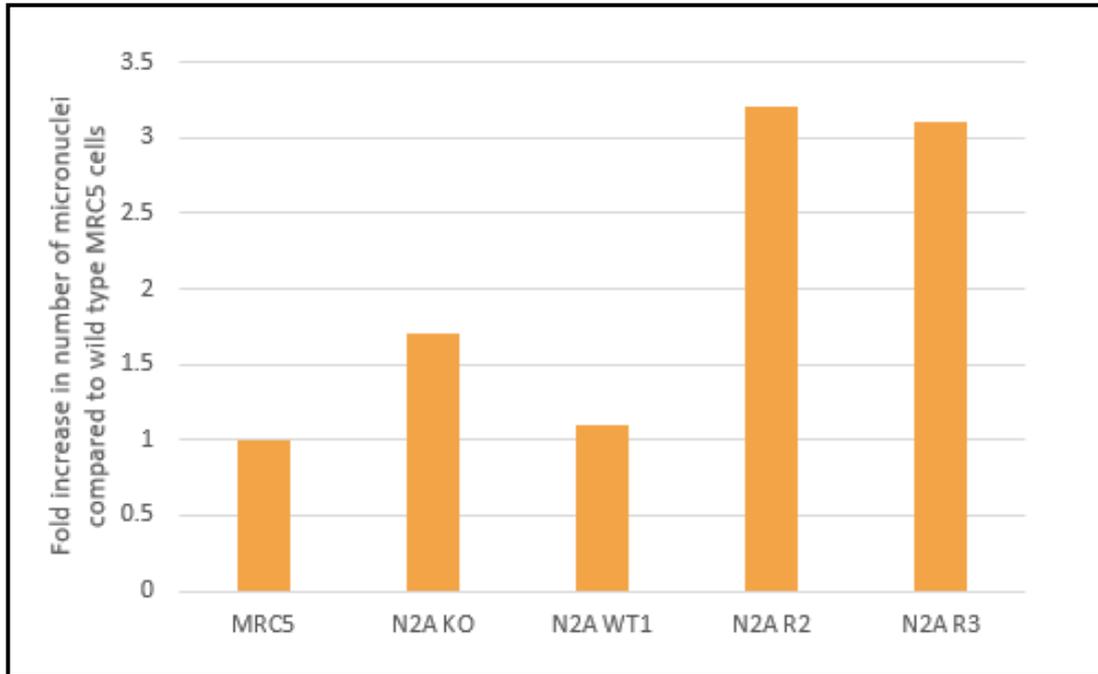
Figure 35 - Fold increase in the number of micronuclei within each of the NSMCE2 cell lines; NSMCE2 knockout (N2AKO) cells as well as N2A KO cells following re-expression of wild type NSMCE2 (N2Awt1, N2Awt6) or ligase-dead NSMCE2 (N2AR2, N2AR3), *when compared to the wildtype MRC5 cells. Cells were analysed through confocal microscopy immunofluorescence and were mounted using Vectashield which contained DAPI. At least 200 cells were analysed per cell line.*

Fig. 35. illustrates that loss of NSMCE2 in its entirety causes a greater than 1.5 fold increase in the number of micronuclei found within the cells. As these structures often form following RS and DNA segregation difficulties, these data directly associate NSMCE2 with either of these processes. Furthermore, loss of only the catalytic NSMCE2 subunit causes a much more severe, 3-fold, increase in micronuclei within the cell lines compared to control MRC5 cells. As well as the increased micronuclei, also note that there was an increase in globular nuclei within the NSMCE2 ligase-dead cell lines, such as the one shown in the N2AR3 cell line in Fig. 33. This data strongly supports the theory that the ligase-dead NSMCE2 cells are having problems with replication and segregation, suggesting that this may be due to RS causing an incomplete S phase before mitosis, with the known roles of the SMC5/6 complex in DNA damage repair and the RS response hard to ignore. However, it is also entirely possible that NSMCE2 may also be directly involved in chromosome decatenation and segregation itself, which would again directly cause the increase in micronuclei. This data again suggests the possibility that the NSMCE2 knockout cell line may be experiencing a mild defect similar to that of the NSMCE2 ligase dead cell lines.

3.4 Protocol Development for the bioSUMO strategy

The NSMCE2 SUMO ligase activity is clearly important for chromosome maintenance, having been associated with DNA repair and RS recovery processes since its discovery, and it is thought that this ligase activity may be how the SMC5/6 complex carries out many of its functions (Andrews et al., 2005; Chavez et al., 2010; Branzei et al., 2006; Kliszczak et al., 2012). For this reason, it is vital that the SUMOylation targets of NSMCE2 are identified and the modifications are characterized. These NSMCE2 knockout and ligase-dead NSMCE2 cell lines provide an opportunity to do so, looking for the loss of SUMOylation targets in these mutants relative to the wild type MRC5 cells. Therefore, we wanted to establish an assay for isolation of SUMOylated proteins to apply in these mutant cell lines. To do this, a protocol was adapted from (Pirone et al., 2016) and was developed.

In general, the protocol isolates SUMOylated proteins by the action of *in vivo* biotinylation of SUMO molecules before they become conjugated to specific proteins within the cell. To do this, cells are transfected with a bio-tagged SUMO conjugate expression plasmid, and once the bio-SUMO protein is expressed within cells, it becomes rapidly biotinylated through the action of the co-transfected *E.coli* BirA enzyme. These biotin-tagged SUMO molecules are then utilized by the cells endogenous SUMOylation system, becoming conjugated to SUMOylation targets within the cell. These modified proteins can then be isolated through streptavidin-based affinity chromatography due to the strong interaction between their biotin-tagged SUMO modification and streptavidin. Once bound, stringent washes are carried out and the SUMO-bound proteins can be eluted before analysis through western blotting. A diagram illustrating the overall protocol can be seen in Fig. 36. The aim of the following experiments was to optimize this methodology using MRC5 cells for future application with the NSMCE2 mutant cell lines.
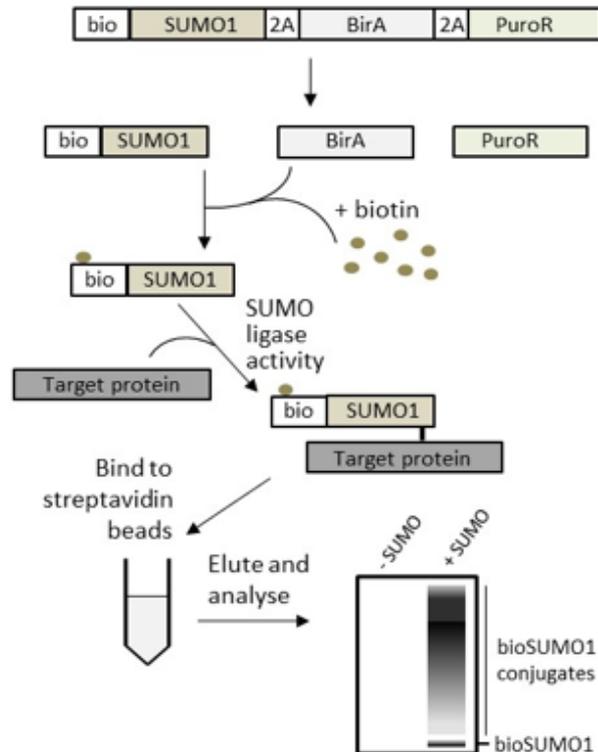
Figure 36 - Diagram outlining the method used to detect SUMOylated proteins within human cells. The bio-SUMO1 conjugate becomes expressed in cells following plasmid transfection. This bio-tagged conjugate becomes biotinylated through the action of BirA meaning when a SUMO ligase attaches this SUMO molecule to a target protein, the target protein has a biotin tag and can be purified with streptavidin beads. The protein elution from the beads can be analysed through western blot. Figure taken adapted (Pirone et al., 2016) by Dr Elaine Taylor.

3.4.1 Initial investigations of the bioSUMO strategy

Initially, we performed this protocol essentially as described in section 2.2.10, to determine whether we could isolate SUMOylated proteins from MRC5 cells, following transfection with either bio-tagged SUMO1, SUMO2 or SUMO3, relative to an empty vector control. The experiment was undertaken using a 1x10cm plate per transfection and the biotin was added into the media 24 hours after the transfection took place. The cells were then collected 24 hours after this. The wash procedure took place using 300µl of each wash buffer in a microcentrifuge tube, which was aspirated for removal. Once protein elution from the beads had taken place, 1/10th of the extract, flow through and eluate were resolved through SDS PAGE gel electrophoresis before western blotting (as methods 2.2.4 and 2.2.5) with Streptavidin HRP antibody in order to highlight any biotinylated SUMO-conjugated proteins. The results can be seen in Fig. 37.
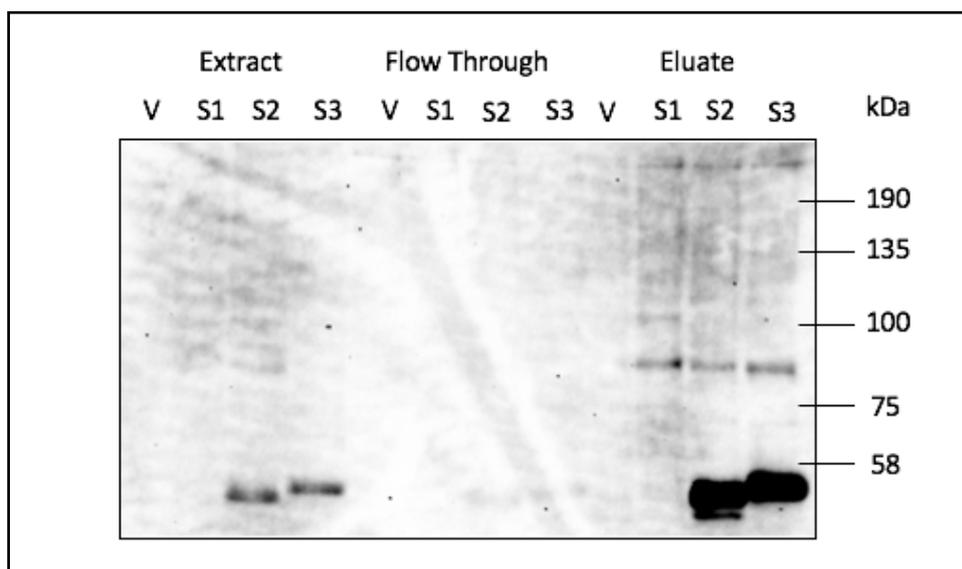
Figure 37 - Western Blot showing SUMOylated proteins isolated from MRC5 cells following transfection with pbio-V (empty vector control), pbio-SUMO-1. pbio-SUMO-2 and pbio-SUMO3. The blot was probed with Streptavidin HRP to visualise all SUMOylated proteins due to their biotin tag. Samples equivalent to 1/10th volume of eluate and of cell extract from one 10cm plates per transfection is shown.

Fig. 37 demonstrates that the methodology and pulldown has been successful. This is particularly clear within the SUMO-2 and SUMO-3 lanes, as the SUMOylated proteins in the extract samples, tagged with either SUMO-2 and SUMO-3 ($\approx$50kDa) have been concentrated in the eluates of these samples. There is no obvious pull down of an individual SUMOylated protein tagged with SUMO-1 from the extract to eluate, however, there is a smear of SUMOylated proteins found in all three of the SUMO eluates from 100kDa upwards as well as a band at ~85kDa in each lane, that were not detected in eluate in the absence of SUMO expression. This ~85kDa band signifies a protein tagged with either SUMO-1, SUMO-2 or SUMO-3 respectively, that has been concentrated from the extract to the eluate using this protocol. It is not possible to visualise free SUMO in this experiment as it has run off the bottom of the gel. As this was only 1/10th of the eluate, the sensitivity was quite high and we assumed that we could achieve much higher yields, enabling the isolation of specific SUMOylated proteins, with the remaining eluate. To achieve this, the remaining sample was resolved through SDS-PAGE gel electrophoresis before western blotting. The NSMCE2 component of the SMC5/6 complex is auto-SUMOylated *in vivo* (Potts and Yu, 2005), so this was a good candidate for analysis, to determine the possibility of detecting

specific SUMOylated proteins within the eluate. The result of this western blot, probed with NSMCE2 antibody can be seen in Fig. 38.
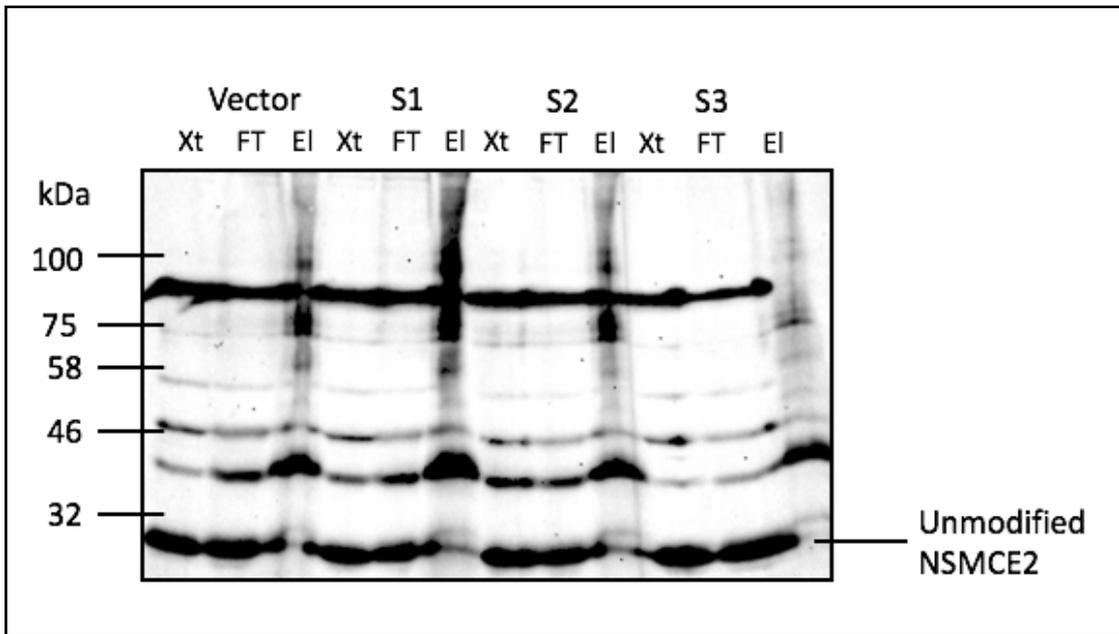
Figure 38 - Western Blot showing SUMOylated proteins isolated from MRC5 cells following transfection with pbio-V (empty vector control),, pbio-SUMO-1. pbio-SUMO-2 and pbio-SUMO3. The blot was probed with NSMCE2 antibody to visualise unmodified and SUMOylated NSMCE2 proteins. Samples equivalent to 9/10th volume of eluate and cell extract from one 10cm plates per transfection is shown. Xt - extract, FT- flow through, El - Eluate.

As shown in Fig. 38, the NSMCE2 antibody cross-reacts with multiple bands in each of the eluate fractions, including the negative control (vector) eluate. This suggests that these relate to non-specific protein interactions with the streptavidin beads. Therefore, to reduce the level of non-specific binding, the experiment was repeated using more stringent wash conditions. These washes took place in a spin column to remove all traces of each wash buffer, with vigorous inversions to ensure thorough washing. An increased volume of 500μl of each wash buffer was also used for each wash. This experiment used only the SUMO-1 and Vector only plasmids transfected into wild type MRC5 cells, as well as MRC5 controls with and without biotin supplementation. The timings for transfection and biotin supplementation were the same as the previous experiment. The results of this western blot when probed with either Streptavidin HRP or NSMCE2 antibody is shown in Fig. 39.
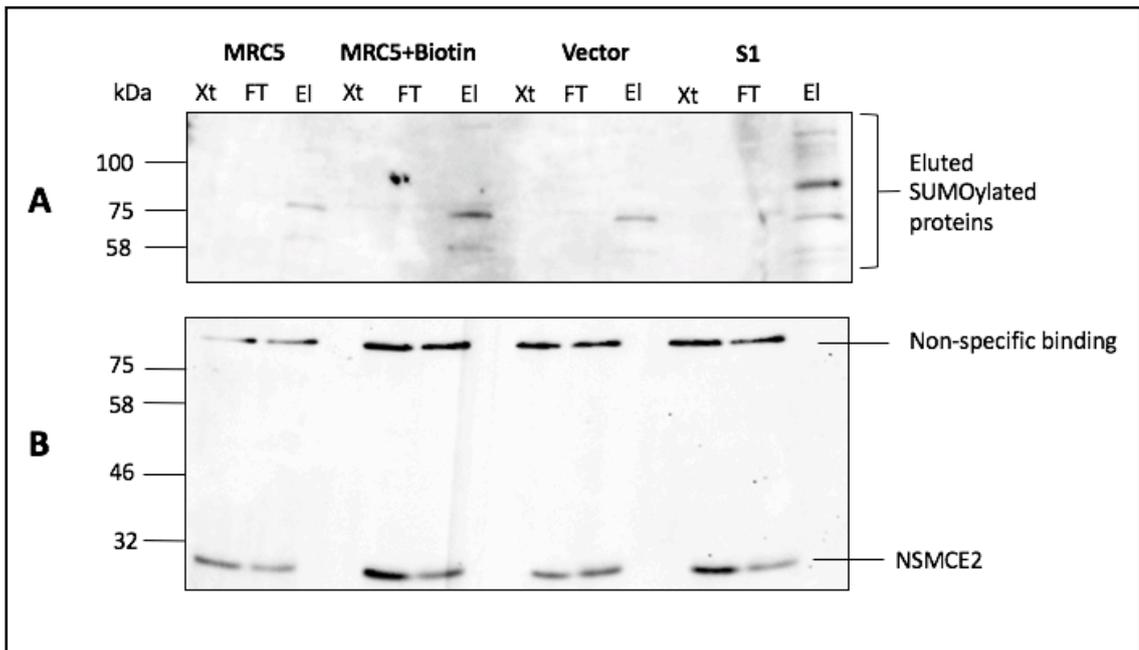
Figure 39 - Western Blot showing SUMOylated proteins isolated from MRC5 cells following transfection with pbio-V (empty vector control) and pbio-SUMO-1. Un-transfected wild type MRC5 cells were also analysed both with and without Biotin addition. The blot was probed with Streptavidin HRP (A,) which detects all biotinylated proteins to allow us to visualise SUMOylated proteins, and NSMCE2 antibody (B) to visualise unmodified and SUMOylated NSMCE2 proteins. Samples equivalent to ½ volume of eluate and ½ volume of cell extract from one 10cm plates per transfection is shown.

Fig 39A illustrates that after more effective washing, a much cleaner eluate is achievable, with some SUMOylated proteins highlighted within the SUMO-1 eluate fraction which are not present within the controls. Although this is a positive result, the detection level is relatively low, and we were unable to detect any SUMOylated NSMCE2 in the SUMO1 eluate in this experiment (see Fig 39B). Given the low level of total SUMOylated protein detected in this experiment it seems likely that any SUMOylated NSMCE2 may be below the level of detection in this experiment. Consequently, the experiment was then repeated for bio-SUMO-1 and an empty vector control, using the same conditions but scaling up the quantity of material used (4x10cm plates per treatment). The results can be seen in Fig. 40.
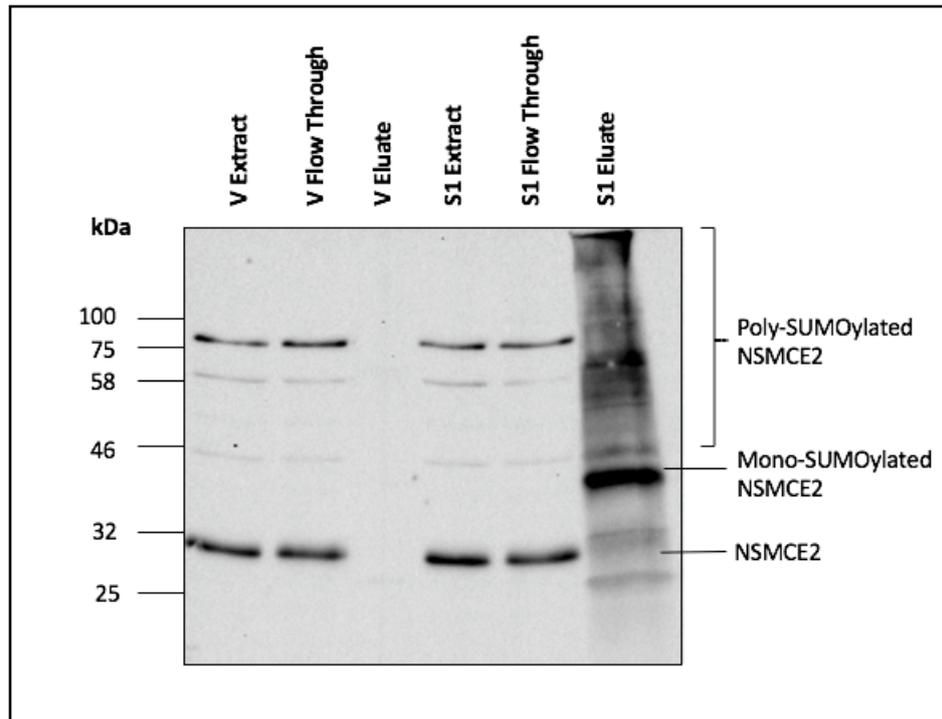
Figure 40 - Western Blot showing SUMOylated proteins isolated from MRC5 cells following transfection with pbio-V (empty vector control), and pbio-SUMO-1. The blot was probed with NSMCE2 antibody to visualise unmodified and SUMOylated NSMCE2 proteins. Samples equivalent to 4/5th volume of eluate and ½ volume of cell extract from four 10cm plates per transfection is shown.

Fig. 40 demonstrates that with four times as much cell material, it was possible to effectively isolate SUMOylated NSMCE2 using the bioSUMO method. The dark band within the SUMO-1 eluate at ≈ 40 kDa is expected to be mono-SUMOylated NSMCE2 whilst the large smear above this likely representing poly-SUMOylated forms. In contrast, no NSMCE2 cross-reacting bands are detected in the vector only eluate sample, confirming the specificity of the isolate procedure.

Unfortunately, although this result was very promising, we were unable to reproduce this result in further attempts. Therefore, in order to confirm the reliability of the methodology we were using, we began to validate every stage to ensure it was possible to achieve the results we expected.

3.4.2 bioSUMO methodology validation

The first thing we checked was the transfection efficiency. The experiment was undertaken using MRC5 cells transfected with a plasmid containing a GFP sequence to determine the expression levels of GFP at different ratios of transfection reagent to DNA. The ratios of reagent to DNA were 2:1, 3:1 and 4:1 along with an untransfected control and transfection efficiency was calculated on the basis of GFP expression. The results are shown in Table 6.

Table 6 - Table to show transfection efficiency of Wild type MRC5 cells when transfected with a plasmid containing GFP at transfection ratios of 2:1, 3:1 and 4:1 and untransfected. Cells were grown in Antibiotic free media to reach 90% confluency before transfection and collection after 24h. Cells in PBS were directly visualised by Flow cytometry using BD Canto II in PBS.

GFP transfection ratio (Transfection Reagent: DNA)	Transfection Efficiency. GFP expression
Untransfected	0.1%
2:1	59.5%
3:1	67.6%
4:1	72.1%

Table 6 shows that the transfection efficiency at each of the tested ratios is above 50%. This would be high enough to visualise the results we were expecting and we were using a ratio of 3:1 so it was ruled out as a reason for the experiment not working.

To limit the variability problems due to the inconsistency we had seen using the Streptavidin HRP antibody, we decided to use an alternative anti-biotin HRP antibody which should reliably detect all proteins with a biotin tag. In order to use this antibody, the detection capabilities of the antibody were first tested. A range of concentrations of Biotin-tagged SUMO were resolved by SDS-PAGE and western blotted using anti-biotin HRP antibody. The result can be seen in Fig. 41.
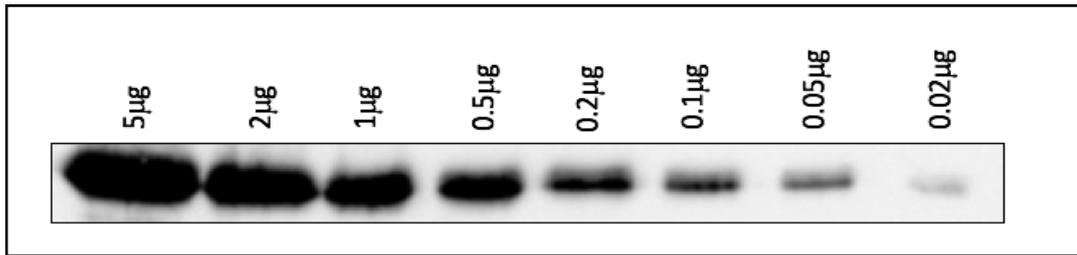
Figure 41 - Western Blot demonstrating the ability of the Anti-Biotin HRP antibody to detect concentrations of Bio-tagged SUMO.

Fig. 41 confirms that the Anti-Biotin HRP antibody can detect biotin-tagged SUMO protein at as little as 0.02µg.

The biotin-streptavidin interaction is known to be extremely strong and so we considered the possibility that one difficulty in detecting SUMOylated proteins, following our isolation procedure, may be related to the difficulty in fully eluting the biotinylated SUMO-tagged proteins from the streptavidin beads (Rybak et al., 2004). Therefore, to determine if a more effective removal method could be found, 1.5µg of recombinant biotin-SUMO1 was bound to 60µl of streptavidin beads overnight in a 3:1 ratio of bind and lysis buffer as described in the original protocol. The beads were then split three ways and three different elution methods were tested for biotin-tagged SUMO1 elution. The first method was as described within the methods section and as used for all previous experimentation, with the addition of 4x loading buffer and 200mM DTT along with heat and agitation, the second involved boiling the beads in 2% SDS, 10mM Biotin and 6M Urea and the third involved boiling the beads in 10mM EDTA and 10mM Biotin in 95% Formamide (Rybak et al., 2004). In addition, in order to maximise protein loading, samples were acetone precipitated and resuspended in a minimal volume prior to SDS-PAGE gel electrophoresis and western blotting (see method 2.2.4 and 2.2.5). The results of this western blot can be seen in Fig. 42.
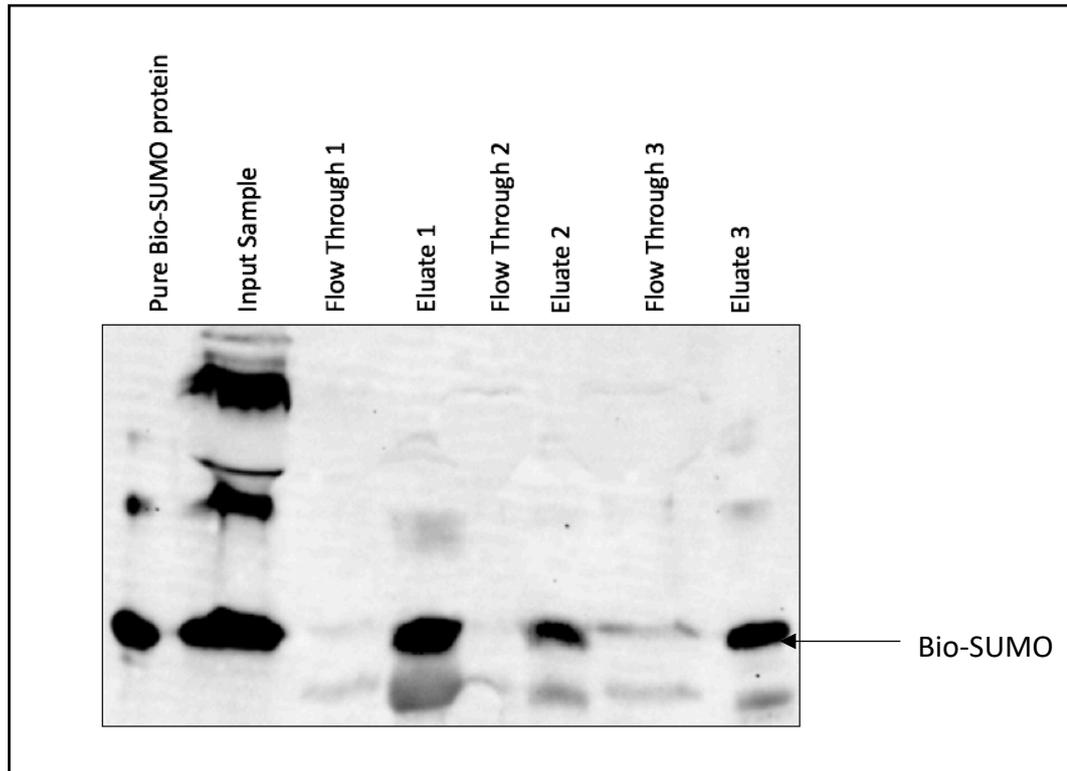
Figure 42 - Western blot to compare efficiency of elution methods for removal of biotinylated proteins from Strepatavidin beads using three different elution methods; 1- 30μl SUMO Elution buffer added, 99℃ for 8 minutes, brief vortex and 99℃ for 5 minutes, 2- 24μl 2% SDS, 10mM Biotin, 8M Urea in PBS added and incubated for 15 minutes at RT, 96℃ for a further 15 minutes following the addition of 6μl 5x SDS PAGE loading buffer, 3- 15μl 10mM EDTA, 10mM biotin in 95% Formamide added and incubated for 5min at 90℃ before 15μl 2x loading buffer was added. Recombinant bio-SUMO protein = 0.5. Input sample = 0.5μg bioSUMO following acetone precipitation to concentrate. Each set of beads for elution had 0.5μg of Bio-SUMO protein bound to 20μl beads O/N. The flow through underwent acetone precipitation before loading.

As shown in Fig. 42, the first elution method, which we had used in previous experiments, was the most effective at removing the bio-SUMO protein from the Strepatividin beads, with the eluate having very similar intensity to the input sample suggesting a very good level of recovery of biotin-SUMO1. This implies that it is not the removal of the proteins from the beads which is preventing detection of SUMOylated proteins in our experimental isolates. At this point, each stage of the protocol had been successfully validated so it was difficult to determine what was causing the variability between repeats.

Following the quality control tests to assess the ability of the bioSUMO method to isolate SUMOylated proteins from cell extract, the experiment was repeated with MRC5 cells transfected with SUMO-1 as the experimental sample and untransfected wild type MRC5 cells as the negative control. Two 10cm plates were used for each condition and the methodology described in method 2.2.10, with the more stringent washes, was scaled up appropriately, apart from the concentration of NEM which was doubled to ensure the maintenance of SUMOylated proteins. Biotin was included in the growth medium from the point of transfection and the cells were collected 32 hours later. Following this, half of the extract, flow through, and eluate from each condition were resolved through SDS PAGE gel electrophoresis before western blotting (see method 2.2.4 and 2.2.5) and the membrane probed with anti-biotin antibody. The result of this experiment can be seen in Fig. 43.
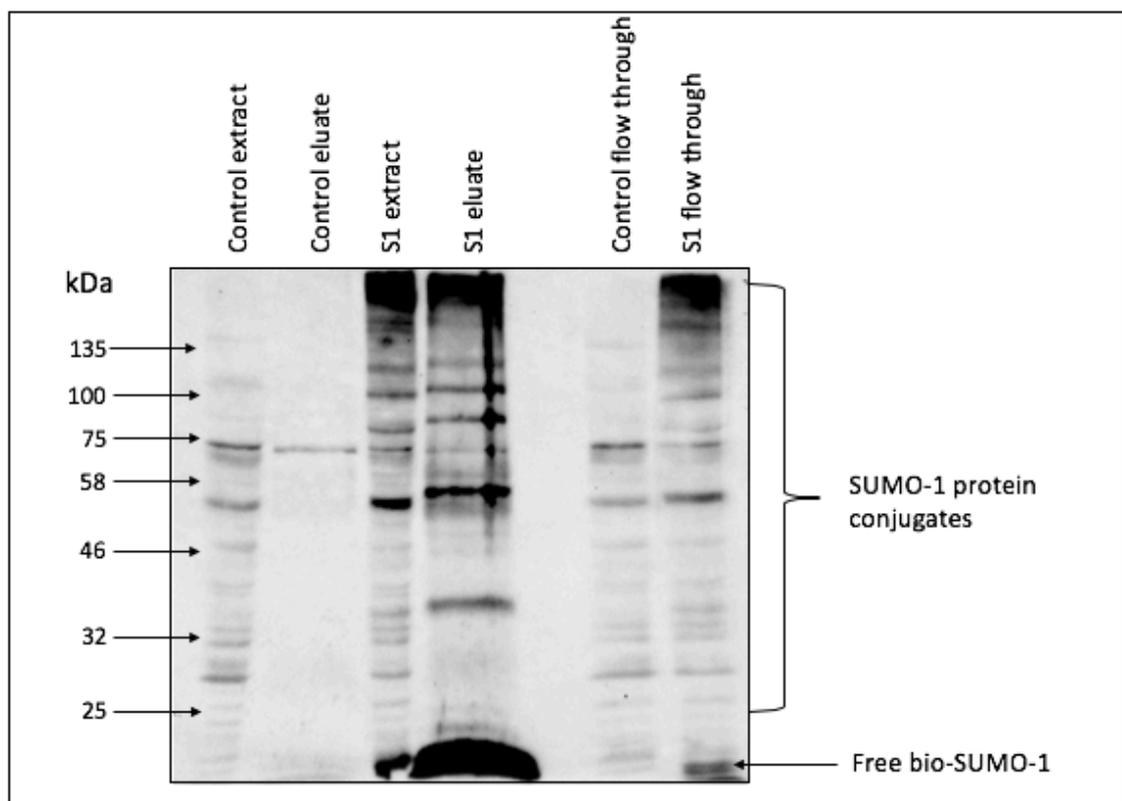
Figure 43 - Western Blot showing SUMOylated proteins isolated from MRC5 cells following transfection with pbio-V (empty vector control) and pbio-SUMO-1. The blot was probed with anti-biotin HRP to visualise all SUMOylated proteins. Samples equivalent to ½ volume of eluate of cell extract from two 10cm plates per transfection is shown.

As shown in Fig. 43, using this method it was clearly possible to isolate SUMOylated proteins from SUMO-1 transfected wild type MRC5 cells (free bio-SUMO1 and SUMO-1 protein conjugates) as shown by the large smear of proteins within the S1 eluate which is not present within the control samples. The other half of these experimental samples were western blotted with anti-RanGAP1 antibody to detect SUMOylated RanGAP1, a relatively abundant SUMOylated protein (Matunis et al., 1996). This enabled determination of the sensitivity of the experimental protocol. The result of this western blot is shown in Fig. 44.
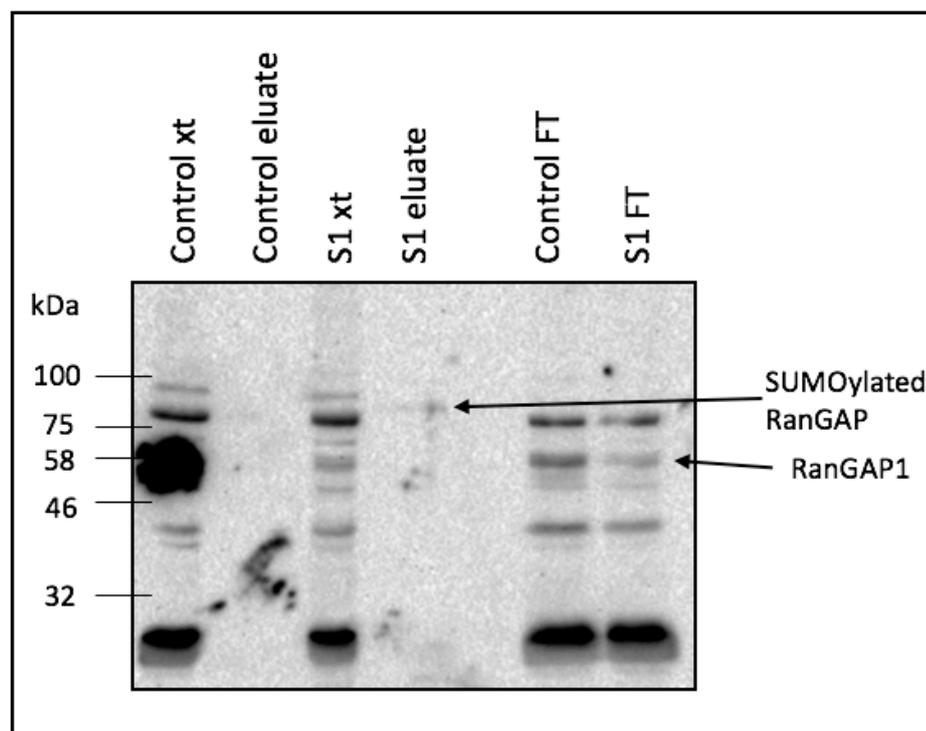
Figure 44 - Western Blot showing SUMOylated proteins isolated from MRC5 cells following transfection with pbio-V (empty vector control) and pbio-SUMO-1. The blot was probed with anti-RanGAP antibody to visualise unmodified and SUMOylated RanGAP proteins. Samples equivalent to ½ volume of eluate and of cell extract from two 10cm plates per transfection is shown.

Fig. 44 illustrates that using these conditions only a small amount of SUMOylated RanGAP was isolated, possibly giving an explanation as to why we were unable to see any SUMOylated NSMCE2 within many of the previous experiments as SUMOylated NSMCE2 is likely to be much less abundant than SUMOylated RanGAP. To overcome this, the experiment was repeated once more using more plates per sample but again

no SUMOylated NSMCE2 could be detected. Due to the time constraints of this project, this experiment could not be continued any further.

Chapter 4: Discussion

RS and GI play a key role in driving the development and progression of cancer. RS is defined as replication challenges such as physical blocks which cause the slowing, stalling or collapsing of replication forks (Zeman and Cimprich, 2014). This stress causes the activation of the S phase checkpoints, which allows the DDR to be carried out to repair any damage caused, as well as allowing the replication fork to reform once the source of the stress has been resolved (Mazouzi et al., 2014). These mechanisms act as a surveillance system with the aim of preserving genome integrity and preventing genetic changes. However, where RS occurs more frequently, such as at common fragile sites, chromosomes are prone to rearrangements during repair of collapsed forks. This can lead to mutations and loss of heterozygosity, inducing a state of GI, which can drive the development of cancer (Halazonetis et al., 2008; Beckman and Loeb, 2006). In precancerous lesions, where a mutation has occurred in an oncogene causing dysregulated proliferation, increased RS and GI causes the activation of p53 which acts as a tumourigenesis barrier. However, ongoing RS and GI provides a continual pressure for p53 mutation, which would lead to the evasion of cell death and so the development of cancer (Halazonetis et al., 2008).

Due to this possible chain of events, it is clearly important that the repair and recovery from RS and its consequences are highly effective. A key molecule known to be involved in this process is the SMC5/6 complex (Lehmann et al., 1995). This protein complex is a member of the structural maintenance of chromosomes family, along with cohesin and condensin, and is made up of 6 key components; SMC5, SMC6, NSMCE1, NSMCE2, NSMCE3 and NSMCE4 (Taylor et al., 2001; Taylor et al., 2008; Harvey et al., 2004; Potts and Yu, 2005). The NMSCE2 component is a vital protein with SUMO ligase capabilities, debated to be the means by which the SMC5/6 complex carries out the majority of its functions (Andrews et al., 2005; Rai et al., 2011).

Since its discovery, the SMC5/6 complex was linked to genome maintenance and repair of DNA damage (Nasim and Smith, 1975; Lehmann et al., 1995). Originally, there was strong evidence from epistasis analysis that the yeast SMC5/6 complex was

involved in the repair of damage through HR, with the complex being directly associated with the RAD51 pathway (McDonald et al., 2003; Lehmann et al., 1995). This was later supported in many cell types including human cells where the inactivation of SMC5/6 caused defects in HR (Potts and Yu, 2007). Furthermore, research suggests that the SMC5/6 complex may act to remove recombination intermediates formed by stalled or collapsed forks through HR mechanisms (Ampatzidou et al., 2006; Branzei et al., 2006), possibly with the help of cohesin (Kegel and Sjögren, 2010).

As well HR, there are many other possible roles for the SMC5/6 complex. For example, the complex has been seen to prevent accumulation of positive supercoiling ahead of the replication machinery to minimize stress as well as remove protein connections between sister chromatids to allow for efficient segregation (Kegel et al., 2011; Outwin et al., 2009). Moreover, two human patient studies have identified that destabilization of the entire SMC5/6 complex or the loss of only NSMCE2, causes the formation of mitotic DNA structures such as micronuclei and nucleoplasmic bridges, often formed as the result of hindered chromosome segregation, as well as increased DNA damage at a cellular level. The NSMCE2 mutant patients also present with primordial dwarfism, a clinical feature often associated with DNA damage disorders (Payne et al., 2014; van der Crabben et al., 2016). Due to the wide range of possible genome maintenance functions suggested for the SMC5/6 complex, it has been postulated that the role of the complex may be to coordinate many of these processes.

As there is a question mark over the current role of NSMCE2, its SUMO ligase function and the SMC5/6 complex as a whole, the aim of this study was to characterize human NSMCE2 CRISPR-Cas9 cell lines which had been produced by this laboratory (NSMCE2 knock out, NSMCE2 knockout with wildtype NSMCE2 re-expression and NSMCE2 knockout cells with ligase-dead NSMCE2 expression) in order to determine a possible genome maintenance and repair roles for these proteins.

4.1 Confirmation of the NSMCE2 cell lines

Throughout the study of the SMC5/6 complex, many different cell types have been used and different methods to remove the function of part or all of the complex have been demonstrated. Initially much of the early work was undertaken using budding and fission yeast, however there was a difference in structure and requirement for the Nse5 and Nse6 subunits between the two yeast strains suggesting that the functions and interactions may be variable between species (Pebernard et al., 2006; Duan et al., 2009) (Stephan et al., 2011b). When the SMC5/6 complex was later isolated in humans it was found to have a very similar structure to the fission yeast (Taylor et al., 2008; Verver et al., 2016a), however SLF1 and SLF2 (the Nse5 and Nse6 equivalents) were not part of the core complex and showed little homology (Räschle et al., 2015). At this time there were also discrepancies between the results of the yeast and human cells. For this reason, it was important to this project that the cell lines we used were human, allowing us to make valid conclusions as to the function of the SMC5/6 complex within human chromosome maintenance and repair.

Previous studies of the human SMC5/6 complex have mainly used RNAi knockdowns of part or all of the complex to try and determine function (Gallego-Paez et al., 2014; Potts et al., 2006; Potts and Yu, 2005; Taylor et al., 2008). However, the use of these techniques often still allows the gene to be partly expressed despite the knockdown, therefore making clear conclusions challenging as the protein is still partly able to function. There is also a risk of large scale off target effects, some of which have been documented within SMC5/6 RNAi knockdown studies (Wu et al., 2012). For these reasons, the more effective method of CRISPR-Cas9 genome editing was used to manufacture the stable NSMCE2 mutant cell lines used in this project. The project also utilised NSMCE2 knockout cell lines in which wild type NSMCE2 expression was restored. This allowed confirmation that any effects seen within the mutant cells were specific to the loss of NSMCE2 or its ligase capabilities, not from off target effects, providing more valid conclusions to be drawn and greater insight into NSMCE2 and its possible roles (Boettcher and McManus, 2015).

To validate these NSMCE2 mutant cell lines, western blotting confirmed that NSMCE2 expression had been completely abolished within the NSMCE2 knockout cell line and restored in the NSMCE2 wild type and ligase-dead re-expression cell lines, albeit slightly over-expressed. There was no effect on the other component expression, as expected, further confirming the validity of the CRISPR-Cas9 knockout expression. Following this, it was important to identify the effect the removal of NSMCE2 or its SUMO ligase expression had on the ability of the whole complex to assemble. It was expected that the rest of the complex would be able to form as NSMCE2 does not sit as part of the main complex, only bound to SMC5, and the immunoprecipitation assay confirmed this (Duan et al., 2009; Andrews et al., 2005; Sergeant et al., 2005). This allowed us to draw conclusions about the specific loss of the NSMCE2 subunit or its SUMO ligase activity, rather than the loss of the entire complex. It was also important to note that the re-expressed NSMCE2, with and without the SUMO ligase function, assembled into the SMC5/6 complex, determining that not only is it expressed, but it is interacting with the complex components as normal. The information gained from these experiments gave us the insight to confidently use each of the NSMCE2 cell lines for further analysis, providing us the ability to determine how NSMCE2 is involved in DNA maintenance and repair in human cells.

4.2 Cell Cycle analysis for each of the NSMCE2 mutant cell lines

Previous to this study, proliferation data had been collected for each of the NSMCE2 cell lines described, indicating that the NSMCE2 knockout cell line had a mild proliferation defect whilst the NSMCE2 ligase dead cell lines had a very severe proliferation defect, proliferating at approximately half the rate of MRC5 cells. This is supported by a previous study of CRISPR-Cas9 NSMCE2 knockout human cells which demonstrated slowed proliferation, although this study could not verify that all effects seen were solely dependent upon NSMCE2 knockout (Verver et al., 2016b). However, it was not clear why the defects seen within our NSMCE2 mutant cells were occurring, whether the slow proliferating cell lines were having issues progressing through a specific cell cycle phase, through the entire cell cycle or whether they were

experiencing large-scale cell death. For this reason, it was vital that cell cycle analysis by FACS was undertaken on each mutant cell line.

Early studies in budding yeast demonstrated that the undefined role of the SMC5/6 complex may be occurring in S phase (Torres-Rosell et al., 2007), with many other studies later describing more specific S phase progression roles. These include; DNA damage repair, recovery from collapsed replication forks by HR, regulating efficient overall segregation and correct chromosome structure when moving from replication into mitosis, and managing replication fork pausing sites particularly in rDNA replication (Ampatzidou et al., 2006; Gallego-Paez et al., 2014; Peng et al., 2018; McDonald et al., 2003; Menolfi et al., 2015). Furthermore, previous FACS analysis undertaken on SMC5/SMC6 siRNA treated human cell lines have revealed a delay and slowed progression through S phase, further supporting an S phase role for the SMC5/6 complex (Gallego-Paez et al., 2014). As it is widely suggested that NSMCE2 carries out many of the SMC5/6 functions, we anticipated that both the NSMCE2 knockout and the NSMCE2 ligase-dead cell lines would follow this trend and both demonstrate cell cycle stage specific S phase defects under FACS analysis.

The FACS profile analysis of the NSMCE2 knockout cells confirmed that the proliferation defect was due to a mild cell cycle stage specific defect, with an increased proportion of cells found within S and G2 phases, as expected. Moreover, both NSMCE2 ligase-dead cell lines had a very large increase in S phase cells, with almost no cells found within G1. Surprisingly, these cell lines also presented a >4N population of cells with a second S phase peak within a second cell cycle. This 8N content indicates that the ligase-dead cells may be undergoing 're-replication' rather than segregating their DNA content and undergoing cytokinesis. This is supported as chromosome segregation has been suggested as a key function of the SMC5/6 complex (Gallego-Paez et al., 2014; Bermúdez-López et al., 2010; Torres-Rosell et al., 2007; Torres-Rosell et al., 2005).

Following the S phase replication roles already suggested for the SMC5/6 complex, we hypothesised that the slow S phase progression noted in both the NSMCE2 knockout

and NSMCE2 ligase-dead cells possibly occurs due to problems incurred during replication or difficulty recovering from RS. This slowed replication could cause unreplicated regions and unresolved RS intermediates to persist which would then lead to the segregation defects thought to be occurring within the NSMCE2 ligase dead cells. As there is thought to be no segregation defects within the NSMCE2 knockout cell line, it is possible that the slowed S phase progression is not as severe as the ligase-dead cells, meaning that replication can be completed in time leaving no unresolved issues. Likewise, it is also possible that the NSMCE2 knockout cell line is just as slow at replication, but the unreplicated regions and RS intermediates left behind are able to be resolved unlike within the ligase-dead cell line. We thought that expression of the catalytically inactivated version of NSMCE2 may be the result of a dominant-negative mutation, therefore allowing the latter effect to take place. The ligase-dead NSMCE2 may be acting to disrupt functions carried out by other components of the SMC5/6 complex or other factors which act in substitute following the absence of NSMCE2, such as other SUMO ligases or resolvase enzymes. These factors may be unable to be recruited to the sites where they are required as non-functional NSMCE2 is present and so is blocking their action. This has been suggested in a previous study which demonstrated that catalytically inactivated yeast NSMCE2 mutant cells had increased occupancy at DNA damage sites when compared to wild type cells, blocking the action of other proteins (Tapia-Alveal and O'Connell, 2011). As well as replication complications alone possibly causing the slowed S phase progression seen both following the loss of the entire NSMCE2 subunit or just its catalytic activity, it is possible that activation of the DNA damage or S phase replication checkpoints may be also contributing to this effect.

The subtle difference in S phase progression as seen between the NSMCE2 knockout and control cells can only be broadly visualised by this FACS presentation of the data. It would be important to follow this data up by pulse labelling populations of these cell lines with 5-ethynyl-2'-deoxyuridine (EdU) in order to determine the quantity of cells in S phase and the specific lengths of time they spend in this phase per cell cycle (Pereira et al., 2017). This would allow more specific accurate quantification of the

differences between the FACS profiles and so allow more precise conclusions to be drawn.

Throughout the study of the NSMCE2 mutant cell lines it became clear that the cells were prone to reversion, altering their proliferation rates and FACS profiles, particularly the ligase-dead cell lines. It is possible that the NSMCE2 ligase-dead cell lines began suppressing the expression of the ligase-dead NSMCE2 as we have noted that the cells seem to progress through the cell cycle better when not expressing NSMCE2 at all. It may also be possible that both the knockout and ligase-dead NSMCE2 cell lines begin to upregulate other cellular SUMOylators which may partially compensate for the loss of the NSMCE2 capabilities, or may upregulate the expression of other proteins which may undertake a similar genome maintenance role as NSMCE2. This meant that all the data had to be carefully studied and repeated in order to draw valid conclusions. All the NSMCE2 mutant cell data presented within this thesis was acquired reproducibly and all questionable data has been omitted but this should be considered within all future analysis of these cell lines.

4.3 Activation of the S phase checkpoints

Throughout its study, the SMC5/6 complex has frequently been associated with genome maintenance, specifically DNA repair and RS tolerance. The complex was connected with DNA repair following initial yeast studies where SMC5/6 knockout cells were very sensitive to damage treatment (Lehmann et al., 1995). Subsequently, many studies have been undertaken analysing its repair role, indicating that the SMC5/6 functions in HR repair with RAD51 in both yeast and human cells, repairing both endogenous and exogenously caused DNA damage (Ampatzidou et al., 2006; Potts et al., 2006; Verkade et al., 1999; De Piccoli et al., 2006; Harvey et al., 2004). Furthermore, its role in recovery from RS was initially shown in *S. pombe* where it was indicated that the complex was vital for the resolution of intermediate structures formed following collapsed forks (Ampatzidou et al., 2006). Following this, roles for the SMC5/6 complex in early as well as late stage RS recovery were then determined. The early stage function involves the stabilisation of the replication fork once it has

stalled to avoid collapse, whilst the late stage function involves the resolution of the collapsed fork and other intermediates by HR to allow fork restart (Irmisch et al., 2009; Branzei et al., 2006; Chen et al., 2013; Bustard et al., 2012). As well as following RS, the SMC5/6 complex has also been associated with controlling normal replication. For example, the complex is thought to be involved in controlling the organisation of DNA replication processing and timing through regulating Topo IIa and condensin binding, influencing supercoiling ahead of the replication fork, and regulating fork pausing in rDNA as well as elsewhere (Gallego-Paez et al., 2014; Peng et al., 2018; Menolfi et al., 2015; Kegel et al., 2011).

Due to the suggested functions in DNA repair and RS recovery, it was considered that the delayed S phase progression in the NSMCE2 mutant cell lines, may have been, at least in part, due to the activation of either of the S phase checkpoints; the DNA damage checkpoint or the replication checkpoint in the absence of NSMCE2 function. These S phase checkpoints are vitally important for ensuring genetic damage and RS are removed, before the cells undergo mitosis and begin the segregation of chromatids into two new daughter cells. Both of these checkpoints are able to halt the progression of cells through S phase, by the activation of ATM and Chk2 or ATR and Chk1 respectively, and so cause the build up of S phase cells found in both the NSMCE2 knockout and ligase-dead cell lines (Maréchal and Zou, 2013). As well as suggested functions following S phase checkpoint activation to resolve any issues faced, the SMC5/6 complex has also been associated with maintenance of these checkpoints. For example, in yeast, it has been suggested that the SMC5/6 complex is required to maintain the S phase DNA damage checkpoint. Mutants were found to activate the checkpoint but then continue to carry out aberrant mitosis as DNA repair was not undertaken, suggesting the checkpoint was not maintained (Verkade et al., 1999; Harvey et al., 2004).

Of course, for DNA damage checkpoint functions to be the cause of the change in cell cycle progression, DNA damage must be occurring. Our experiments showed that the accumulation of the damage markers, phopho-p53 and γ-H2AX, were increased in only the NSMCE2 ligase-dead mutants. This implies that only the ligase-dead cells are

accumulating DNA damage whilst the NSMCE2 knockouts are not. This is supported by the activation state of the S phase DNA damage checkpoint, where the checkpoint is only activated within the NSMCE2 ligase-dead cell lines. These results suggest that the activation of the DNA damage checkpoint is probably not the cause of slowed S phase progression in the knockout cell lines, although it may be at least contributing to this phenotype in the ligase-dead mutants.

The increase in DNA damage within the NSMCE2 ligase-dead cells is not unexpected, due to the DNA repair and homologous recombination functions of the SMC5/6 complex described earlier. However, it is surprising that this is not also the case in the knockout cell line. As previously described, this could be due the expression of the catalytically inactivated version of NSMCE2 being the result of a dominant-negative mutation, and so disturbing the action of other proteins which would replace the function of NSMCE2 in its absence. This could explain why no DNA damage is noted within the NSMCE2 knockout cell lines as other proteins are able to compensate for its loss.

Unlike the S phase DNA damage checkpoint, unexpectedly there was no activation of the replication checkpoint in any of the NSMCE2 CRISPR-Cas9 mutant cells, with no Chk1 activation being noted by western blot by any of the cells. This indicates that the S phase replication checkpoint is not causing the delayed S phase progression within any of the mutant cell lines. This led us to question whether the obvious replication problems the cells were facing, as shown by proliferation assay and FACS analysis, were problems which were not sensed by the replication checkpoint or whether the SMC5/6 complex and NSMCE2 itself are required for activating the checkpoint. To test this, FACS analysis as well as western blotting were undertaken for each of the NSMCE2 cell lines following 16 hour 2mM Hydroxyurea (HU) treatment which should cause RS and activate the replication checkpoint if it were possible to do so. Both methods implied that each of the NSMCE2 cell lines could activate the replication checkpoint so it is more likely then that the cause of the slowed S phase progression seen by these cells cannot be sensed by the replication checkpoint, or the activation of the checkpoint was below the detection level of our protocol.

On the basis of what is already known about the RS response functions of the SMC5/6 complex, it is thought more likely that the replication issues faced by these cell lines were directly causing the slowed S phase progression themselves but cannot be sensed or responded to by the replication checkpoint. This suggestion is similar to a study in *S. pombe* where Smc6 mutants accumulated RS intermediates as the late stage function of the SMC5/6 complex could not prevent fork collapse. These intermediates were not recognised by the G2 DNA damage checkpoint, so it is possible then that this type of RS may also not activate the replication checkpoint either (Ampatzidou et al., 2006). This indicates further interaction between the difficulty recovering from RS and DNA damage formation within these NSMCE2 mutant cell lines, as well as the fact that genetic damage often causes the RS itself. It is suspected that the initial problem, which may be the main cause of the slowed S phase faced by these mutant cell lines, is within the RS tolerance/repair pathways, as both the suggested SMC5/6 early and late stage functions cannot be carried out so replication forks cannot continue when faced with replication challenges. Due to this, unrecognised by the replication checkpoint, DNA damage accumulates as the replication forks collapse and form RS intermediates. This then causes the activation of the DNA damage response pathway, where the damage would normally be repaired in an SMC5/6 dependent manner. However, within the NSMCE2 knockout cells it is possible that other cellular proteins compensate for the loss of NSMCE2 and remove the DNA damage and repair the collapsed replication forks in its place, causing no substantial accumulation of DNA damage and activation of the S phase DNA damage checkpoint. As discussed earlier, due to the possible dominant-negative mutation, this may not occur within the NSMCE2 ligase-dead mutants and so the DNA damage remains and the DNA damage checkpoint is activated. It is possible the increased DNA damage itself aids the slowed S phase progression by causing more RS within the NSMCE2 ligase-dead cells compared to the NSMCE2 knockout cells. However, it is also likely that the S phase DNA damage checkpoint may be an additional stimulus.

To determine if activation of the DNA damage checkpoint does aid, at least in part, the slow S phase progression within the NSMCE2 ligase-dead cells, it would be important to carry out a treatment of ATM inhibitors (ATMi) to each cell line before cell cycle

analysis. As ATMi would inhibit the activation of the DNA damage checkpoint, if the cells were being halted at this checkpoint, the cell cycle profile would alter to be more similar to the NSMCE2 knockout cell lines. However, I believe it is more likely that it is the increased DNA damage and unresolved RS itself which is contributing to the increased slowing in S phase within the ligase-dead cells compared to the NSMCE2 knock out cells, with the checkpoint possibly marginally having an influence upon the cell cycle. In order to further corroborate the theories postulated within this thesis, it would also be necessary to investigate the replication profile, such as speed, fork stalling and fork recovery, of each the NSMCE2 mutant cell lines. This would be possible through DNA combing (Bianco et al., 2012). I suggest there would be a larger amount of stalled forks accumulated as well as reduced recovery within the NSMCE2 ligase dead cell lines compared to the knockout as the knockout is able to recover through other undisturbed cellular pathways. It would also be interesting to determine the ability of each NSMCE2 mutant cell line to recover from RS, such as through a mild hydroxyurea treatment followed by FACS analysis after different periods of recovery time, to establish if the ligase-dead cell lines do in fact struggle to recover from RS more than the NSMCE2 knockout cells.

4.4 Segregation of duplicated DNA

The mechanism for chromosome segregation is a complex process, but put simply, involves the removal of connections between the sister chromatids and the physical movement of the replicated chromosomes to the poles of the mitotic cell, before cytokinesis can occur forming two new daughter cells. During cytokinesis, a furrow forms down the centre of the newly replicated cell, contracting through the action of an actin and myosin band, until a membrane forms separating the two sets of DNA. Following this, an abscission step occurs where the two cells can detach from each other (Damelin and Bestor, 2007; Guertin et al., 2002).

The process of removing connections between the sister chromatids is vital to avoid chromosome breakages and imbalances during chromosome segregation. There are two main types of connections found between sister chromatids which need to be

resolved; protein cohesin connections and DNA mediated linkages such as catenations and other connections such as ongoing replication and unresolved RS characteristics (Bermúdez-López et al., 2010). Cohesin physically tethers the two chromatids together and removal first occurs throughout the arms of the paired chromatids, in a Wap-1 dependent manner, before the removal occurs at the centromeric/pericentromeric regions independently of Wap-1 (Leman and Noguchi, 2014). Additionally, catenations, which interlock the DNA through chain links as sister chromatids pair up, are thought to be mainly be removed through the action of TopoII and condensin at a checkpoint known as the decatenation checkpoint (Charbin et al., 2014; Holm, 1994). Cells can continue to carry out inappropriate cell division in the presence of catenations so this checkpoint is essential for ensuring genome stability (Damelin and Bestor, 2007). As well as these specific connections, sister chromatids can be connected through chromosome junctions; collapsed forks, RS intermediates and unexpected ongoing DNA replication. These connections are frequently seen where DNA damage is unable to be repaired or in replication mutants (Bermúdez-López et al., 2010)

The SMC5/6 complex has been associated with removal of each type of physical connection between sister chromatids to allow segregation. It has been suggested in *S. cerevisiae* that the complex controls the distribution and action of Topo II as well as condensin within mitosis, indicating a role within decatenation (Gallego-Paez et al., 2014; Pryzhkova and Jordan, 2016). As well as this, the SMC5/6 complex has a similar binding pattern to cohesin throughout mitosis and some SMC5/6 mutants demonstrate incomplete mitosis due to cohesin connections still being present (Gallego-Paez et al., 2014; Outwin et al., 2009). More recently, it has been indicated in *S. cerevisiae* that the SMC5/6 complex may be more closely involved in the removal of DNA mediated linkages between sister chromatids produced due to incomplete replication and unresolved RS structures (Bermúdez-López et al., 2010). This was corroborated by Branzei et al., 2006 and Farmer et al., 2011 who showed that SMC5/6 mutant cells undergo inappropriate cell division as these connections are not removed. As there is a lack of clarity for the role SMC5/6 plays in the removal of connections between sister chromatids and DNA segregation as well as the possibility

there is species specific differences, it is important we characterize the capabilities of our human NSMCE2 mutant cell lines to complete replication, particularly considering the >4N DNA content shown by the NSMCE2 ligase-dead cell lines.

This study has found that when decatenation is inhibited through ICRF-193 treatment, NSMCE2 ligase-dead cells were unable to activate or maintain the decatenation checkpoint since they do not arrest cell cycle progression. However, this was not found in the NSMCE2 knockout cell line. This finding gives support that the ligase-dead NMSCE2 may be 'getting in the way' of other cellular proteins which may be compensating for the loss of NSMCE2 activity, and indicates that NSMCE2 may be directly involved with activating and maintaining the decatenation checkpoint and perhaps in decatenation itself.

Using immunofluorescence to visualise the cell size of each CRISPR-Cas9 NSMCE2 mutant cell line revealed that there was an average increase in size of 1.2 times between the wild type and knockout cell lines. This is consistent with taking longer to move through the cell cycle as the cells continue to grow until they reach mitosis, particularly since there was no increase in DNA content shown through the FACS analysis. Moreover, the NSMCE2 ligase-dead cells increased in size by an average of 1.4 times compared to wild type cells. This corresponds to continued cell growth due to the increased cell cycle length before, in many cases, failed cell division, since cells accumulate 8N DNA content. This supports the possibility that there is a segregation defect within the ligase-dead cells, possibly due to the inability to decatenate the DNA or activate the decatenation checkpoint. It is possible that there is also a chromosome segregation defect within the NSMCE2 knockout cells but it is at a much lower level as the increased DNA content was not detectable by the FACS analysis.

As well as the possibility that the complex is involved in removing specific catenations, the inability to remove connnections between the DNA could be due to unresolved RS characteristics, such as cruciform structures which are formed upon the stalling or collapsing of replication forks, known to be resolved by the SMC5/6 complex (Gelot et al., 2015). The inability to remove RS characteristics, DNA mediated linkages, and so

separate sister chromatids, can cause cells with an incomplete S phase to move into early entry mitosis and so cause aneuploidy as they have problems with segregation (Mankouri et al., 2013). This could lead to the 8N DNA content noted in the NSMCE2 ligase-dead cell line as the cells re-replicate the remaining abnormal DNA. This possibility is supported by a study which showed that some SMC5/6 mutants underwent chromosome nondysjunction and entered mitosis before finishing replication (Torres-Rosell et al., 2007). The main questionmark above this theory, is that the prescence of the RS intermediates would cause the activation of the replication checkpoint which is not seen, although as discussed previously, this defect may not be sensed by the checkpoint.

To investigate this further, immunoflourescence was used to study the quantity of micronuclei within each of the NSMCE2 cell lines. Micronuclei are extra-nuclear bodies of DNA known as the footprint of chromosome missegregation. They are often formed when cells enter mitosis without completing S phase due to RS intermediates remaining or other defects in chromosome segregation (Mankouri et al., 2013). There was a 3-fold increase in micronuclei content within the NSMCE2 ligase-dead cells further supporting their defect in chromosome segregation and possibly suggesting that RS characteristics, which leave DNA connections, may be causing these defects. However, interestingly, there was also a small, but significant, increase in micronuclei content in the NSMCE2 knockout cells compared to the wild type. This supports the conclusion that the knockout cells may also have a mild defect in chromosome segregation, possibly due to unresolved RS leaving DNA mediated connections between the sister chromatids. It is possible that whilst other proteins are mainly able to compensate for the loss of NSMCE2, the functional replacement is not perfect, leaving some mild phenotypes still present.

This data indicates that the NSMCE2 subunit of the SMC5/6 complex may have a role in either decatenation directly, activation of the decatenation checkpoint or RS intermediate removal which can cause defects in chromosome segregation and cell division. I suggest that the most likely scenario is that problems faced during replication which cause the slowed progression in both the NSMCE2 ligase-dead and

knockout cell lines, leave RS intermediates, such as cruciform structures, within the ligase-dead cells as they cannot be repaired. These intermediates mean DNA connections between the sister chromatids remain, therefore causing cells to enter mitosis with an incomplete S phase, producing chromosome segregation problems. This is exacerbated as the NSMCE2 subunit of the SMC5/6 complex may also be normally involved in the removal of these and other connections between sister chromatids. It is possible that as the loss of NSMCE2 function is proposed to be mainly compensated for within the NSMCE2 knockout cells, the RS structures are not as prevalent, therefore little to no segregation defect is seen. It is also possible that instead of or as well as the accumulation of RS intermediates, the NSMCE2 ligase-dead cells are just slower at replication than the knockout cells, therefore leaving unreplicated regions of DNA which remain connected during mitosis. These connections would also inhibit or aid inhibition of appropriate chromosome segregation.

Further work needs to be undertaken to investigate further and determine whether there is an increase in RS within the ligase-dead cell lines which could cause the segregation defects, such as by DNA combing. It would also be interesting to visualise the chromosome segregation through live cell microscopy with GFP expression to further investigate whether connections are present between the chromatids, their possible nature and how they affect the way in which the chromatids try and separate. To determine whether the NSMCE2 subunit also plays a role in removing protein cohesin linkages between sister chromatids, inhibition of separase which is required for cohesin removal, may also be carried out on each of the cell line before FACS analysis is carried out, to determine if there is a difference in the ability to then segregate their DNA after treatment (Hauf et al., 2001).

4.5 The SUMOylation ability of NSMCE2

The SUMOylation capabilities of NSMCE2 have always been important when studying the SMC5/6 complex as it often suggested that this may be the method by which the complex carries out many of its DNA repair and genome maintenance roles (Andrews

et al., 2005). Over the years of study, the NSMCE2 ligase capabilities have been associated with RS recovery, the removal of DNA cruciform structures and other replication intermediates, telomere elongation and homologous recombination as well as many other chromosome maintenance processes (Wu et al., 2012; Rai et al., 2011; Chavez et al., 2010; Branzei et al., 2006; Potts and Yu, 2007). Despite the ever-broadening knowledge of the importance of NSMCE2 SUMOylation in different cellular processes, few specific targets have been identified.

Recently, a bioSUMO method has been developed to enable easy isolation of SUMOylated proteins from cell extracts (Pirone et al., 2016). It was thought that if this technique could be applied within wildtype MRC5 as well as the NSMCE2 mutant cell lines, it would enable the identification of NSMCE2 specific SUMOylation targets as these SUMOylated targets would not be present within the NSMCE2 knockout cell line compared to the wildtype cells. Therefore, the goal of this experimentation was to optimize the bioSUMO technique within wildtype cells using the known autoSUMOylation of NSMCE2 as a measurable readout (Andrews et al., 2005).

Throughout this experimentation, we managed to efficiently express SUMO-1 that had undertaken in vivo biotinylation and was conjugated to cellular proteins within wild type MRC5 cells. We were then able to consistently recover this mixture of bio-SUMO-conjugates using a stringent purification protocol. However, unfortunately, after many attempts of optimizing and repeating this experiment, we were unable to detect individual SUMO modified targets using western blotting. Many quality control methodology validating experiments were undertaken, testing the transfection efficiency as well as the binding and elution of SUMOylated proteins to the Streptavidin beads, which each confirmed the protocol was working in the manner it should. This provided further uncertainty as to why we could not achieve the results we expected. Due to time constraints, we were unable to continue developing this protocol further, however, it was possible to isolate a very small amount of RAN GAP1 within one of the last experiments undertaken. As this is a largely SUMOylated protein within cells and there was only a very small amount of the SUMOylated protein detectable, this indicated several possibilities which could be used to optimize and

troubleshoot the protocol further (Matunis et al., 1996). These are; that the SUMO tag on the proteins was very labile and so was becoming removed, either from all cellular SUMOylated proteins or specifically from proteins we were attempting isolation of, during the binding, washing or elution process, that we were not using enough cellular material in order to detect the low levels of SUMOylated proteins found within cells or that the western blot technique was not sensitive enough and so mass spectrometry should be undertaken.

Previous development of similar protocols within this lab has identified that in less than 8M urea SUMO/Ubiquitin tags can be labile and so could be becoming removed from tagged proteins during the binding stage. In light of this, this strategy would need to be repeated using higher concentrations of urea or guanidine during all stages to ensure the non-removal of the SUMO tags. If this still did not achieve the expected result, it would also be suggested to undertake mass spectrometry on the protein extracts in order to achieve a more sensitive result and so confirm the validity of this protocol.

Conclusion

The purpose of this thesis was to validate and characterise CRISPR-Cas9 mutated NSMCE2 cell lines which had been created previously by this laboratory, enabling the ability to suggest possible roles for NSMCE2 and the SMC5/6 complex in chromosome maintenance and repair. It has been found that there was a mild proliferation defect within the NSMCE2 knockout cell line and a substantial proliferation defect within the NSMCE2 ligase-dead cell line both caused by slowed S phase progression. The NSMCE2 ligase-dead cell lines also demonstrated >4N content by FACS analysis. The remainder of the project investigated the cause of these defects, determining that the replication checkpoint was not involved in the slowed S phase progression although the DNA damage checkpoint may be, particularly in the NSMCE2 ligase-dead cell lines. It has been identified that the most likely source of the proliferation defects was due to unresolved replication stress characteristics, unsensed by the replication checkpoint, which then cause further DNA damage and further slowed S phase progression. A

dominant negative mutation is expected to have caused the increased proliferation defect within the NSMCE2 ligase-dead mutant, as the ligase-dead NSMCE2 may disrupt other proteins compensating for its loss, such as proteins involved in replication stress characteristic resolution. This data also proposes that the NSMCE2 ligase-dead cell line has difficulty in segregating sister chromatids, causing the >4N DNA content. This may be due to the unresolved RS characteristics leaving DNA connections between the sister chromatids, or the NSMCE2 subunit of the SMC5/6 complex may also be directly involved in decatenating chromosomes before segregation or activating the decatenation checkpoint. Further work has been suggested to aid the specific identification of the chromosome maintenance and segregation actions undertaken by NSMCE2. A bioSUMO method for isolating specific NSMCE2 SUMOylation targets has been developed, with further technique recommendations made to ensure accurate results.

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