THE ROLE OF NSMCE1 IN MAINTAINING GENOMIC STABILITY

A Thesis

Presented to

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I declare that this thesis and the work presented therein are my own and has been generated by myself as the result of my own original research and that the work contained within has not been submitted elsewhere.

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Abstract

Genome instability is a major driving force behind the development and progression of cancer therefore the mechanisms that prevent genome instability are crucial to help prevent cancer. In addition, many cancer treatments exploit cancer cells' inability to respond normally to genome instability resulting in cell death. It is therefore vitally important to understand the cellular mechanisms for maintaining genome stability. Here we investigated the role of NSMCE1, a component of the SMC5/6 complex, which is important for several aspects of genome stability maintenance. We used mutant *NSMCE1* cell lines to investigate the role of NSMCE1 in SMC5/6 complex formation using immunoprecipitation and western blot analysis. We explored the importance of NSMCE1 for cell cycle progression and genome stability using flow cytometry and DNA damage assays, as well as exploring the ubiquitin modification of NSMCE1 by purification of ubiquitin-modified proteins following formation of epitope-tagged ubiquitin in cells. Our results confirm that NSMCE1 is integral to the formation of the SMC5/6 complex and that it is also essential for cell proliferation. We have demonstrated that a functional NSMCE1 RING domain is not required for SMC5/6 complex formation but is necessary for normal cell growth and division.

Table of contents

1.0 Introduction

1.1 Cancer and Genome stability.

Cancer is defined as abnormal and unregulated cell growth and is one of the top causes of death in developed countries. According to data published by Cancer Research UK, between 2014 and 2016 there were over 350,000 new cases every year in the UK alone, with only a 50% survival rate on average (Cancer survival statistics, 2020). Despite different cancers originating in different tissues, with each having a variety of triggering or contributing factors, certain characteristics are common amongst all cancers. Thanks to the work of Hanahan and Weinberg, six primary hallmarks of cancer were established in 2000: sustained proliferation, growth suppressor evasion, replicative immortality, apoptosis avoidance, angiogenesis induction and metastasis (Hanahan and Weinberg,2000). While each of these characteristics will contribute to the development of cancer, they are not all required simultaneously. Instead, these hallmarks represent an inter-related and dynamic set of cellular changes leading to cancer (Floor et al., 2012). Further analysis and investigation led Hanahan and Weinberg to extend their original model of hallmarks of cancer to include additional factors. One such factor was genomic instability (GI) as an enabling characteristic of cancer, alongside other factors such as deregulated cellular energetics (Hanahan and Weinberg,2011)

GI relates to the frequency of mutations acquired over the course of a cell's lifetime which can be influenced by a variety of factors. Mutations to DNA can change cellular behaviour, which can have varying effects, either benign or malignant. Since GI increases the probability of mutations occurring this greatly increases the chance that aberrant cell behaviours will occur. These aberrant cell behaviours will commonly include hallmarks of cancer, making GI a key contributor to carcinogenesis and thus an important avenue of research.

In many cases, GI is likely to be the initial driving force behind carcinogenesis. This is often seen in patients with inherited DNA repair defects, where an increased mutation frequency increases the propensity for genetic changes that give rise to cancer hallmarks. This is illustrated in patients suffering with the hereditary condition Lynch syndrome, a condition in which the individual has inherent GI as a result of mutations occurring in one or more DNA mismatch repair proteins which leads to increased microsatellite instability and results in a cancer predisposition, (Pino et al., 2009). BRCA1 and BRCA2 are additional well studied examples of how inherited mutations impair DNA repair processes leading to higher levels of GI which ultimately drives forward carcinogenesis for example through knock-on p53 impairment following BRCA depletion (Roy et al., 2011).

Over the years multiple cancer genome sequencing projects have been conducted to determine if DNA repair defects were in fact the initiating mutation in all tumorigenesis. Surprisingly, studies into several

cancers found that DNA repair factors such as *BRCA2* were often found to not be mutated, at least initially, during tumourigenesis (Stephens et al., 2005). These discoveries provided evidence against the mutator hypothesis, that implies increasing mutation rates to be a direct result of defective DNA repair, in many sporadically arising cancers (Negrini et al., 2010). In fact, these investigations determined that oncogene activation or tumour suppression loss was often the driving force behind increased cell dysregulation leading to increased genomic instability (Negrini et al., 2010). Once oncogenes are activated and become established, the resultant increase in cell proliferation leads to several problems, one of which is genome instability as a result of replication-induced DNA damage which, when cumulative gives rise to the development of other cancer hallmarks (Halazonetis et al., 2008).

1.2 DNA damage and repair

All living organisms depend upon the integrity of their genome in order to manufacture RNA and proteins that support normal cellular functions. However, the DNA comprising the genome undergoes almost constant modification and, over the course of their lifetime, cells incur a high number of mutations through accidental replication errors and as a result of exposure to endogenous and exogenous damage sources. Damage to DNA occurs in many different forms and therefore a variety of different mechanisms exist to detect and correct these various kinds of DNA damage. Cancer often arises from the failure of DNA repair and other DNA damage response pathways to counteract the damage. While degraded proteins can readily be recycled and resynthesized using information from the DNA template, damage to DNA is dependent on being repaired by the very proteins it helps create and it is therefore vital for organisms to have a wide variety of effective DNA repair proteins and mechanisms to ensure adequate maintenance is carried out. (Hakem, 2008)

Endogenous sources of DNA damage arise from within the cell without external influences. These types of damage can be because of mechanical issues with DNA replication such as misincorporation of nucleotides or DNA replication slippages, particularly at repetitive sequences. Replication errors of this sort are corrected by the mismatch repair system (MMR), which detects and repairs mismatches and small insertion/deletion loops (Li, 2007). Microsatellites mostly occur within non-coding regions of our DNA, but they can occur in certain exon sequences. These microsatellites or simple tandem repeats are widely regarded as being responsible for a degree of GI and while occurrences vary, “slipping” of these sections if not repaired by mismatch repair can result in microsatellite instability (Jiricny, 2006, Li, 2007). If left unrepaired apoptosis will usually occur as if left unrepaired this would lead to incorrectly synthesised DNA and a mutated phenotype eventually resulting in disease through increased GI (Zhivotovsky, B. and Kroemer, G., 2004; Li, 2007) .

Endogenously arising damage can also be a consequence of reactions between DNA and free radicals such as reactive oxygen species (ROS). By 2013 over 100 base lesions and backbone modifications had been identified as resulting from oxidative stress (Cadet and Wagner, 2013). Formed in mitochondria through the waste products of chemical reactions these ROS commonly take the form of hydroxyl radicals ·OH, and they are widely regarded as being responsible for aging. Though ROS are not exclusively damaging to cells and are components in several useful pathways including the maintenance of homeostasis (Di Meo et al., 2016), in large quantities ROS can react with DNA and RNA, modifying nucleotide bases and causing lesions in the sugar-phosphate backbone which results in errors with transcription and translation. These DNA modifications resulting from ROS often cause single strand breaks, which occur in DNA at a rate of tens of thousands in each cell per day (Moore et al., 2000). If left unresolved single strand breaks can lead to increased GI and risk worsening into double strand breaks (DSBS). Cells are therefore equipped with specialised enzymes and ligases which work together to rectify the break ensuring the 5' and 3' ends of the break are modified to be complimentary so the lesion can be appropriately sealed through single strand break repair or base excision repair pathways. (Cooper and Hausman, 2000)

Exogenous factors can cause many other problems with DNA. DNA can encounter other forms of damage such as the formation of cytobutane pyrimidine dimers produced primarily in skin cells through UVB light exposure (and possibly UVA (Mouret et al., 2006) which induces the formation of linkages between consecutive pyrimidine bases, cytosine and/or thymine. The formation of these dimers can lead to replication and transcription errors and polymerase stalling and ultimately requires repair through nucleotide excision repair (NER), a multifaceted process initiated by damage recognition which involves the unwinding of the DNA at the site of damage and excision of a short 23 nucleotide damage-containing oligonucleotide before re-synthesis of the correct DNA sequence required to fill the gap (Sugasawa et al. 1998; Shivji et al., 1994).

These examples of inherent and acquired GI are but a few of the many factors that can lead to increased mutations in DNA. While all organisms incur mutations over the course of their lifetime from endogenous and exogenous sources, the more exposed an organism is to mutagenic factors such as from an inherited disease or lifestyle choice the greater that organism's GI which may eventually result in cancer.

1.2.1 Double strand breaks (DSB)

Double strand breaks (DSB) arising from factors such as severe mechanical stress, unresolved single strand breaks, or exposure to DNA damaging agents such as ionising radiation, are one of the most detrimental forms of DNA damage which often results in apoptotic responses from the cell if the break

is not easily rectified. DSBS are very damaging to cells as the task of re-connecting the two ends, which may not have remained near each other, makes the task more complicated and potentially error prone. Double strand break repair (DSBR) is often resolved in one of two ways, homologous recombination repair or non-homologous end joining (Scully et al., 2019)

1.2.2 Non-homologous end joining

When DSBS occur, cells can utilise non-homologous end joining (NHEJ) to re-ligate broken DNA ends with as little as a single nucleotide of complementary sequence. This process is very effective at rapidly repairing DSBS without the need for a homologous template which is vital for repairing damage during G1, where our cells spend most of their time. The NHEJ repair process involves a multitude of proteins but initially requires recruitment of the KU-heterodimer to both sides of the break which acts as a scaffold (Davis and Chen, 2013) which allows the association of other factors to the damage site including the DNA-dependent protein kinase DNA PKcs, structure-specific endonucleases such as Artemis and DNA ligase IV (Kurosawa and Adachi, 2010) which together act to seal the break. This mechanism is depicted in Figure 1.

1.2.3 Homologous recombination HR.

While NHEJ is very fast which is advantageous in repairing DSBS it is also very error prone, therefore in the presence of a template from a sister chromatid homologous recombination (HR) is the more favourable mechanism to use. HR has several sub-pathways depending on the level of damage requiring repair (Hartlerode and Scully, 2009). Figure 1 depicts one such pathway in which the MRN complex containing Mre11, Rad50 and Nbs1 associates with CtIP, a tumour suppressor and endonuclease which assists in excising damaged DNA around a DSB (Makharashvili and Paull, 2015). The MRN complex itself conveys both single stranded DNA endonuclease activity and double stranded DNA 3’ → 5’ exonuclease activity which assists in the cleaning up of damaged termini (Lamarche et al., 2010). Once the damaged termini have been rectified BRCA2 and/or Rad51 family proteins form nucleoprotein scaffolds (Wright et al., 2018) to support and direct further protein responses which leads to ATM phosphorylating checkpoint kinase 2 (Chk2) which leads to an upregulation of cell cycle checkpoint pathways leading to a delay in cell cycle progression and the recruitment of downstream DNA repair proteins. The open section of damaged DNA allows for strand invasion of the overhanging 3’ end into a nearby undamaged DNA molecule such as a nearby sister chromatid. The 3’ end uses the undamaged DNA as a template to synthesise a new DNA which is then used to repair the damaged strand.

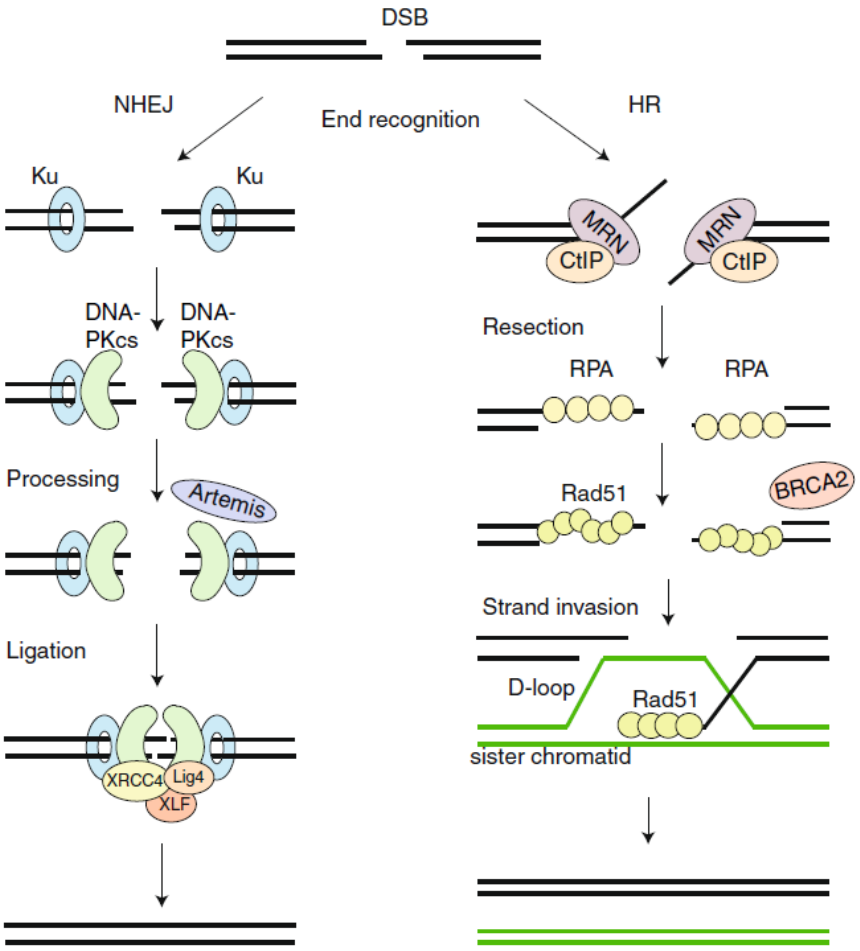


Figure 1 – Non homologous end joining and homologous recombination. NHEJ begins with the recognition of the double strand break by the Ku-heterodimer which recruits cofactors leading to the ligation of the break. HR relies on damage recognition by the MRN complex and the usage of nearby sister chromatids to form a template to repair the damaged DNA following strand invasion by a single stranded hanging DNA chain. Figure reproduced with permission from Brandsma et al 2012

1.3 DNA damage checkpoints

The reliability of successful genome duplication and the following cell division is maintained, in part, by the regulation of cell cycle progression at a series of checkpoints to ensure the ensuing daughter cells are viable. The cell cycle is primarily driven using tightly controlled cyclin-dependent kinases which phosphorylate specific substrates to drive the cell cycle forwards though replication and mitosis. If the DNA incurs damage or complications arise during DNA replication, mechanisms exist to pause the cell cycle allowing for repair to be carried out, DNA replication to be completed or for the cell to be terminated should the damage be irreparable. There are a multitude of proteins responsible for mediation of cell cycle checkpoints with two main initiator proteins responsible for the DNA damage and DNA replication checkpoint pathways, Ataxia telangiectasia mutated (ATM) and Ataxia

Telangiectasia – Rad3-Related (ATR) which are both protein kinases of the conserved PI3K-related kinase family (Awasthi et al., 2015).

ATM acts mainly in response to DNA double-strand break damage such as that caused by ionizing radiation and follows two primary responses in relation to this, as seen in Figure 2. Following its recruitment to DSB sites by the DSB sensor MRN complex, ATM becomes activated and is phosphorylated at S1981, within its FAT domain, which stabilises its association at a site of DNA damage (Awasthi et al., 2016) and contributes to the relaxation of heterochromatin (Goodarzi et al., 2008) to allow easier access to the DNA by the MRN complex. If the break is clean or without large overhanging broken nucleotide sequences, ATM can recruit several complex proteins to initiate HR. If the break is more complex with larger damaged nucleotide sequences exposed, ATM phosphorylates checkpoint kinase 2 (Chk2) to upregulate cell cycle control and inhibit DNA synthesis while repairs are carried out. Failure to repair the damage results in further phosphorylation and activation of proteins such as p53 which can cause the cell to undergo apoptosis (Vousden and Lu, 2002).

ATR acts in response to structural damage that is encountered during DNA replication caused by replication errors or exogenous sources like UV-light as seen in Figure 2. Unlike ATM which is recruited directly, ATR cannot directly associate with DNA and so it liaises with the replication complexes which have encountered difficulties though phenomena such as DNA loops or pyrimidine dimers. This association is accomplished via ATR-associated ATRIP (ATR-interacting protein) which interacts directly with replication protein A, a protein that associates with single stranded DNA (Zou and Elledge, 2003). ATR then interacts with associated DNA damage clamp proteins RAD9, RAD1 and HUS1 which leads to the phosphorylation and activation of ATR which activates downstream proteins such as BRCA1 which will subsequently assist in HR to allow replication to continue in one of two ways. Either BRCA1 is activated alongside CHK1, the primary target of ATR, which triggers a global cellular response to delay S-phase progression, allowing time for the damage to be rectified or bypassed before resuming fork progression (Lopes et al., 2001). Alternatively NBS1 is activated, alongside CHK1, to change the repair systems over to those involved in HR.

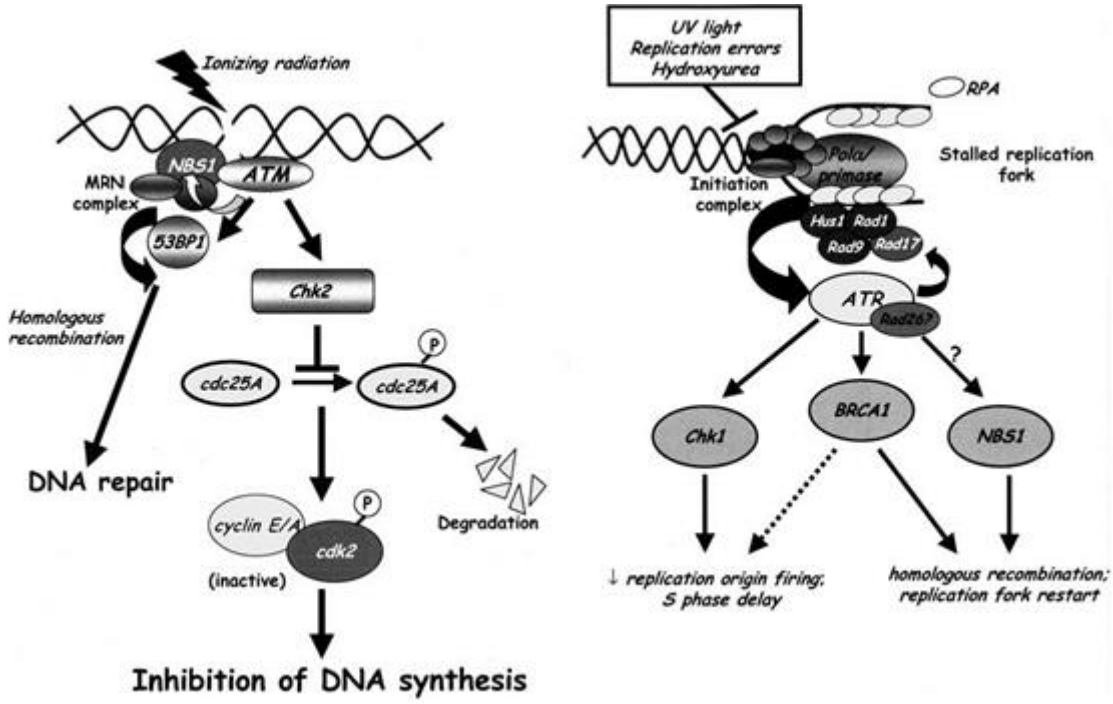


Figure 2 – Roles of ATM and ATR in mediating cell cycle checkpoints and damage response. ATM dependent pathway (left) assists in response to ionizing radiation damage and mediates the HR of DNA or degradation in the case of irreparable DNA damage. ATR dependant pathway (right) mediates the already established DNA replication complexes and halts the cell cycle if replication is stalled to allow for HR. Figure reproduced with permission from Abraham, 2001.

1.4 Structural Maintenance of Chromosomes Proteins

Structural maintenance of chromosome proteins are a group of essential ATPases which function throughout the cell cycle to ensure efficient and problem-free segregation of chromosomes. At least six SMC proteins are present within many eukaryotes and are highly conserved from yeast to mammals (Harvey et al., 2002) and this conservation also extends to several prokaryotic species which can have either one or two SMC genes. Prokaryotic SMC proteins can however be quite divergent between species, as in the case for *Escherichia coli (E. coli)* MukB, which presents vast differences in its terminal domains compared to other prokaryotes and is therefore much more greatly diverged from SMC sequences than in other species (Melby et al., 1998).

SMC proteins, being between 110 and 170 kDa in size are relatively large and comprise an NTP-binding domain followed by two long coiled coil segments linked by a hinge and terminating in a globular COOH-domain (Melby et al., 1998). The proteins fold back on themselves at their hinge region, the point through which individual SMC proteins interact to form specific pairings, causing their respective coiled coil domains to interact along their length, forming an antiparallel structure of around 50nm and bringing the N and C-terminal globular regions together to form a functional ATPase head domain. SMC proteins in eukaryotes are found as heterodimers, with SMC1 and SMC3 comprising the core of

cohesin complexes, SMC2 and SMC4 form the core of condensin complexes, with SMC5 and SMC6 forming part of a third eukaryotic SMC complex, SMC5/6.

1.4.1 Cohesin

Cohesin in humans contains at its core SMC1 and SMC3 though the complexes can have varying subunits depending on whether the complex is contributing to mitosis or meiosis. The primary role of cohesin is to expedite the cohesion of sister chromatids up until the transition into anaphase, although there has been evidence of cohesin contributing to other mechanisms such as chromosome condensation and DSBR (Mehta et al., 2013). The exact model for how cohesin operates remains unclear though the most favoured model is the one ring model in which SMC1 and SMC3 along with its other units form a triangular ring around sister chromatids holding them in place (Haering et al., 2008). An example of this chromatid trapping is depicted in Figure 3 in which the hinge region of cohesin allows for the encompassing of chromatin during DNA replication. This binding of sister chromatids is important for chromosomal organisation following their synthesis up until they are lined up in mitosis at metaphase, at which time separase, a cysteine protease, hydrolyses the Scc1 cohesin subunit which allows for the release of DNA which is essential for anaphase to begin (Hauf, 2001).

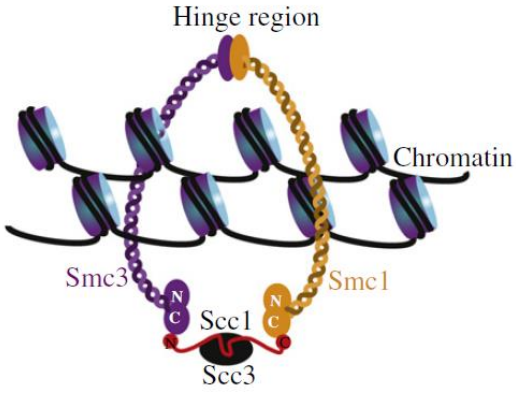


Figure 3 – One ring model illustration. SMC3 And SMC 1 embrace sister chromatids, holding them tightly which helps in the organisation of chromosomes during mitosis. Figure reproduced with permission from Mehta, Rizvi and Ghosh, 2012.

1.4.2 Condensin

Condensin has two variants in mitotic cells, condensin I and condensin II, both of which contain SMC2 and SMC4 and are similar in overall structure to the related cohesin complex, as depicted in Figure 4. The role of condensins are primarily to facilitate the compaction and condensation of DNA into chromosomes for easy organisation during mitosis. Despite playing similar roles the two complex variants are not co-located. Condensin I is located within the cytoplasm and only gains access to chromosomes after the dissolving of the nuclear envelope, whereas condensin II is present within the nucleus (Hirota, 2004). Condensin II initiates the condensation of chromosomes as the nuclear envelope dissolves which allows condensin I to further assist in the compacting of the chromosomes in order to fully resolve the two sister chromatids. While cohesin is responsible for closely binding sister chromatids, condensin works by closely binding loops of DNA on the same chromatid which facilitates the compaction and organisation of the chromosomes.

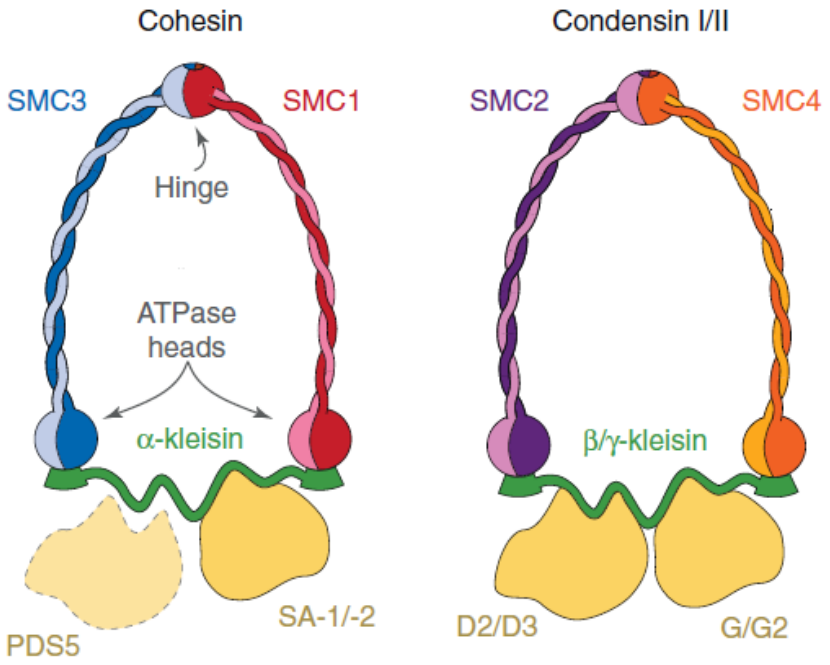


Figure 4 – Comparison of Cohesin and Condensin architecture and components. Both proteins exhibit the same hinge region linking their complex specific SMC components with tail ends bound to their cell specific sub-units. Figure reproduced with permission from Cuylen and Haering, 2011.

1.5 SMC5/6 Complex. Structure and composition.

The SMC5/6 complex as shown in Figure 5 is amongst the least understood of the SMC protein complexes but has been clearly shown to share similar structural characteristics with cohesin and condensin (Sergeant et al., 2005). While the components of cohesin and condensin are well established in addition to their meiotic and mitotic roles there is still much we do not know about the SMC5/6 complex and its components. The SMC5/6 complex is comprised similarly to cohesin and condensin, with SMC5 and SMC6 forming two long coiled segments linked at their hinge regions and associated with several non-SMC elements NSMCE1-4.

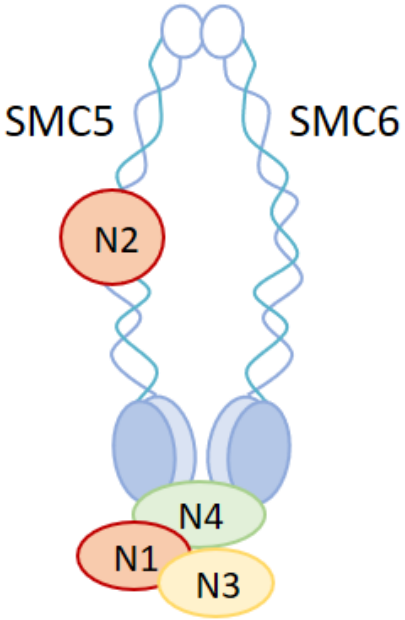


Figure 5 – Structure of SMC5/6 complex. SMC proteins adhered directly at hinge region with NSMCE2 bound to SMC5's helical domain. Subcomplex of NSMCE4, NSMCE3 and NSMCE1 complete the complex by bridging the SMC5 and SMC6 terminal domains.

NSMCE4, a kleisin subunit which has analogues in cohesin and condensin, bridges the SMC5 and SMC6 head domains holding them together maintaining the complex shape. (Palecek et al., 2006). Associated closely with NSMCE4 are NSMCE1 and NSMCE3. NSMCE1, containing a really-interesting-new-gene (RING) domain, has been implicated as being like E3 ubiquitin ligases (Doyle et

al., 2010). NSMCE1 forms a subcomplex with NSMCE4 and with NSMCE3, a ubiquitously expressed melanoma antigen gene family (MAGE) protein which has been shown to contribute to DNA repair, specifically by HR (Pebernard et al., 2004). NSMCE3 being a MAGE protein further supports NSMCE1 as being a likely E3 ubiquitin ligase given the close association of MAGE proteins with other Ub ligases (Doyle et al., 2010). Finally, NSMCE2, a SUMO ligase which SUMOylates substrates through interactions with the SMC5/6 complex and assists in response to DNA damage, is uniquely bonded to SMC5 half way up its antiparallel coiled coil domain.

1.6 Discovery of the SMC5/6 Complex

Human SMC5/6 complex components have orthologues in several yeast species. The first SMC6 orthologue identified was in the fission yeast *Schizosaccharomyces pombe.* Originally referred to as *rad18* and later renamed as *smc6, rad18* was found to be one of several genes which, when mutated, conveyed sensitivity to radiation within this fission yeast (Nasim and Smith, 1974). In later years, additional components of the SMC5/6 complex in *S. pombe* were identified including *smc5* (originally designated *ppr18*) and *nse1, 2, 3 and 4 (*McDonald et al., 2003, Taylor et al., 2008*). nse5* and *nse6* were also identified as Smc5/6 complex components in *S. pombe.* Although *nse5* and *nse6* were deemed non-essential genes mutants of *nse5/6* provided evidence that they assist in the DNA repair role carried out by the SMC5/6 complex (Pebernard et al., 2006).

Similarly to these experiments previously described, *Saccharomyces cerevisiae's* SMC5/6 complex components were initially identified on the basis of mutant sensitivity to DNA damaging agents. Evidence was gathered for *NSE2*, also named *MMS21*, through genetic screening in 1977 (Prakash, S, 1977), in which its disruption was found to convey sensitivity to methyl methanesulfonate (MMS), an alkylating agent which produces DNA damage (Lundin, 2005). Later, orthologs were found for *SMC5/6* and *NSE1-4 a*lthough they shared little sequence identity to those in *S. pombe*. One final difference discovered between these two yeast species is was that functional homologues of *nse5* and *nse6* in *S. pombe* were identified and deemed essential in *S. cerevisiae* (Hazbun et al., 2003) unlike in *S. pombe*.

Early research into *S. pombe* mutants of SMC5/6 components showed sensitivity to DNA damaging agents which implicated the complex's involvement in DNA repair (Lehmann et al., 1995). Evidence gathered from mutants generated by Lehmann indicated that SMC6 acts via a pathway independent of NER, playing a role in repairing DSBS in conjunction with RAD51, a protein already linked with HR (Sung, 1994). Epistatic analysis with the other SMC5/6 DNA repair defective mutants also revealed that this DNA repair defect was within the same pathway as HR factor *rad51* which indicated a role in recombinational repair of DSBs (McDonald et al., 2003; Andrews et al., 2005).

Experiments into the essential nature of SMC6 found that while hypomorphic mutants of *SMC5/6* in *S. pombe* were DNA-repair defective and sensitive to DNA damaging agents, null mutants showed limited ability to divide beyond a few cell cycles (Harvey et al., 2003). This work suggested that the absence of Smc6 may be responsible for the accumulation of unrepaired DNA damage. In *S. cerevisiae,* SMC5/6 has been shown to prevent the accumulation of joint molecules, a hazardous intermediate, and to work closely with Esc2 during HR to ensure tight regulation of the pathway (Sollier et al., 2009, Xaver, Huang, Chen and Klein, 2013). Additionally, SMC6 has been demonstrated to be critical in regulation of DNA replication and its recruitment is upregulated at collapsed replication forms because of replication stress (Ampatzidou et al., 2006). It was postulated that SMC5/6 may directly suppress rDNA recombination since rDNA instability increased when SMC5/6 function was inhibited (Murray and Carr, 2008). Evidence supporting this demonstrated that removal of *S. cerevisiae* SMC5/6 complex caused an increase in the number of recombination events occurring at ribosomal DNA sites, providing evidence that the complex was indeed responsible for restraining the recombination process (Peng et al., 2018).

Within yeast the SMC5/6 complex has demonstrated a versatile set of interactions in addition to its involvement in HR and roles such as through the regulation of Mph1, a DNA helicase to sites of RNA-DNA hybrid interaction damage. In *S. cerevisiae,* knockouts of *SMC5/6* resulted in synthetic lethality through lack of Mph1 regulation indicating its role in mediating this DNA damage repair pathway. (Lafuente-Barquero et al., 2017). It has also been suggested that SMC5/6 may be responsible for alleviating replication-induced tension within DNA which becomes critical within longer chromosomes which naturally take longer to replicate, as within SMC6 mutants longer chromosomes often experience increased replication times and the accumulation of abnormal structures (Betts Lindroos et al., 2006)

1.7 Human SMC5/6 and its functions

Human SMC5 and SMC6 are each comprised of around 1100 amino acids and share sequence similarity with their yeast counterparts. One study identified the similarity between human *SMC5* and *SMC6* and those found *S. pombe* and *S. cerevisiae,* and found overall sequence similarities of around 27%, with the most conserved sequences found within the terminal domains (Taylor et al., 2001). *NSMCE1* orthologs in humans were identified following experiments between human cells and fission yeasts to confirm the interaction with the SMC5/6 complex which affirmed their relationship as a non-SMC component of the complex (Harvey et al., 2003). Similarly to the work done to identify human NSMCE1, NSMCE2's role in humans was confirmed by demonstrating how the protein was in fact a SUMO ligase in humans and yeast contributing to the DNA repair aspects of the SMC5/6 complex (Potts and Yu., 2005). Further work identified the final orthologs of *NSMCE3* and *NSMCE4* in human

cells confirming their presence in the SMC5/6 complex in humans as in yeast cells (Taylor et al., 2008). Conversely to yeasts, NSE5 and NSE6 have been shown to have equivalents in human cells, SLF1 and SLF2, but they are not considered part of the core complex although they do however appear to act as a recruitment aid to sites of DNA damage (Raschle et al., 2015)

Further evidence of SMC5/6's role in HR appeared to be uncovered through investigating sister chromatid exchange and HR through the recruitment of cohesin by SMC5/6 to sites of DSBS (Potts et al., 2006). These results were however later found to be in error due to the off-target effects of the siRNA used in the initial knockdown experiments (Wu, et al. 2012). Despite this, in addition to the work conducted by Potts et al., Wu et al. indicated the SMC5/6 complex is only active following double-strand DNA damage incurred after DNA replication through experiments utilising MMS to cause DNA damage and as expected where sister chromatids were present damage was not repaired within the confines of the experiment. It has been demonstrated that knockdowns of any of the SMC5/6 complex components except for NSMCE2 causes complex destabilisation (Taylor et al., 2008).

Building on the evidence of Potts et al., the essential nature of SMC5/6 in HR was further described through analysis of the plant *Arabidopsis thaliana.* In these plant studies mutations in the plants *SMC6* gene variants, homologs for the human SMC protein, caused levels of HR to reduce by half when compared to the wild-type control following exposure to DNA damaging agents (Watanabe et al., 2009). The similarity between these experiments in human and plant cells shows that the SMC5/6 complex and its components have highly conserved roles throughout species with regards to HR between sister chromatids.

This destabilisation has been linked with chromosome breakage syndromes causing severe lung disease when missense mutations occur in *NSMCE3* which results in decreased stability of the complex and problems with HR which results in impaired lymphocyte functionality (Van der Crabben et al., 2016). More recent investigations into human SMC5/6 to uncover the structural importance of various complex components was done through the generation of cell lines in which various subunits of the SMC5/6 complex could be degraded via the addition of 3-indole-acetica acid, a plant hormone. The results of this experiment confirmed that SMC6 or NSMCE4 degradation causes destabilisation of the complex which results in the activation of p53 and subsequent cell death following few cell divisions (Venegas et al., 2020) a direct parallel to evidence obtained in budding and fission yeasts (Lehmann et al., 1995).

Different non-SMC proteins have been shown to influence a variety of developmental conditions. In mouse models where cells were NSMCE2 deficient, these cells experienced increased levels of chromosomal rearrangement which would be a likely contributor to increased DNA damage

accumulation (Jacome et al., 2015). As a result of this experiment it has been theorised that NSMCE2 could act as a cancer suppressor in addition to other roles such as links to insulin resistance in cases of hypomorphism (Payne et al., 2014).

1.8 NSMCE1. An enigmatic, integral component of the SMC5/6 complex

Amongst all SMC5/6 complex components, NSMCE1 has remained one of the least understood components to date. Human NSMCE1 contains a conserved RING domain that shares some similarity to E3 ubiquitin ligases. In fact many studies into the structure of NSMCE1 have identified its zinc finger RING domain (Fujioka et al., 2002), and postulated the potential enzymatic activity of NSMCE1 as an E3 ligase (McDonald et al., 2003; Pebernard et al., 2004).

Sequence analysis comparing *S. pombe Nse1* to other species including human, mouse and *S. cerevisiae* shows the RING domain (amino acids 184-219 in *S. pombe* Nse1) to be highly conserved between these species (Pebernard et al., 2008). The amino acid sequences as displayed in Figure 6 show several conserved cysteines and histidine residues which likely convey predicted E3 ubiquitin ligase activity of NSMCE1.

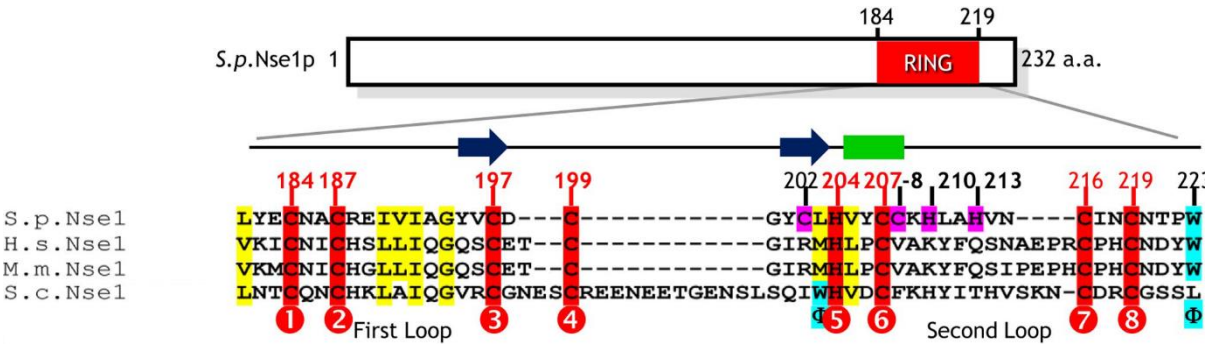


Figure 6 – Structural analysis of NSMCE1 RING-domain. Schizosaccharomyces pombe NSMCE1 compared with, Homo sapien, Mus musculus and Saccharomyces cerevisiae. Conserved cysteine and histidine residues highlighted in red show a total of 8 highly conserved regions indicating a high level of positive selection pressure to maintain these amino acids in these locations. Sequence diagram reproduced with permission from Pebernard et al ., 2008.

When compared to its *S. pombe* counterpart however it has been modelled that human NSMCE1's cross-braced RING-like structure is more tightly packed than in budding yeast with fewer cysteine and histidine residues and it was suggested that human NSMCE1 may primarily be responsible for structurally stabilising the NSE1-3-4 trimer required for DNA damage response. (Pebernard et al., 2008). Additionally, despite this conserved sequence similarity and similarity to E3 ubiquitin ligases, most assays conducted in yeast to detect such activity by Nse1 were unsuccessful (Pebernard et al., 2008). As previously mentioned, this lack of *in vitro* E3 ligase activity for Nse1 led to speculation that

its functionality was more structural within the Nse1-3-4 trimer in supporting the larger SMC5/6 complex (Pebernard et al 2008).

Within yeast Nse1 has been shown to be an essential component, since removal of the protein leads to terminal phenotypes in yeast which indicates its functionality is non dispensable (McDonald et al., 2003). To further outline the structural importance of *S. pombe* Nse1, different point mutations generated within the Nse1 RING domain conveyed low levels of temperature sensitivity but were still viable which demonstrate how the RING domain of Nse1 does has some enzymatic role but is not required for the roles performed by the other SMC5/6 complex components (Pebernard et al., 2008). The loss of *NSMCE1* destabilises the larger SMC5/6 complex in humans which we know from other studies such by Taylor et al., 2008 and Van der Crabben et al., 2016 conveys a multitude of knock on effects from increased levels of chromosomal recombination and the activation of apoptotic pathways which suggests NSMCE1 is crucial in structurally maintaining the complex rather than merely being resigned to an enzymatic role (Taylor et al., 2008) .

In 2010, Doyle et al., reported weak E3 ubiquitin ligase activity for human NSMCE1 in *in vitro* assays, in the presence of conjugating enzymes which was enhanced by the presence of NSMCE3. The results of their experiment shown in Figure 7 were the result of extracting biotin-ubiquitinated proteins in the presence of with and without NSMCE3. This activity was conversely not able to be demonstrated in fission yeast which suggests the level of activity of NSMCE1 may be extremely low in both human and yeast cells. Aditionally, Doyle et al identified crystal structure of NSMCE1 derived through co-expression with NSMCE3. This crystal structure identified NSMCE1's RING domain forming a cross-brace structure between two zinc ions which interestingly did not interact with NSMCE3 but rather with its own winged-helix motifs which was predicted to not impede the potential binding of NSMCE1 to E2 ubiquitin- conjugating enzymes. This information tells us that despite NSMCE1's unsual structure, it does have enzymatic

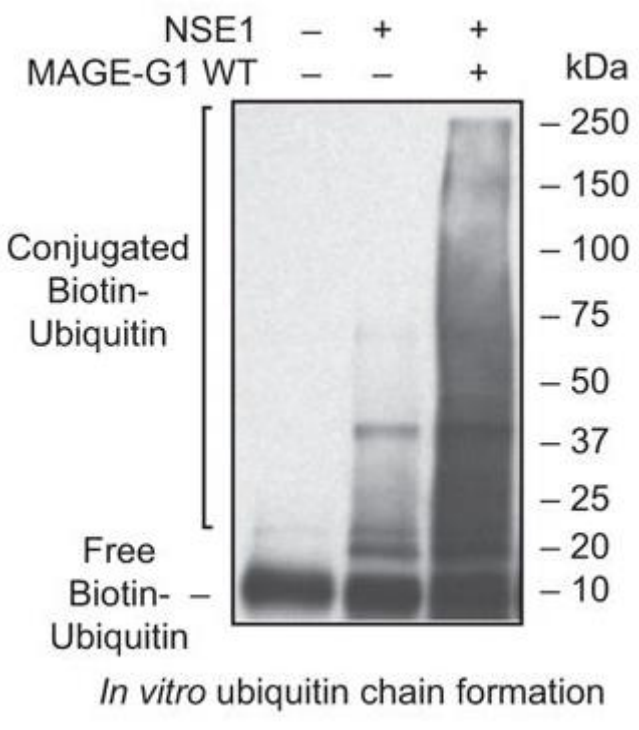


Figure 7- Biotin-ubiquitin pulldown experiment on streptavidin. Results show in the absence of NSE1 no biotin-ubiquitin proteins are detectable compared to where NSE1 is present. Biotin-ubiquitinylated proteins are enhanced in the presence of MAGE-G1 (NSMCE3). Diagram reproduced with permission from Doyle et al, 2010.

activity which seems to be dependant on interactions with NSMCE3 in addition to contributing to SMC5/6 structural stability.

1.9 Aims of Project

In order to determine the importance of NSMCE1 in the SMC5/6 complex and the cellular consequences of *NSMCE1* disruption, various cell lines had been generated prior to the work presented in this thesis. These cell lines were derived from MRC5-VI cells, a transformed lung fibroblast line, in which NSMCE1 production was abolished via CRISPR gene editing, targeting exon 2 of *NSMCE1*, to produce *NSMCE1*-Knockout cells (N1-KO). N1-W4 and N1-W14 cell lines were the result of stable transfection of *NSMCE1* into these N1-KO cells, to re-introduce wild type NSMCE1. Finally, modified RING-mutant *NSMCE1* which was modified through a double substitution of Zn^{2+} coordinating Cys residues to Alanine (NSMCE1-C191A,C194A) this was stably transfected into knockout cells to produce our N1-RING cell line, where NSMCE1 is expressed but is theoretically enzymatically inactive.

By characterising these various cell lines, we aimed to gather evidence of the effects of *NSMCE1* disruption by comparing the cellular phenotypes of these *NSMCE1* mutant cell lines and the parental MRC5-VI cells and also the N1-KO cells re-expressing wild type NSMCE1. Our ultimate aim was to confirm the structural significance of NSMCE1 for the SMC5/6 complex and hopefully obtain evidence or indication of NSMCE1's enyzmatic function through comparison between the parental and mutant cell lines.

2.0 Materials and methods

2.1 Composition of solutions used in this study

Solution	Composition
Phosphatase Inhibitor Resuspension Buffer (for total cell extracts)	50 mM Tris pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 20 mM NaF, 10 mM β-glycerophosphate, 2 mM Na_2VO_3, Protease inhibitor cocktail (Expedeon®) at 1/100
Lysis Buffer (for total cell extracts)	50 mM Tris pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 µl/ml Base muncher (Expedeon®), Protease inhibitor cocktail (Expedeon®) at 1/100 , 0.1% SDS
IP lysis buffer	50 mM Tris pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 µl/ml Base muncher (Expedeon®), Protease inhibitor cocktail (Expedeon®) at 1/100 , 0.1% NP40
5x Loading Buffer for SDS-PAGE electrophoresis	0.25% bromophenol blue 0.5 M dithiothreitol, 50% glycerol, 10% SDS
6x Loading Buffer for Gel electrophoresis	30% glycerol, 0.25% xylene cyanol FF, 0.25 bromophenol blue
SDS-PAGE running buffer	24.8 mM Tris-base, 192 mM Glycine, 0.1% SDS
Transfer Buffer (for semi-dry transfer)	48 mM Tris-base, 39 mM Glycine, 0.038% SDS, 20% Methanol
PBS	50mM Potassium Phosphate, 150 mM NaCl, pH 7.2
PBS-T	PBS plus 0.02%, 0.1% or 0.5% Tween
ECL Reagent	20 ml of 100 mM Tris pH 8.0 with 6 µl Hydrogen Peroxide (30% solution), 50 µl 90 mM p-coumaric acid, 100 µl 250 mM luminol
Mild Stripping Buffer	200 mM glycine pH 2, 0.1% SDS
MP1	50 mM glucose, 25 mM Tris, pH 8 and 10 mM EDTA

MP2	200 mM NaOH and 1% SDS
MP3	3 M Potassium Acetate and 2 M Acetic acid
Immunofluorescence Blocking Buffer	3% BSA in 1X TBS
Immunofluorescence Fixing Solution	4% PFA in 1x TBS
Immunofluorescence Permeabilisation Buffer	TBS with 0.1% TritonX-100
Immunofluorescence Antibody Buffer	0.5% BSA in TBS
Biotin Stock Solution	1 mM Biotin dissolved in Culture Medium
UBL Binding Buffer	3 M Urea, 1 M NaCl, 0.25% SDS in 1xPBS
UBL Wash Buffer 1 (WB1)	8 M Urea, 0.25% SDS in 1xPBS
UBL Wash Buffer 2 (WB2)	6 M Guanidine Hydrochloride in 1xPBS
UBL Wash Buffer 3 (WB3)	6.4 M Urea, 1 M NaCl, 0.2% SDS in 1xPBS
UBL Wash Buffer 4 (WB4)	4 M Urea, 1 M NaCl, 10% Isopropanol, 10% Ethanol, 0.2% SDS in 1xPBS
UBL Wash Buffer 5 (WB5)	8 M Urea, 1% SDS in 1xPBS
UBL Wash Buffer 6 (WB6)	2% SDS in 1xPBS
UBL Elution Buffer	4x Loading Buffer for SDS-PAGE electrophoresis, 200 mM DTT
Immunofluroescence Wash	1x TBS

2.2 Primary Antibodies

Antibody	Routine Incubation Conditions	Origin (Secondary Antibody)	Concentration	Supplier	Blocking Agent
Chk1	O/N 4°C	Mouse	1:1000	Cell Signaling Technology®	5% Milk PBS-0.1% Tween
Chk1 (p) (S345)	O/N 4°C	Rabbit	1:1000	Cell Signaling Technology®	3% BSA, PBS-0.1% Tween
Chk2	O/N 4°C	Mouse	1:1000	Cell Signaling Technology®	5% Milk PBS-0.1% Tween
Chk2 (p) (T68)	O/N 4°C	Rabbit	1:1000	Cell Signaling Technology®	3% BSA, PBS-1% Tween
GAPDH	RT, 1h	Mouse	1:4000	Abcam	5% Milk PBS-0.1% Tween
H2AX	O/N 4°C	Mouse	1:1000	Upstate Biotechnology	5% Milk PBS-0.1% Tween
Histone H3	RT, 1h	Rabbit	1:2000	Abcam	5% Milk PBS-0.1% Tween
Histone H3 (pS10)	O/N 4°C	Rabbit	1:1000	Millipore	IF blocking buffer
NSMCE1	O/N 4°C	Rabbit	1:1000	Raised in-house	5% Milk PBS-0.1% Tween
NSMCE2	O/N 4°C	Rabbit	1:1000	Raised in-house	5% Milk PBS-0.1% Tween

NSMCE3	O/N 4°C	Rabbit	1:1000	Raised in-house	5% Milk PBS-0.1% Tween
NSMCE4	O/N 4°C	Rabbit	1:1000	Raised in-house	5% Milk PBS-0.1% Tween
p53	O/N 4°C	Mouse	1:2000	Cell Signaling Technology®	5% Milk PBS-0.1% Tween
p53 (p) (S15)	O/N 4°C	Mouse	1:1000	Cell Signaling Technology®	5% Milk PBS-0.1% Tween
SMC5	O/N 4°C	Rabbit	1:1000	Raised in-house	5% Milk PBS-0.1% Tween
SMC6	O/N 4°C	Rabbit	1:1000	Raised in-house	5% Milk PBS-0.1% Tween
Biotin HRP	O/N 4°C	N/A	1:2000	Cell Signaling Technology®	5% Milk PBS-0.1% Tween
Streptravidin HRP	O/N 4°C	N/A	1:2000	Cell Signaling Technology®	5% Milk PBS-0.1% Tween
Anti-Mouse HRP	O/N 4°C	Rabbit	1:1000	Dako	5% Milk PBS-0.1% Tween
Anti-Rabbit HRP	O/N 4°C	Goat	1:1000	Dako	5% Milk PBS-0.1% Tween
Anti-Rabbit Alexa Fluor 568	O/N 4°C	Goat	1:500	ThermoFisher Scientific	3% BSA TBS

2.3 Molecular Biology Methods

2.3.1 Restriction Digests

Preparative-scale Restriction digests were carried out routinely in 40 µl total volume containing 10 µg DNA with 10-20 units of restriction enzymes and 1x Cut smart buffer (NEB). Diagnostic digests were carried out in 20 µl total volume with 0.5-1 µg DNA with 3-6 units restriction enzymes and 1x Cut smart buffer (NEB). All digests were incubated at 37°C for 1-3 hours.

2.3.2 Gel electrophoresis

To separate DNA fragments, agarose gel electrophoresis was carried out in gels made from 0.8 g agarose in 100 ml 1xTBE (0.1 M Tris-base, 0.1 M Boric acid, 2 mM EDTA). The gel was placed in the electrophoresis apparatus and covered in 1x TBE. The desired DNA samples were mixed with 6x loading buffer and loaded into wells. The gel was run at 100 v for between 30-45 min. Gels were stained for 30 minutes in 50ml 1xTBE containing 2 µl GelRed Nucleic Acid Gel stain and visualised on the UV transilluminator.

2.3.3 Gel extraction

DNA fragments were excised from gels under UV light using a scalpel, weighed and processed according to the QUIAGEN gel extraction kit protocol.

To concentrate the DNA the extracted material was re-suspended in 1 ml ethanol with 10 µl sodium acetate and centrifuged (13,000 rpm, 10 minutes). The ethanol was removed, and the pellet washed in 100 µl 70% ethanol and re-centrifuged before removing the supernatant. The final DNA pellet was re-suspended in the desired volume.

2.3.4 Ligation

Ligations into the Bio-Ub vector were set up using a 3:1 ratio of DNA insert to vector. The ligation was set up in 10 µl containing 5 µl 2x ligase buffer with 0.5 µl Bio-Ub vector and 4 µl DNA insert with 0.5 µl T4 DNA ligase (NEB Quick ligase), which was incubated at RT for 5 minutes.

2.3.5 Mutagenesis

Mutagenesis of NSMCE1 to create a RING domain mutant, was performed using the NEB Q5 site-directed mutagenesis kit. 11 µl nuclease free water was combined with 12.5 µl Q5 master mix and 12.5 pmol EP60 (ATCGCTCACAGCCTCCTCATCCAG) and EP61 (ATTGGCGATCTTCACCGCGTCGGG) DNA primers. This mixture was split into two 12 µl aliquots to which 5 ng of plasmid template NSMCE1/pCL-FLAG and FLAG-NSMCE1/Bio-Ub plasmids were added respectively, with thermal cycling carried out as follows

Cycles	Temperature (°C)	Run-time (s)
1	98	30
18	98	10
	65	20
	72	260
1	72	120

2.3.6 Mutagenesis Ligation

Ligation mixture containing 5 µl 2XKLD Reaction Buffer, 1 µl 10x KLD Enzyme mix and 3 µl Nuclease-free water was combined with 1 µl PCR product, mixed well and left for 5 minutes.

2.3.7 Transformation into competent *Escherichia coli* DH5α

Transformations of competent *E. coli* were carried out using 5 µl ligated DNA added to 50 µl competent *E. coli* which was mixed gently, and the cells incubated on ice for 20 minutes before being heat shocked for 30 s at 45 °C. The cells were left on ice for 2 minutes before being plated on 100 µg/ml ampicillin plates and incubated overnight at 37 °C

2.3.8 Alkaline lysis miniprep

To extract small scale plasmid DNA, 2-5 ml LB with carbenicillin (100 mg/ml) containing a single colony was grown at 37 °C until cloudy, 1 ml of this taken and centrifuged 16000x *g*, 30 s), and the supernatant discarded. The pellet was re-suspended in 100 µl MP1, 200 µl of MP2 was added, followed by 150 µl MP3. The mixture was inverted several times and centrifuged (16000x *g*, 3 minutes). The supernatant was transferred to a new micro centrifuge tube and mixed with 1 ml ethanol and centrifuged (16000x *g*, 10 minutes). The supernatant was removed, and DNA pellet re-suspended in 30 µl dH_2O.

2.3.9 Qiagen midiprep

To purify plasmid DNA from bacterial cultures (100 ml), Qiagen midipreps were conducted according to the manufacturer's instructions but with some modifications. Briefly, cells were harvested (4200x *g*, 10 minutes) and re-suspended in 4 ml buffer P1 before the addition of 4 ml lysis buffer P2 then 4 ml neutralisation buffer P3. After mixing, samples were centrifuged (5000 rpm, 15 minutes) and the supernatant decanted into a new tube. Plasmid DNA was precipitated by addition of 10 ml isopropanol and centrifugation (4200x *g*, 30 minutes). The pellet was re-suspended in 0.5 ml TE and, to remove any endotoxins present, 4.5 ml QBT buffer containing 10% Triton X-100 was added (30 minutes, 4 °C). The mixture was then applied to an equilibrated QIAGEN-tip 100. The column was washed (2x 10 ml

buffer QC) and DNA eluted with 5 ml buffer QF. The DNA was then precipitated by addition of 3.5 ml isopropanol 4200x *g*, 30 minutes). The DNA pellet was washed with 70% ethanol, briefly air-dried then re-suspended in 400 µl buffer EB.

2.4 Protein methods

2.4.1 Cell extract preparation

2.4.1.i Standard total cell extract lysis

For general protein extraction, cells were harvested from flasks, pelleted by centrifugation (1500x *g*, 1 minute) and washed in 0.5 ml 1x PBS . 100 µl lysis buffer per $5x10^5$ cells was added to each pellet ensuring complete resuspension and lysis. The extracts were centrifuged (16000x *g*, 5 minutes) and the protein concentration assessed by Bradford assay before addition of 25 µl 5x SDS-PAGE loading buffer and boiling at 93 °C for 3 minutes.

2.4.1.ii Direct cell lysis

To minimise mechanical stress to cell samples and preserve protein phosphorylation during extract preparation, cells harvested from flasks were pelleted (1500x *g*, 5 minutes, 4°C) and washed in 1 ml cold 1x PBS before being resuspended in 100 µl resuspension buffer with phosphatase inhibitors per $5x10^5$ cells. 25 µl 5x SDS-PAGE loading buffer was added and mixed before boiling at 95°C for 3 minutes. 1 µl diluted Base Muncher (1 in 5) was added to each sample and incubated for 5-10 minutes at RT before boiling for 3 minutes at 95°C.

2.4.2 Bradford Assay

Protein concentrations of samples were obtained using Bradford Assays. 2 µl of cell extract samples were added to 600 µl Bradford Ultra reagent in a cuvette and mixed. These samples were compared, relative to a control using a spectrophotometer by measuring absorbance at A_{600}.

2.4.3 SDS-PAGE Electrophoresis

To prepare SDS-PAGE gels the mini-gel apparatus (Biometra) was assembled following the cleaning of the glass plates and rubber gasket with IMS. 5.5 ml of resolving gel was poured into the apparatus and allowed to set, overlaid with butanol to ensure no air bubbles. The unset acrylamide and butanol were poured off and the stacking buffer was added followed by the well comb.

Once set, the gel(s) were assembled in the electrophoresis tank with 1x SDS-PAGE running buffer, ensuring no air bubbles were present beneath the gel and all lanes were clear of acrylamide residue. The samples were loaded into the wells and the gel run at 120 V through the stacking gel and 165 V through the resolving gel for approximately 1-1.5 hours.

	11%	14%	Stack
	µl	µl	µl
ddH_2O	3.6	2.6	2.1
30% Acrylamide mix	3.7	4.7	0.5
1.5M Tris (pH 8.8)	2.5	2.5	0.38 (1M Tris pH6.8)
10% SDS	0.1	0.1	0.03
10% APS	0.1	0.1	0.03
TEMED	0.004	0.004	0.003

2.4.4 Western Blot Semi-dry transfer

One piece of nitrocellulose membrane and three pieces filter paper were soaked in transfer buffer and assembled in a layered configuration, ensuring air bubbles were removed by rolling out after each layer component in the order as follows; 2 layers filter paper followed by the nitrocellulose, the SDS-gel, washed briefly in transfer buffer, and finally the last filter paper. The apparatus was assembled and proteins transferred for 50 minutes at 20 V.

2.4.5 Western Blot antibody staining

Following western blot transfer the nitrocellulose blot was blocked for 1 hour in appropriate blocking buffer, depending upon the primary antibody to be used, before incubation in primary antibody for 1-3 h at RT or overnight at 4 °C. The blot was washed (4x 5 minutes) with 1x PBS/ 0.1% Tween before incubation with the secondary antibody in blocking buffer for 1 hour. The secondary antibody was then discarded and the blot washed as previously.

2.4.6 Enhanced chemiluminescence (ECL) development of Western Blots

Development of western blots was carried out by incubation of the washed membrane in 20 ml of 100 mM Tris 8.0 containing 6 µl 30% hydrogen peroxide, 50 µl 90 mM p-coumaric acid and 100 µl 250 mM luminol, for one minute before drying, wrapping in plastic wrap and imaging on the Chemi-Doc imager (Bio-Rad).

2.4.7 Western Blot Stripping

To remove bound antibodies prior to re-probing, nitrocellulose blots were washed 2x 5 minutes in mild stripping buffer. The blot was then washed 3x 5 minutes in 1x PBS/ 0.1% Tween.

2.4.8 Immunoprecipitation

Immunoprecipitation of extracted protein samples was carried out using IP lysis buffer with protein concentrations calculated via Bradford assay. Equal amounts of protein for each extract (126 µg) were added to individual microcentrifuge tubes containing 200 µl washed anti-SMC6 beads and 200 µl washed IgG beads to act as a control, and the tubes left to mix for 2 hours at 4°C. Each set of antibody beads was washed several times with lysis buffer before elution of bound proteins with 50 µl 0.2 M glycine at pH 2.0. The eluate was immediately transferred to fresh microcentrifuge containing 7 µl Tris pH 8.8 to neutralise the pH and ¼ volume of 5x SDS PAGE gel loading buffer was added.

2.4.9 Bio-Ubiquitylated protein purification

To purify biotin-tagged ubiquitylated proteins, cells were grown to 70-80% confluency using 6cm plates and transfected with Bio-Ub plasmid, alongside an untransfected control [2.5.1]. Biotin was added to the medium at concentration of 50 µM. Cells were harvested approximately 48 hours later. The plates were washed twice in cold PBS before scraping cells into microcentrifuge tubes in residual PBS and centrifugation (1500x *g*, 5 minutes). The cell pellets were re-suspended in 0.25 ml UBL Lysis buffer per sample and sonicated (2-3x, 12% amplitude) to shear DNA and remove viscosity. The samples were centrifuged (16000x *g*, 5 minutes) and the supernatant collected and diluted with 3 volumes of UBL binding buffer with 50 µl of each collected for SDS-PAGE analysis. The remaining sample was bound to 30 µl of equilibrated Neutravidin beads for 2-3 hours, on a wheel. Post-binding, the beads were pelleted (4000 rpm, 1 minutes) and 50 µl unbound extract collected for SDS-PAGE analysis. The beads were then washed in 1.5 ml of the UBL wash buffers described below; 2x Wash buffer 1, 1x Buffer 2, 1x Buffer 3, 1x Buffer 4, 1x Buffer 1, 1x Buffer 5, 1x Buffer 6. Proteins were eluted in 50 µl UBL Elution buffer by boiling (99°C for, 8 minutes, followed by brief mixing then 99°C, 3 minutes). To remove any remaining beads, the elution samples were spun through spin columns (Pierce) into labelled tubes (16000x *g*, 30 s).

2.5 Cell culture techniques

2.5.1 Cell culture conditions

SV40-transformed MRC5 cells and the derivative cell lines used in this project were cultured in growth medium (DMEM/4.5 Glucose with L-glutamine, 10% Foetal Bovine Serum) and grown at 37 °C in 5% CO_2. When confluent, cells were split and either sub-cultured or discarded depending on requirements. The medium was aspirated off and cells washed in 10 ml 1x PBS. PBS was aspirated off and the flask incubated for 5 minutes at 37 °C following the addition of 7 ml of a 5% trypsin solution until all cells were in suspension. This cell suspension was collected and spun through 5 ml fresh

growth medium (160x *g*, 5 minutes). The medium/trypsin mixture was aspirated off and the cells re-suspended in an appropriate volume of medium. The cells could then be replaced in flask or used to set up for experiments

2.5.2 Transfections

For specific protein production, cells were grown to 80% confluency in antibiotic-free medium and transfected using FuGENE® HD Transfection Reagent according to the manufacturer's protocol. For each plate, an appropriate amount of plasmid DNA was mixed with serum free media before the addition of an appropriate quantity of FuGENE®. The mixture was left for 5 minutes at RT and added dropwise to each plate.

2.5.3 Flow Cytometric Analysis

For visualisation of cell cycle progression in our cell populations flow cytometric analysis was carried out. Cells were harvested from their growth medium ensuring equal populations from each sample. Cells (1x 10^6) were washed in cold PBS and centrifuged at (170x *g*, 5 minutes) and re-suspended in 0.5 ml PBS before being added dropwise to 4.5 ml 70% ethanol (4 °C) while vortexed. Cells were stored at 4 °C until needed.

Before analysis, fixed cells were pelleted (170x *g*, 10 minutes) and re-suspended in 5 ml cold PBS for 20 minutes at RT to facilitate re-hydration. 0.5 ml PBS containing 5µg/ml Propidium Iodide and 100 µg/ml RNase were then added. After 30 minutes, flow cytometric analysis was then be carried out using a Beckman Coulter CytoFLEX™ Flow Cytometer with CytExpert™ Software.

2.5.4 Immunofluorescence

To allow for visualisation of each of the cell lines used in this project with regards to their DNA content and potential abnormalities, immunofluorescence was carried out. Cells were grown on 18mm cover slips in a 12 well plate up to a concentration of 4x10^4. Growth medium was aspirated and each well washed 3x 1 ml 1xTBS. Cells were fixed in 1ml 4% PFA for 15 minutes, on a shaker, then the fixing solution was aspirated off and cells washed 3x in 1ml 1xTBS. Cells were permeabilised using 1 ml 0.2% Triton in TBS for 5 minutes on shaker. Coverslips were rinsed 2x in 1 ml 1xTBS and cover slips blocked in 200µl 3% BSA for 30 minutes. Block solution was aspirated off and coverslips rinsed 3x in 1 ml 1xTBS. 1:100 of appropriate primary antibody (Histone H3 phosphoserine) added onto parafilm with cover slips transferred face down onto the antibody, left at 4 °C overnight in a moist environment. After primary antibody incubation, coverslips were transferred back to their wells, face up, and rinsed 5x 5 minutes in 1x TBS with mixing. 200 µl of fluorescent secondary antibodies at 1:500 was added to each cover slip and incubated for 60 minutes at RT in the dark. Secondary antibody was removed and

coverslips rinsed 5x 5 minutes 1x TBS. 4 µl Vectashield containing DAPI was added to glass slides and cover slips immediately placed cell side down onto the mount media ensuring no air bubbles. Coverslip edges were sealed with clear acetone-based nail polish and finished slides stored in the dark at 4 °C until visualisation on an LED Epi fluorescent microscope / DeltaVision Ultra High Resolution Microscope using ZEN imaging software.

3.1 Results

All the cell lines used to obtain results in this investigation were derived from our MRC5-VI parental cells, a transformed lung fibroblast line. Our *NSMCE1*-Knockout cells were created through abolished NSMCE1 production via CRISPR gene editing, targeting exon 2 of *NSMCE1*, this resulted in a viable yet noticeably slow growing cell line. Our N1-W4 and N1-W14 cell lines were the result of stable transfection of *NSMCE1* into theN1-KO cells, to re-introduce wild type *NSMCE1* which seemingly restored normal cell functions despite N1-W14 slightly over-expressing *NSMCE1*. Finally our N1-RING cell line was created through modified through a double substitution of Zn^{2+} coordinating Cys residues to Alanine (NSMCE1-C191A,C194A) stably transfected into our N1-KO cells to produce our N1-RING cell line, where NSMCE1 is expressed but is theoretically enzymatically inactive.

3.1 Analysis of SMC5/6 complex expression

In order to analyse the cellular levels of NSMCE1 and the other components of SMC5/6 complex, following the disruption and re-introduction of *NSMCE1* in the various *NSMCE1*-mutant cell lines , the protein content was studied via western blotting of total cell extracts, prepared as described in [2.4.3-2.4.6]. Each of the different *NSMCE1* cell lines were analysed and compared against the parental MRC5-VI cells. Antibodies, specific to each of the SMC5/6 complex components were used to detect their complementary proteins, while GAPDH was used as a loading control.

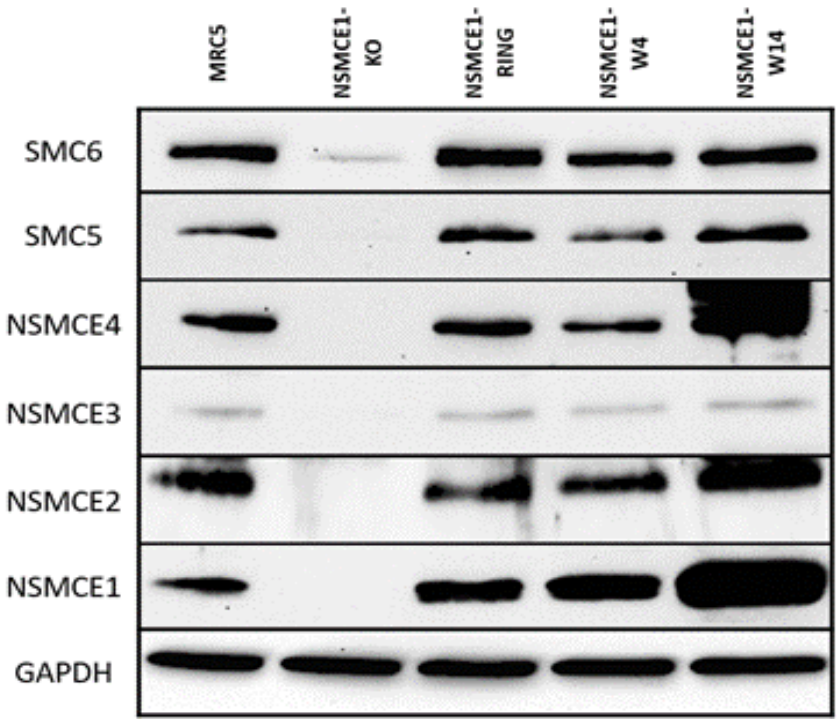


Figure 8 – Western blot of NSMCE1 cell lines showing levels of SMC5/6 components in wild type (MRC5-VI), Knockout (N1-KO), and N1-KO cells stably expressing wild type (N1-W4, N1-W14) or modified NSMCE1 (N1-RING). GAPDH present as loading control. This blot was carried out several times with the most representative blot shown.

Figure 8 shows that NSMCE1 production in the N1-KO cell line appears to have been substantially reduced in comparison to the parental MRC5-VI cell line, since no NSMCE1 was detected in N1-KO cells in this Western blot analysis. In addition, it is apparent that the levels of the remaining SMC5/6 complex components are also significantly reduced in the N1-KO cells.

This is consistent with previous siRNA knockdown studies that demonstrated all of the SMC5/6 complex components except NSMCE2 are required for complex stability (Taylor et al. 2008). It is interesting to note here that on both NSMCE2 and NSMCE4 blots additional cross-reacting bands of reduced electrophoretic mobility were detected which may suggest post-translational modifications such ubiquitylation which could bear further scrutiny with regards to what these bands are and how they may change in response to *NSMCE1* modification. In addition, this analysis confirms that in N1-KO cells stably transfected with *NSMCE1*, the production of NSMCE1 is restored to a level that is either comparable to (N1-W4) or greater than(N1-W14) endogenous levels. In both N1-W4 and N1-W14 cells, where *NSMCE1* translation is restored, the levels of the other SMC5/6 complex components is also restored. Stable introduction of RING-mutant *NSMCE1* in the N1-KO background (N1-RING) also restored the levels of the remaining SMC5/6 complex components to endogenous levels. Together, these observations suggest that the presence of the NSMCE1 protein but not the functionality of the RING domain is necessary to maintain the levels of the other SMC5/6 components.

This data confirms the validity of these *NSMCE1* cell lines, in terms of abolition of NSMCE1 production in the N1-KO and the re-introduction of *NSMCE1* following stable transfection, in the N1-W4, N1-W14 and N1-RING cell lines. The data also highlights that the loss of *NSMCE1* results in a reduction of other SMC5/6 complex component levels, compared to the MRC5-VI parental cells. It is most likely that the absence of NSMCE1 is causing instability of the remaining SMC5/6 complex components, increasing their turnover and degradation, although it is possible the N1-KO could instead be influencing SMC5/6 complex levels at the level of gene expression. The data confirms that the loss of these protein levels can be salvaged via re-introduction of either wild type *NSMCE1* or RING-mutant *NSMCE1* when compared to the N1-KO.

3.2 SMC5/6 complex formation in *NSMCE1*-mutant cell lines.

The previous experiment found evidence that a loss of *NSMCE1* causes reduced levels of multiple SMC5/6 complex components, which may reflect an effect on the stability of the SMC5/6 complex in the absence of NSMCE1. Following this, we endeavoured to analyse SMC5/6 complex formation in each of our various cell lines.

This analysis of complex formation was achieved via immunoprecipitation of SMC6, along with its associated proteins, from total cell extracts prepared from each of our *NSMCE1* cell lines and the parental MRC5-VI cells. This procedure, as outlined in [2.4.8-2.4.9] used anti-SMC6 antibodies, cross-linked to protein-A sepharose beads to specifically immunoprecipitate SMC6 and its associated proteins. Non-specific IgG beads were used as a negative control to confirm the specificity of any anti-

SMC6 association. Once precipitated, the proteins were analysed via western blot using antibodies against the various SMC5/6 complex components.

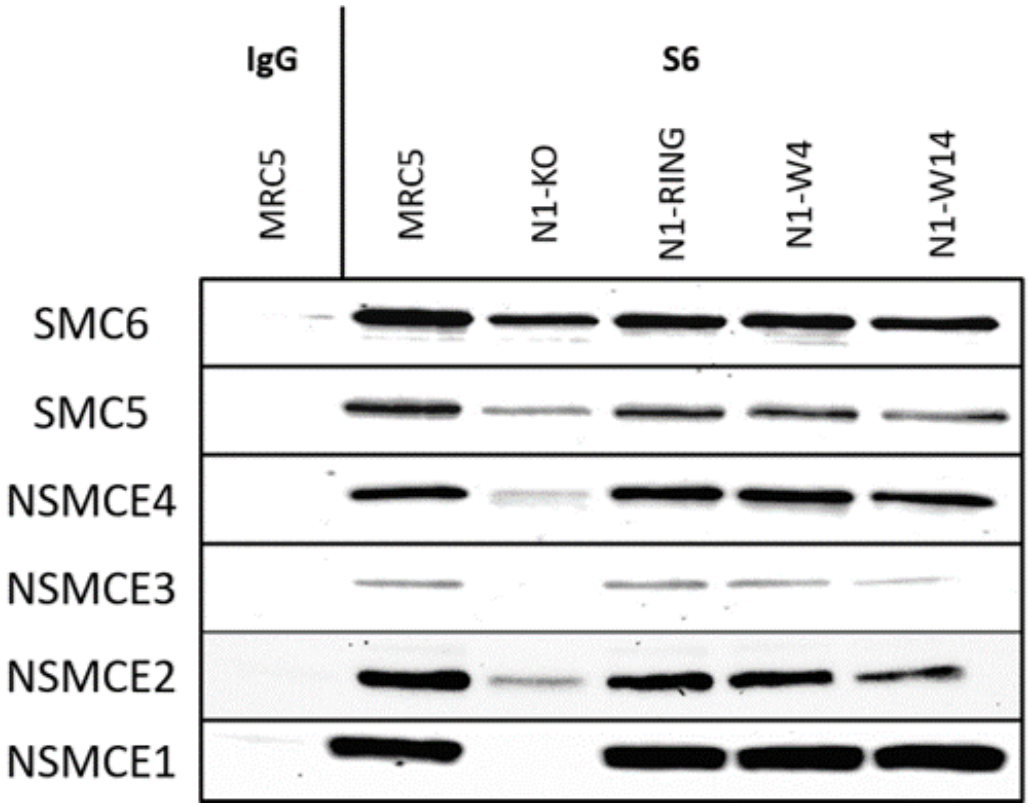


Figure 9 – Western blot analysis of SMC5/6 proteins following immunoprecipitation of SMC6 . SMC6-associated proteins were analysed following immunoprecipitation of SMC6 from MRC5-VI, N1-KO, N1-RING, N1-W4 and N1-W14 cell extracts. Immunoprecipitation with non-specific IgG serves as a negative control. This blot was carried out several times with the most representative blot shown.

The immunoprecipitation data for the MRC5-VI cell extracts, seen in Figure 9, shows that SMC6 is successfully pulled down by the anti-SMC6 antibody associated beads and that the other SMC5/6 complex proteins are co-immunoprecipitated alongside SMC6. This co-precipitation confirms the stable association of NSMCE1-4 and SMC5 with SMC6 in wild type cells, under these conditions. This immunoprecipitation experiment in conjunction with our analysis of SMC5/6 protein levels re-affirms the evidence that the absence of *NSMCE1* results in a significant reduction of the other SMC5/6 complex components. As shown by our immunoprecipitation, small amounts of SMC6, SMC5, NSMCE2 and NSMCE4 remain in the N1-KO cells after the removal of *NSMCE1*. These reduced protein levels of SMC5/6 components can still associate and form a small amount of a partial complex even in the absence of NSMCE1 and NSMCE3.

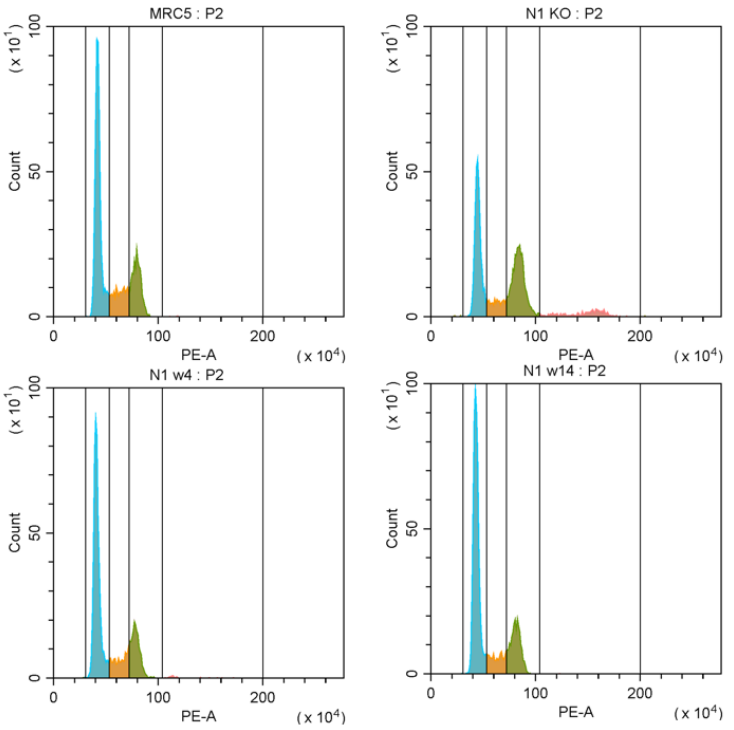
From Figure 9, re-introduction of wild-type or RING-mutant *NSMCE1* in the N1-KO cell line, restored normal protein levels of the other complex components. Following this, Figure 9 demonstrates that the levels of these SMC5/6 complex proteins corresponds with restored levels of SMC5/6 complex after the re-introduction of *NSMCE1*. The immunoprecipitation of SMC6 shows that the SMC5/6

complex components co-precipitated with SMC6, are at comparable levels to the parental MRC5-VI cells when wild type *NSMCE1* is restored in the N1-KO background. Interestingly, analysis of the N1-RING cell line indicates that levels of SMC5/6 complex are also restored following the introduction of RING-mutant *NSMCE1* and is comparable to both the MRC5-VI cell line and the re-established N1-W4 and N1-W14. Together these two experiments confirm that *NSMCE1* deletion and re-introduction influences overall levels of SMC5/6 complex , and that NSMCE1-RING is just as proficient as wild type NSMCE1 in maintaining the levels of SMC5/6 complex. Given that the N1-RING cell line has been demonstrated to have a proliferation defect like that of N1-KO cells, but clearly does not disrupt SMC5/6 complex stability in the same manner, this raises a question as to what is the function of the NSMCE1 RING domain?

3.3 Cell cycle analysis of the NSMCE1 mutant cell lines

Following this initial analysis of the *NSMCE1* cells, we endeavoured to shed further light on the proliferation defect in these cells by analysing the cell cycle distributions of the various cell lines. As indicated by previous studies, the *NSMCE1* cell lines used in this project proliferate at a slower rate than the parental MRC5-VI cells. Thereafter to determine if the generated cell lines had noticeable differences in their cell cycle distributions due to a defect at a particular stage of the cell cycle, we used flow cytometric analysis, to discriminate between cell populations at different stages of the cell cycle.

In order to perform the flow cytometry to analyse cell cycle progression based on the DNA content, we fixed and stained each of our cell lines (MRC5-VI , N1-KO, N1-W4, N1-W14 and N1-RING) with propidium iodide, which stains the DNA, as described in 2.5.3.



Cell cycle phase (%)

Cell Line	G1	S	G2/M	>4N
MRC5	58.38	16.73	22.97	1.66
NSMCE1 KO	39.27	10.68	36.83	11.53
NSMCE1 RING	46.61	11.56	37.38	3.46
NSMCE1 w4	60.74	14.12	20.84	3.39
NSMCE1 w14	63.52	11.70	22.96	1.44

Figure 10 – ***Flow cytometric analysis to analyse variations in cell cycle distributions in the various NSMCE1 cell lines. Cell lines analysed include MRC5-VI, N1-KO, N1-W4, N1-W14 and N1-RING. The DNA content was assessed based on the propidium iodide signal intensity. The table summarises the percentages of cells at varying stages of the cell cycle for each cell line. The data represents a sample size of 10.000 cells for each cell line.***

From the data shown in Figure 10, the MRC5, N1-w4 and N1-w14 cell lines show close similarities in their cell cycle distributions with approximately 60% of cells in G1 phase, 12-17% in S phase and 20-23% in G2/M. In contrast to this, The N1-KO cells exhibit a significantly reduced proportion of cells in G1 phase, with approximately 40% in G1 in comparison to around 60% when wild type *NSMCE1* is expressed, as well as an increased percentage of cells in G2/M, at nearly 37% as compared with approximately 22%. Most interestingly from this data, it becomes apparent that around 12% of the N1-KO cells contain a greater than 4N DNA content. The N1-RING cell line also appears to have a bias towards G2/M phase, with slightly fewer G1 cells (47% and slightly more G2/M cells (37%) as compared with wild type cells, although these cell cycle changes are less pronounced than with the N1-KO cells.

The data gathered in this experiment suggests that the loss of *NSMCE1* in the N1-KO cell line creates a disruption in the cell cycle causing cells to accumulate in either G2 or mitosis. This might suggest that the cells are incurring some form of DNA damage, activating a checkpoint and slowing their advancement through the cell cycle. Conversely the N1-W4 and N1-W14 appear almost wild type in terms of their cell cycle distributions indicating that re-introduction of the wild type NSMCE1 removes the apparent cell cycle defects demonstrated in the N1-KO cells. This reinforces the evidence that the phenotypic effects of *NSMCE1* disruption observed in the N1-KO cells, are a result of the *NSMCE1* loss

and not a result of off-target gene editing, since reintroduction of *NSMCE1* effectively rescues the mutant phenotype. The N1-RING cell line however seems to only partially correct the problems caused by the loss of *NSMCE1* which, together with the proliferation defect in these RING mutant cells, suggests that NSMCE1 has an additional function, other than as a structural component maintaining the SMC5/6 complex, and that this function is dependent on the NSMCE1 RING domain.

This analysis confirms that the proliferation defects observed in both the N1-KO and N1-RING mutant cell lines relates to a defect in cell cycle progression at the later stages of the cell cycle, in G2/M. This problem in cell cycle progression appears to be exacerbated in the N1-KO cells in comparison to the RING-mutant where it is evident that a proportion of the cells fail to undergo normal cell division with a great number of cells containing a >4N DNA content.

3.4 DNA damage analysis in NSMCE1 mutant cell lines

As the results of the flow cytometric and proliferation analysis indicate proliferation and cell cycle progression defects within our N1-KO and N1-RING cell lines, and given SMC5/6's acknowledged role in maintaining genome stability, we endeavoured to determine if the observed effects on cell cycle progression were a consequence of DNA damage or checkpoint activation. In order to assess endogenously arising DNA damage levels in the various *NSMCE1* cell lines we analysed phosphorylation of H2AX, a histone protein modification used as an indicator of DNA DSBS (Foster and Downs, 2005). The phosphorylation of p53 was also investigated to determine if a wider range of downstream DNA damage responses were triggered. Furthermore, we analysed the activation of CHK2, a multifunctional protein which contributes to repairing DSBS by aiding in the relaxation of heterochromatin and recruitment of proteins such as recombinases to assist with repair (Zhang et al., 2004). In addition, activated CHK2 can cause cell cycle arrest and trigger apoptosis should DNA damage be irreparable. Finally, we investigated the activation of CHK1, a protein that helps regulate DNA replication in addition to halting the cell cycle at the G2/M transition in response to problems encountered during replication such as detection of stalled or unstable replication forks (Lopes et al., 2001). There is some evidence CHK1 is required for M phase late DNA damage responses and M-phase progression, but the precise role remains unclear (Zhang and Hunter, 2013). Total cell extracts were

prepared from each of the *NSMCE1* mutant cell lines as described [2.4.1.i] and the respective protein/phosphoprotein levels were determined by western blotting.

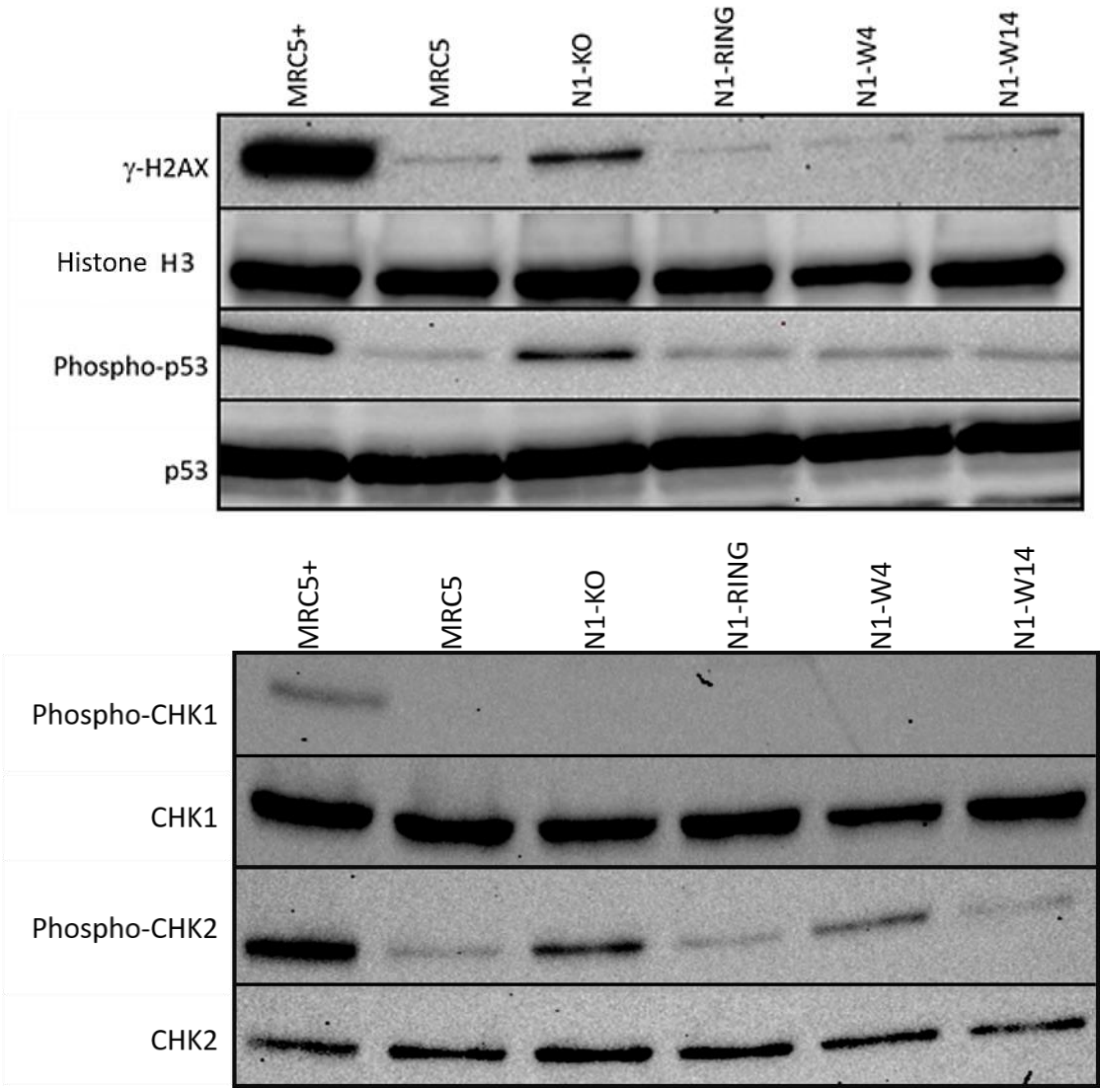


Figure 11 – DNA damage response activation in the various NSMCE1 cell lines. Western blot showing levels of protein phosphorylation and activation. Cell lines analysed include MRC5-VI, N1-KO, N1-W4, N1-W14 and N1-RING and MRC5cells (MRC5+) treated with etoposide as a positive control for DNA damage and checkpoint activation. This blot was carried out several times with the most representative blot shown.

From this analysis displayed in Figure 11 it is clear the N1-KO cells are the only cell line displaying elevated levels of DNA damage and checkpoint activation, with increased phosphorylation of H2AX, p53 and CHK2 being detected in these cells relative to the untreated parental MRC5-VI cell line. These findings are consistent with elevated DNA DSB formation in N1-KO cells, leading to activation of the ATM-dependent DNA damage checkpoint, for which CHK2 phosphorylation serves as a marker. Contrarily despite the proliferation defect and cell cycle progression changes observed in the N1-RING cell line, these cells do not show any obvious indication of increased DNA damage responses or checkpoint activations in this analysis. The differences observed between the N1-KO and N1-RING cell

lines suggests that the increase in DNA damage and checkpoint activation, observed in N1-KO, results from the loss of the SMC5/6 complex as a whole, as opposed to the anticipated disruption of E3 ubiquitin ligase activity in the N1-RING mutant. Analysis of the N1-W4 and N1-W14 cells shows that the re-introduction of NSMCE1 into KO cells also rescues this DNA damage phenotype confirming that the phenotypic differences observed in the N1-KO cell line are genuinely dependent on the loss of the NSMCE1 protein and, subsequently, the SMC5/6 complex in its entirety. It is noteworthy that some CHK2 phosphorylation was observable in N1-W4 cells though no apparent change in H2AX and p53 which leaves it unclear how meaningful or reproducible this CHK2 phosphorylation is. It is also interesting to note that while p53 damage-dependent phosphorylation is normally accompanied by p53 protein stabilisation, and therefore an increase in p53 protein levels, there is no evident increase in stabilised p53 levels in the N1-KO cells despite p53 clearly being activated when compared to the damaged MRC5+ control and undamaged MRC5 cells. The reason for this is unclear but suggests the damage incurred by the N1-KO cells is sufficient to activate some pathway of p53 though not to the same extent experienced by the MRC5+ cells which would be suffering increased DNA damage and checkpoint activation as a result of their treatment with etoposide.

Interestingly none of our cell lines show any obvious signs of replication issues given the absence of CHK1 phosphorylation, even in the N1-KO and N1-RING cell lines. This absence of activated CHK1 is consistent with our evidence that the N1-KO and N1-RING cells are encountering problems towards the G2/M stages of the cell cycle.

3.5 Immunofluorescence analysis of NSMCE1 cell lines

To further analyse the cell cycle perturbation and indications of genome instability we have observed in our NSMCE1-mutant cell lines through our previous experiments, we performed immunofluorescence analysis. The aim of these experiments was to determine whether any of our modified cell lines were displaying observable phenotypic abnormalities when compared to the parental MRC5-VI cells, as well as to determine the mitotic index for each cell line since our previous data indicated a bias towards G2/M in the N1-KO and N1-RING.

3.5.1 Preliminary immunofluorescence analysis (DAPI and Phospho-histone H3 (Ser10) staining)

Cells (N1-W14, N1-KO and N1-RING) were grown on coverslips, stained, then analysed by immunofluorescence imaging as described in /section/. DNA was stained using 4’,6-diamidino-2-phenylindole (DAPI), while phospho-histone H3 (Ser10) antibody was used to detect mitotic cells since

from early prophase through to early anaphase phosho-H3 is detectable on chromosomes which becomes quickly dispersed in the cytoplasm through anaphase and telophase (Li et al., 2005).

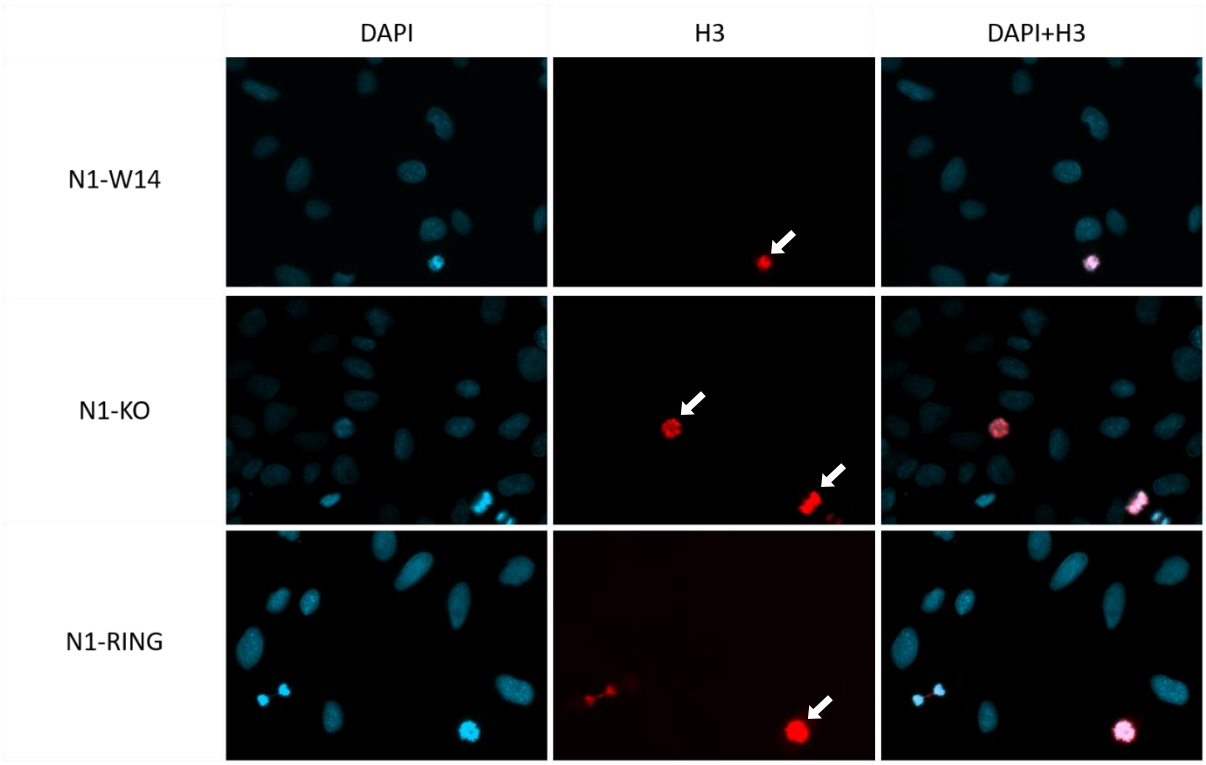


Figure 12 –Representative image of N1-W14, N1-KO and N1-RING cells stained with DAPI (blue) & anti-phospho-histone H3 (Ser10) (red). Arrows indicate cells stained positive for phospho-H3(S10) in prophase-metaphase, late anaphase/telophase cells were not counted.

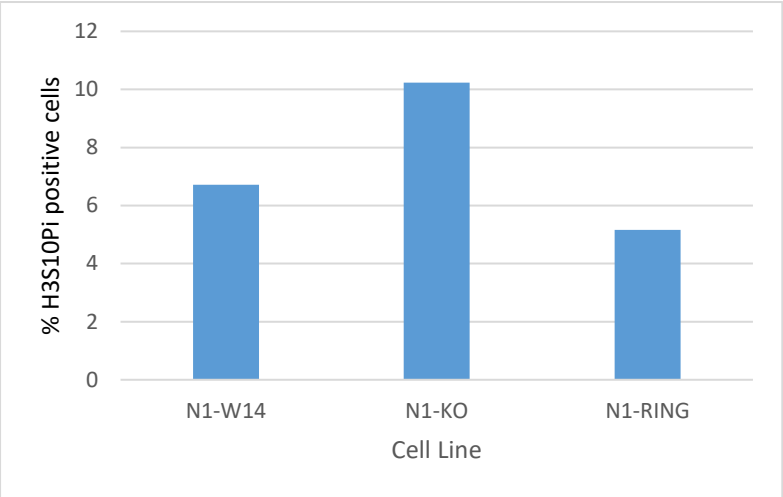


Figure 13 – Comparison of % H3S10pi positive cells of N1-W14, N1-KO and N1-RING cells. Data represents an average cell count for each cell line of 350 cells with mitotic cells identified on the basis of significant phospho-histone H3 (Ser10) staining.

In this preliminary analysis, the mitotic index was determined for each cell lines from counting cells with strong histone-H3 staining (as indicated by the arrows in Figure 12). Mitotic cells were quantified for the N1-W14 cells, (the NSMCE1-rescue cell line) which serves as the positive control, and for the N1-KO and N1-RING mutant cell lines. Average cell counts based on the analysis of approximately 350 cells per cell line are presented in Figure 13.

Since around 95% of the cell cycle is spent in interphase, we would expect that in any given cell population around 5% of the cells would be in mitosis. As depicted in Figure 13, the N1-W14 and N1-RING cells had mitotic indexes of 6.7% and 5.1% respectively which are relatively similar to expected values for a given cell population. In comparison, the N1-KO cells have a greater mitotic index of 10.2%, which is consistent with our previous flow cytometric analysis showing a shift of the N1-KO cell population towards G2/M. From this preliminary analysis, the N1-RING cells did not have a noticeably high mitotic index which suggests that the mutation of the NSMCE1 RING domain does not cause the same disruption to the cell as complete removal of NSMCE1.

Furthermore, from this analysis we were able to observe certain cell abnormalities present with the N1-KO and N1-RING cells which were not apparent within the N1-W14 cells. The cellular anomalies investigated included micronuclei, anaphase lag and nucleoplasmic bridges, representative images of each of these counted anomalies can be seen in Figure 14.

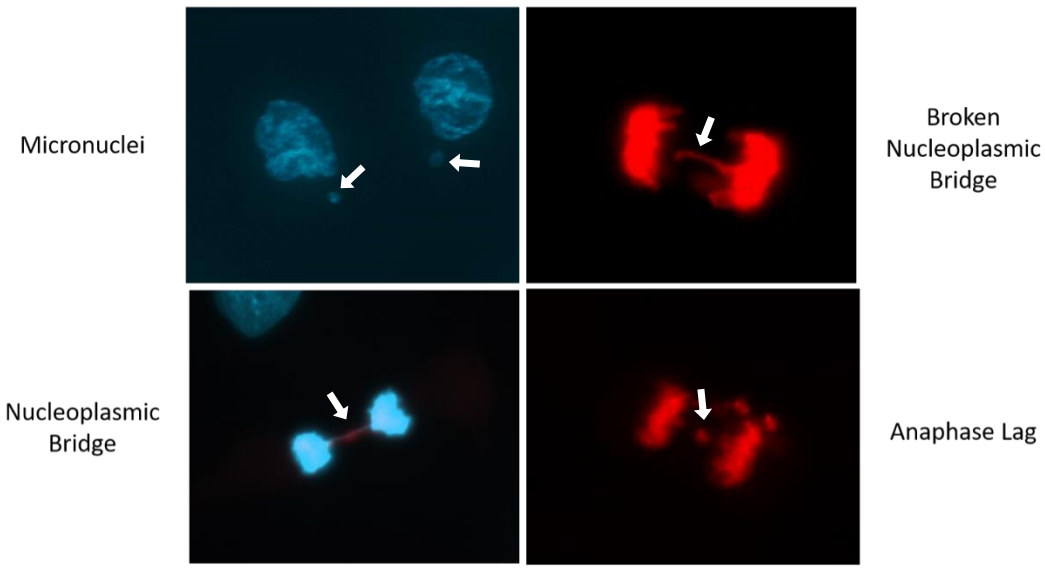


Figure 14 – Representative images of each type of cell anomaly observed within the cell samples. Images stained with DAPI (blue) and anti-phospho-histone H3 (Ser10) (red). Micronuclei, broken nucleoplasmic bridge and anaphase lag are indicated by white arrows and images were taken from N1-KO samples. Nucleoplasmic bridge (bottom left) taken from N1-RING and is highlighted with an arrow.

In this analysis (of 387 cells) no cell abnormalities were detected in our N1-W14 positive control cells. Data shown in Figure 15 demonstrate how in the N1-KO cells (420 cells) the greatest number of abnormalities was detected with 1.4% of the cell population displaying micronuclei with around 0.5% of the population containing either nucleoplasmic bridges or showing anaphase lag, more than the N1-W14 positive control. In the N1-RING cell line (329 cells) around 0.3% of the cell population displayed either anaphase lag or micronuclei, less than the N1-KO but more so than the N1-W14 positive control.

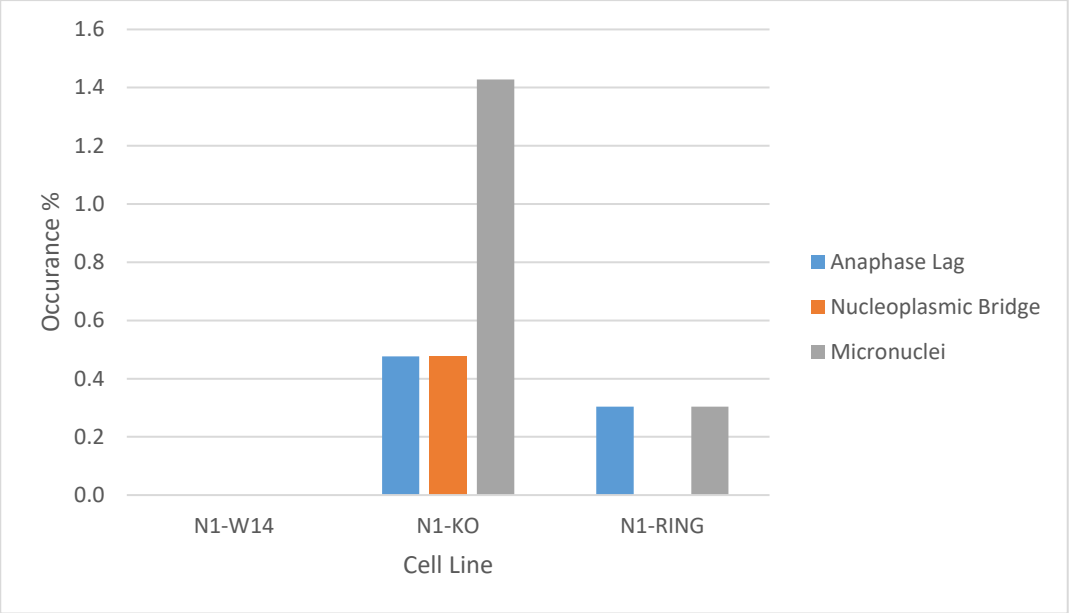


Figure 15- Comparison of the percentage of observed cell anomalies present in each of the cell lines. Anomalies counted following DAPI and anti-phospho-histone H3 (Ser10) staining. Data presented representsmore than 320 cells for each cell line.

3.5.2 Further immunofluorescence analysis

The immunofluorescence analysis trialled above was then repeated in a second experiment using MRC5-VI, N1-W14, N1-KO and N1-RING cell lines. In this experiment, images were collected using a DeltaVision Microscope which allowed for a larger sample size to be taken with higher image clarity.

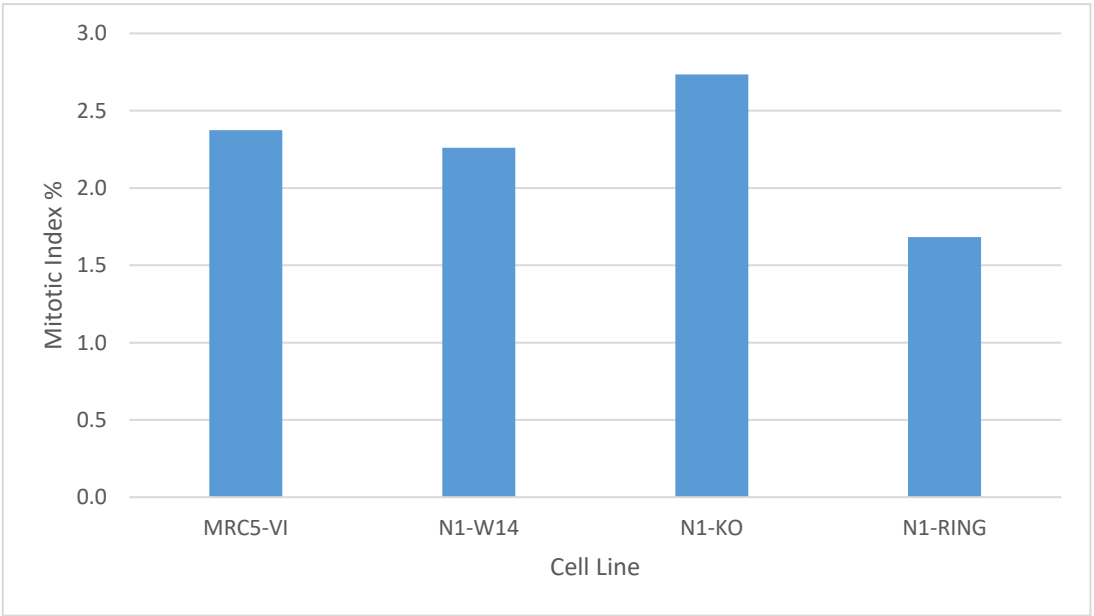


Figure 16 - Comparison of mitotic index (%) of MRC5-V1, N1-W14, N1-KO and N1-RING cells. Data represents an average cell count for each cell line of 500 cells with mitotic cells identified on the basis of significant phospho-histone H3 (Ser10) staining.

As with the previous experiment a mitotic index was determined for these cells, based on the proportion of cells staining positive for phospho-histone H3 (Ser10) (Figure 14). Figure 16 shows the MRC5-VI cells having a mitotic index of 2.4% compared with 2.3% for N1-W14, the N1-KO cells showed the highest overall mitotic index at 2.7% with the N1-RING showing a mitotic index of 1.7%. Overall, when comparing the N1-W14, N1-KO and N1-RING with the previous experiment, these values are lower. In addition, although the N1-W14, N1-KO and N1-RING cells display the same trend as seen in the previous experiment (a higher proportion of N1-KO cells in mitosis of N1-W14 and a lower proportion of N1-RING cells in mitosis of N1-W14) in this experiment the difference are very small. Further experiments would be required to ascertain if these differences are reproducible.

By using a DeltaVision microscope to obtain stack images which could be compressed into a single plane, we were able to count cell anomalies as to a much higher degree of accuracy than in the previous experiment. In this analysis shown in Figure 17 (337 cells for MRC5-VI and 1,200 cells for N1-W14) both cell lines exhibited a low level of micronuclei formation of approximated 0.25% with no apparent mitotic anomalies detectable in these cells during this analysis. In contrast in the N1-KO cells (1,499 cells) a larger proportion of 1.13% of these cells showed micronuclei with 0.07% displaying nucleoplasmic bridges. The N1-RING cells (654 cell) displayed 0.46% of its cells containing micronuclei with no other anomalies being detected. In both the initial experiment and this further investigation the evidence of some micronuclei detectable across cell lines shows this can occur in healthy cells, however given the substantial (approximately five-fold) increase displayed in the N1-KO cell line, this suggests the absence of NSMCE1 exacerbates the occurrence of micronuclei, in addition to causing other mitotic anomalies.

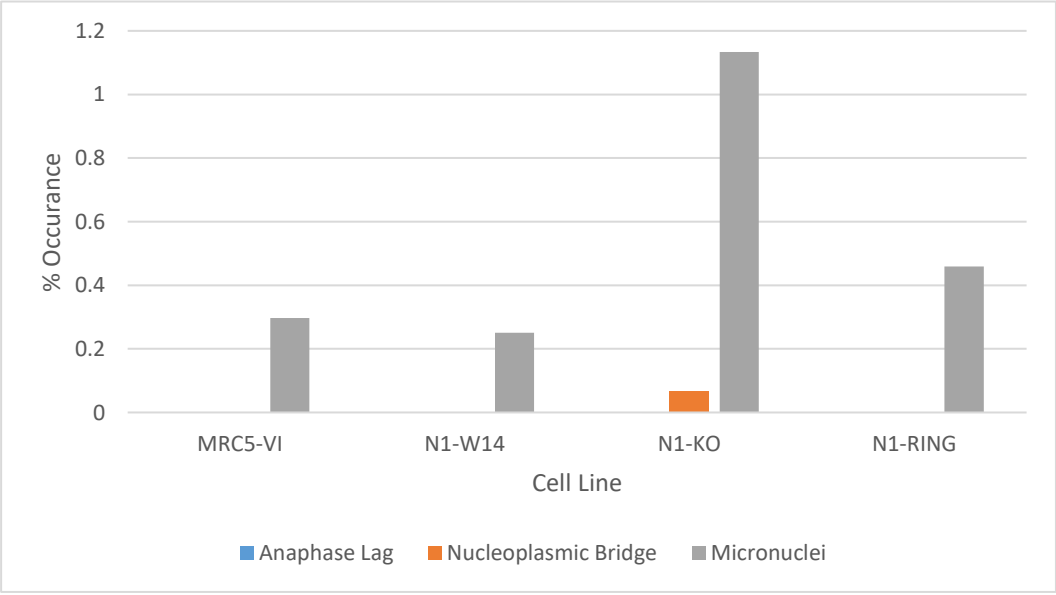


Figure 17- Comparison of the percentage of observed cell anomalies present in MRC5-V, N1-W14, N1-KO and N1-RING. Anomalies counted following DAPI and anti-phospho-histone H3 (Ser10) staining.

3.6 NSMCE1 & Ubiquitin modification.

Our previous experiments over the course of this investigation aimed to confirm the validity of our cell lines in addition to highlighting the effects of NSMCE1 disruption on cell proliferation. This s analysis has shown some functional importance of the NSMCE1 RING domain. The enzymatic role of NSMCE1 however is understood to a lesser extent than its structural role in maintaining the SMC5/6 complex. While NSMCE1 demonstrates a great likeness to E3 ubiquitin ligases due to its variant RING domain, to date there have been mixed reports on its enzymatic activity. One such investigation in *S. pombe* showed some cellular importance through NSMCE1's maintaining of mitotic fidelity (Tapia-Alveal and O'Connel,2011).

As such, given the indication that NSMCE1 may be an E3 ubiquitin ligase, we endeavoured to investigate this, with hopes to uncover some evidence of its enzymatic function. Given the reported difficulty in demonstrating Ub ligase activity for NSMCE1 in *in vitro* studies, our investigation assumed that NSMCE1's activity may rely upon a cellular context and in conjunction with the SMC5/6 complex as a whole. As such, such we addressed this hypothesis using a method to investigate NSMCE1 activity within cells.

Given the absence of any known targets of NSMCE1 ubiquitylation, we focused on ubiquitin modification of NSMCE1 itself as a plausible readout for auto-ubiquitylation activity. To explore potential ubiquitin modification of NSMCE1 we followed a Bio-ubiquitin pulldown method outlined in Pirone et al.,2017 as displayed in Figure 18 which involves transient transfection of a bio-ubiquitin plasmid into our MRC5-WT cells to produce biotin-tagged ubiquitin *in vivo*. This biotin-tagged ubiquitin would theoretically be attached to proteins via cellular ubiquitin ligases and could then be used to pull down these proteins on streptavidin beads, with the aim being to see if disruption of NSMCE1 would affect the levels of proteins being pulled

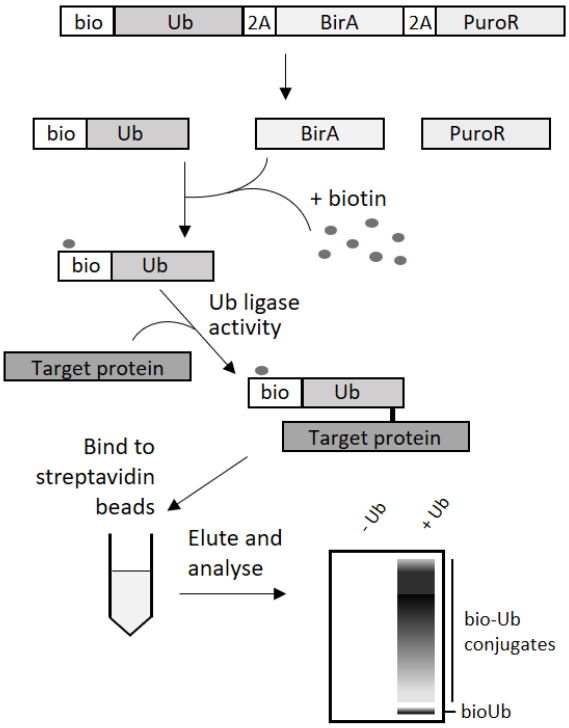


Figure 18 – Outline of the Bio-ubiquitin pulldown methodology. Bio-ubiquitin is biotinylated by a co-expressed BirA biotin ligase and this biotinylated ubiquitin is then covalently attached to cellular proteins by endogenous ubiquitin ligases. The resulting biotin-ubiquitin-tagged proteins can then be purified by their high affinity interaction with NeutrAvidin beads.

down. All the experiments presented here took place in wild-type MRC5-VI cells to establish a working protocol. In addition, since we anticipated endogenous NSMCE1 modification would be beyond the level of detection for western blot and as such our stratergy entailed the over-production of these proteins. To do this, we first created bio-UB/FLAG-NSMCE1 and bio-Ub-FLAG-NSMCE1-RING plasmids for transfection into cells. These plasmid constructs were made by replacement of the puromycin resistance marker of the bio-UB plasmid (Pirone et al. 2017) with the *NSMCE1* ORF, followed by site directed mutagenesis to insert the NSMCE1-C191A,C194A double Cys to Ala changes into the RING domain.

Initial trial transfection of these plasmids to confirm the production of NSMCE1 and bio-ubiquitin (Figure 19) showed the process was sucessful at conjugating biotinylated-Ub onto a range of cellular proteins as expected, detected through anti-biotin antibody staining of total cell extracts. This trial experiment also showed the FLAG-N1 and FLAG-N1 RING were also sucessfully produced, at higher levels than endogenous NSMCE1, via staining with α-NSMCE1 antibody. This initial experiment showed the expected bands for FLAG-NSMCE1 as well as some higher molecular weight bands that may represent potential Ub-modified forms of FLAG-NSMCE1, which prompted us to conduct the full pulldown.

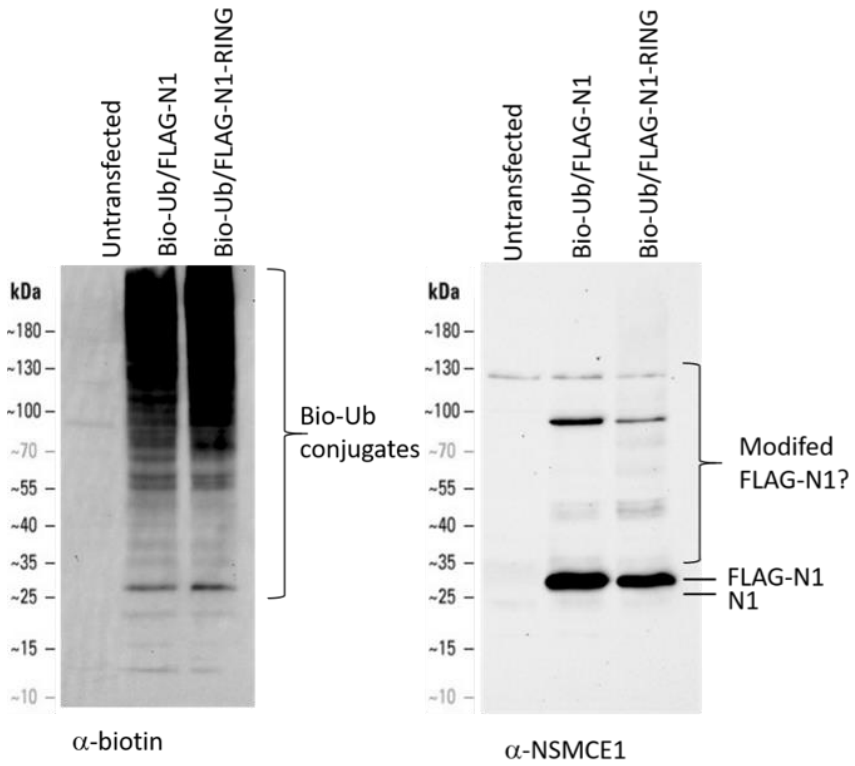


Figure 19 – Test transfection of Bio-Ub and FLAG-N1/N1RING plasmids into MRC5-WT cells. Antibody staining of whole cell extracts with α-biotin indicated high levels of biotinylated ubiquitin being successfully conjugated onto proteins. α-NSMCE1 stain indicated FLAG-N1 and FLAG-N1-RING production were additionally successful.

Having established that transient transfection and production of the bio-ubiquitin/FLAG-N1 in our MRC5-V1 cells was successful, we carried out the Bio-Ub pulldown in full to establish whether we could successfully isolate ubiquitin-modified NSMCE1. The data depicted in Figure 19 shows untransfected cells do not produce the bio-tagged ubiquitin which is to be expected as they lack the bio-ubiquitin coding sequence and the BirA biotin ligase and, as such, biotin-tagged ubiquitylated proteins are not pulled down by the Neutravidin beads. The remaining transfection conditions we tested included FLAG-N1 with Bio-Ub, FLAG-N1-RING with Bio-Ub and Bio-Ub alone in MRC5-V1 cells.

Cells transfected with bio-Ub alone show evidence of successful Bio-ubiquitin conjugation on cellular proteins as these proteins were isolated on Neutravidin bead pulldowns as expected when probed with the α-biotin antibody as shown in Figure 20. There was however no detectable bio-Ub modification of NSMCE1 in this experiment at endogenous NSMCE1 levels.

Bio-Ub/FLAG-N1 transfected cells show some evidence of Bio-Ub conjugation but overall levels appear very low in this transfection with lower levels of Bio-Ub conjugates being detectable when compared to the Bio-Ub alone. FLAG-NSMCE1 is evidently overexpressed as shown by the higher molecular weight band detected by α-NSMCE1 with some possible evidence of modified NSMCE1, though as no detectable bio-UB was extracted on Neutravidin it appears unlikely that this represents ubiquitylated NSMCE1. Bio-Ub/FLAG-N1-RING shows good conjugation of Bio-Ub alongside the FLAG-N1-RING protein. There is some evidence of NSMCE1 modification in the Neutravidin extraction indicating successful Bio-Ub modification.

Only overexpressed FN1-RING was detectably ubiquitinated in this experiment as is evident from the Neutravidin pull down. Despite this, given that the total pull down for bio-Ub/FLAG-N1 was considerably less than the RING it is inconclusive whether ubiquitination is elevated in the presence of N1-RING or just representative of normal ubiquitination at elevated levels.

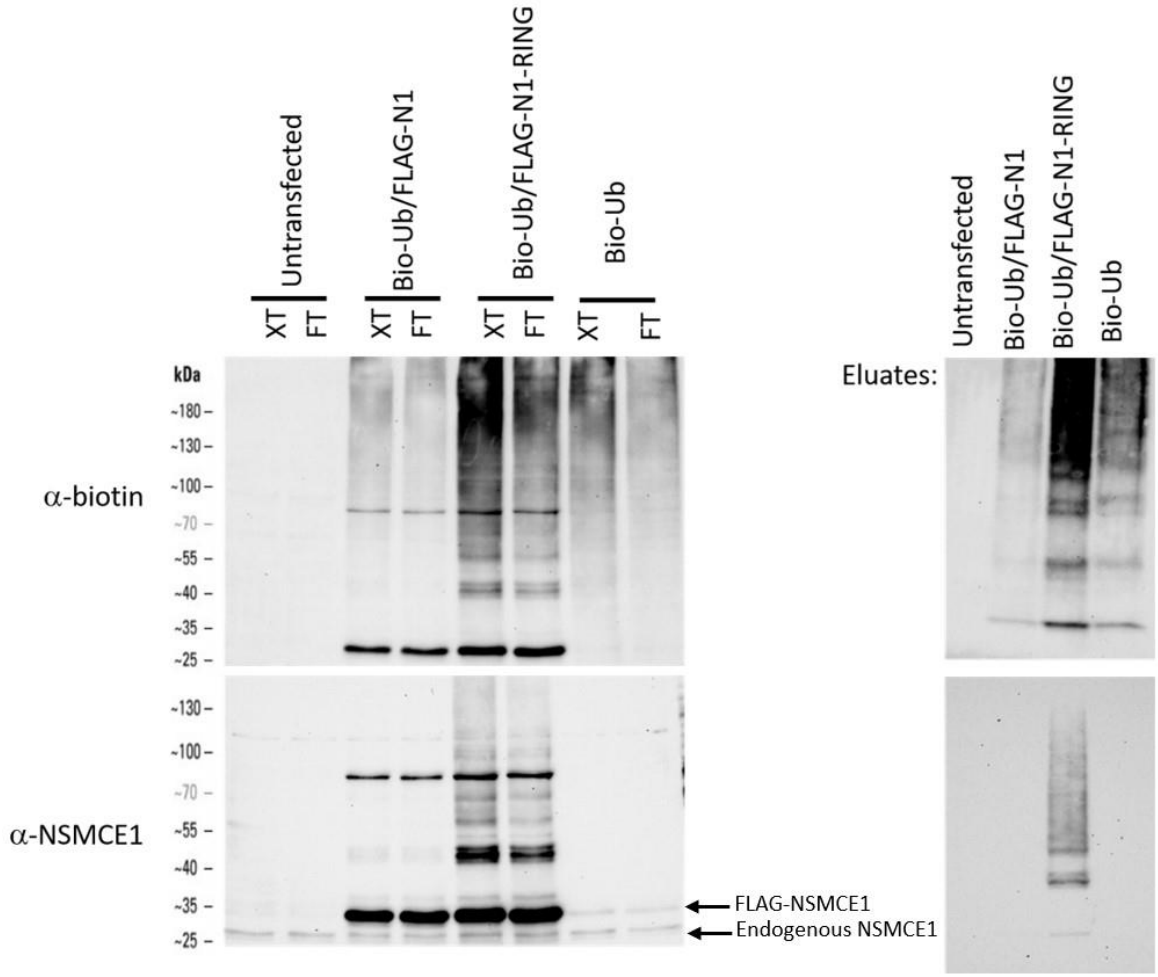


Figure 20 – Western Blot analysis of un-transfected and transfected MRC5-VI cells using Bio-Ubiquitin/FLAG-N1, Bio-Ubiquitin/FLAG-N1-RING, and Bio-Ubiquitin. Extract (XT) sample indicate proteins present in the initial cell extract with Flow-through (FT) showing remaining proteins un-bound to the NeutrAvidin beads. Eluates indicate Bio-Ub modified proteins which remained bound to the NeutrAvidin beads. Top blot indicates Bio-ubiquitin conjugated proteins were present in each cell extract, flowthrough and elute but not in untransfected cells as expected. Endogenous NSMCE1 was detectable in all extracts, and flowthroughs, with FLAG-NSMCE1 detectable in the Bio-Ub/FLAG-N1 and Bio-Ub/FLAG-N1-RING. Diagram suggests some bio-ubiquitin modified NSMCE1 extracted Bio-Ub/FLAG-N1-RING eluate as it was detectable via α-NSMCE1 antibody.

4.0 Discussion

4.1 NSMCE1 is integral for levels of the SMC5/6 complex

NSMCE1 is unique in its role as a structurally important component of the larger SMC5/6 complex. While containing a RING domain characteristic of E3 ubiquitin ligases, to date it has not reliably displayed this activity *in vitro*. Through our investigations into NSMCE1 we demonstrated the structural importance of the protein in maintaining the levels of the SMC5/6 complex through analysis of total cell extracts in conjunction with an immunoprecipitation assay. Our western blots of SMC5/6 protein levels suggest several possible things occurring in response to the loss of NSMCE1. The SMC5/6 complexes may become destabilised following the loss of *NSMCE1* within the N1-KO cells, leading to increased turnover of the complex components, which is salvageable through re-introduction of wild type or RING-mutated *NSMCE1*. Or, given that the other complex components can still be detected, it suggests that the loss of *NSMCE1* causes reduced gene expression of the other SMC5/6 complex components, as reduced but still visible levels were detectable in our N1-KO which would suggest NSMCE1 has a non-structural role in SMC5/6 complex regulation.

Immunoprecipitation data for SMC6 associated proteins indicates that most of the SMC5/6 complex components (NSMCE2, NSMCE4, SMC5 and SMC6) are still present in a complex within N1-KO cells although at greatly reduced levels compared to the parental MRC5 cell line, while neither NSMCE1 and NSMCE3 are detectable in N1-KO cells, in our analysis. Re-introduction of *NSMCE1*, with or without a mutated RING domain, salvages the complex to mostly wild-type levels, confirming that the reduction in complex levels is entirely dependent on the absence of NSMCE1. The complete loss of NSMCE3 within the N1-KO cell line suggests that the stability of these proteins is extremely interdependent. This is consistent with the close association of MAGE proteins with Ub ligases and may provide further support for NSMCE1 being a E3 ubiquitin ligase (Doyle et al.,2010).

Thanks to Sergeant et al., 2005 we have a good understanding of the structure of the SMC5/6 complex. Based on this understanding SMC5, NSMCE4 and NSMCE2 should theoretically be able to form an incomplete sub-complex with SMC6 without the need for NSMCE1 or NSMCE3 being present, given that NSMCE2 binds half way up the SMC5 coiled domain and NSMCE4 is responsible for bridging the SMC5 and SMC6 head domains. However, our data suggests that NSMCE1 is in fact integral to the levels of SMC5/6 complex within cells despite not having an obvious interaction with proteins other than NSMCE4 and NSMCE3. One explanation for this may be that NSMCE1 induces a conformational change in NSMCE4 which allows it to stably bridge the SMC5/6 gap since without NSMCE1 the reduced levels of the complex components we observe suggests that the remaining complex components are being expressed to a lesser extent or degraded. This data fits with previous studies who all

demonstrated how loss of other complex components including NSMCE1 and NSMCE3 leads to reduced levels of the SMC5/6 complex with varying levels of detriment to the cell (Taylor et al.,2008,Van der Crabben et al.,2016 and Venegas et al.,2020).

Because our data indicates the NSMCE1-RING mutant restores levels of the SMC5/6 complex like its wild-type counterpart, this argues that, in the case of this mutation, changes to the RING domain do not result in reduced levels. Previous studies into NSMCE1-RING mutants in *S. pombe* have demonstrated that certain modifications to the NSMCE1-RING site can affect complex stability through affecting its binding with NSMCE4 but not with NSMCE3. These results indicated the effects on binding stability was dependent on the type of mutation incurred by NSMCE1-RING but that in most cases binding was not completely ruined (Pebernard et al., 2008). Unlike in *S. pombe* however a study into NSMCE1-RING-domain mutations in *S. cerevisiae* revealed that single substitution mutations did not have any effects on SMC5/6 complex stability yet double substitutions produced cells where NSMCE1 was unable to bind NSMCE4 and NSMCE3 unlike in *S. pombe* where NSMCE3 binding was mostly unaffected (Wani et al., 2017).

Since these studies were performed in yeast it is unclear whether other changes the in human NSMCE1-RING domain would convey the same sensitivities or show similar binding defects as demonstrated in yeast. However, it is evident that our two point, mutations, NSMCE1-C191A,C194A double Cys to Ala changes affecting the Zn-co-ordinating residues, does not produce binding deficiencies and SMC5/6 structural instability unlike the double Zn-finger substitutions in budding yeast.

Following the understanding that NSMCE1 itself has a sizeable impact on SMC5/6 complex levels our analysis into the cell cycle progression indicated that the loss of the complex leads to notable changes in growth and cell cycle distributions. Cells lacking NSMCE1 showed an overall 37% bias towards G2/M, with 12% of the cell population displaying a greater than 4N DNA content, indicating cells were struggling to correctly segregate their DNA. This greater DNA content could represent possible re-replication of DNA as a result of SMC5/6 disruption and a de-regulation of singular origin point firing or as a result of downstream complications in chromosome segregation resulting in failed mitotic segregation, given that SMC6 has been implicated in salvaging collapsed replication forks as well as alleviating replication tension (Ampatzidou et al., 2006, Betts Lindroos et al., 2006) likely achieved in conjunction with the remaining SMC5/6 complex. Without the complex being stably present within the cell as a result of NSMCE1 loss, cells would have to utilise other repair methods to bypass errors such as replication fork collapses and increases in DNA tension during replication, which potentially could result in DNA damage and abnormal DNA structures being created (Bets Lindroos et al., 2006).

These abnormal structures could easily cause complications in chromosome segregation through chromosome tangling, which would explain the variable DNA content detected within the cells of this N1-KO population due to potential chromosome breakages resulting in uneven distributions of DNA.

Interestingly while N1-W4 and N1-14 cell lines were mostly comparable to the MRC5 parental cell line, confirming rescue of this cell cycle phenotype by re-introduction of wild type NSMCE, the N1-RING cells displayed a significant 37% bias towards G2/M similarly to the N1-KO cells, though considerably less of these N1-RING cells had a greater than 4N DNA content at only 3%. Since our previous experiment has shown the SMC5/6 complex stability can be salvaged through re-introduction of NSMCE1 harbouring this RING mutation, this data suggests that the N1-RING domain clearly conveys some significant functional importance that impacts on cell cycle progression. This similarity between the N1-KO and N1-RING confirms NSMCE1 has a functional role that impacts on the cell cycle, however we are unable to conclude from our data whether or not the activity of NSMCE1 more prevalent in mitosis or may, as previously stated, be required in S-phase to prevent accumulative problems that eventually impact mitosis. Hypothetically if NSMCE1's enzymatic action is only active during mitosis this could account for the variability in previous studies failing to demonstrate the proteins ubiquitin ligase activity, since the majority of the cell population would be in interphase (Pebernard et al., 2008).

4.2 DNA damage response after NSMCE1 disruption

Our investigation into endogenously arising, DNA damage and checkpoint activation as a result of NSMCE1 disruption yielded insightful results though not without some mystery. Of our test samples, N1-KO was the only cell line to show p53 activation which is supported by recent publications that show loss of SMC6 and NSMCE4 (which we know are destabilised by the loss of NSMCE1(Venegas et al.,2020)) causes p53 upregulation which is not surprising in a cell that has lost a protein complex responsible for DNA repair. H2AX was similarly only phosphorylated in the N1-KO cell line which is an indicator of the presence of DSBS which further indicates that NSMCE1 loss severely affects the functionality of the SMC5/6 complex. The accumulation of unrepaired DSBs is consistent with evidence of a role for SMC5/6 in DSBR (Bailly et al., 1994; McDonald et al., 2003). In addition to H2AX phosphorylation, CHK2 which halts the cell cycle to provide time to repair DNA damage and notably assists in helping DSBR was also phosphorylated further indicating the presence of DNA damage and DSBS within the N1-KO cells.

In addition to our investigation into cell cycle progression, our analysis of CHK1 activation revealed that problems arising from the loss of NSMCE1 does not activate replication checkpoints as may be expected. With CHK1 being a protein that helps regulate DNA replication and halt the cell cycle at G2/M in response to replication fork instability and DNA damage, it would appear from our data that

the DSBS generated are activating the control points within G2/M or causing problems within mitosis itself but that replication itself is not inhibited and so damage is occurring later. In fact previous studies which analysed checkpoint maintenance involving CHK1 and SMC6 in yeast have suggested that SMC6 is required not only for DNA repair but maintaining checkpoint responses specifically to ensure the response is not terminated early following initiation by other factors such as CHK1 (Verkade et al., 1999; Chen et al., 2013).

4.3 Physiological impact of SMC5/6 destabilisation

Our investigations into physiological manifestations in cells because of NSMCE1 disruption yielded promising insights into how the loss of the SMC5/6 complex affected mitosis. Preliminary and secondary investigations demonstrated N1-KO cells contained a greater proportion of cells in mitosis which supports our previous data when compared with MRC5-VI, N1-W14, and N1-RING cells, however significant variability of the results warrants further study. Interestingly N1-RING cells consistently showed the lowest proportion of cells in mitosis based on histone H3 (Ser10P) staining. Since the N1-RING cells showed bias within our flow cytometric analysis towards G2/M, the lack of histone H3 (Ser10P) staining suggests the N1-RING cells may be accumulating before mitosis in G2. We would suggest future analysis focus in on where exactly the N1-KO and N1-RING mutants are blocked in the cell cycle with attention focused on the G2/M transition.

Staining revealed several common defects within our N1-KO and N1-RING populations. Firstly, anaphase lag was detected in both these populations but at moderately higher levels in the N1-KO than the N1-RING cells. However in both cases this supports the evidence that these cells are taking longer to complete mitosis if they are struggling to separate their chromosomes which implies NSMCE1 or possibly the SMC5/6 complex as a whole plays a role in mediating chromosome segregation during telophase.

Micronuclei were detectable in all of our cell lines but were more prevalent in both the N1-KO and N1-RING, though in much larger quantities in the N1-KO occurring in almost 1.5% of the total cell population. This damage may be due to insufficient DNA repair because of the loss of the SMC5/6 complex or due to the nucleoplasmic bridges, detectable only in the N1-KO which if snapped would explain these micronuclei being formed from chromosome fragments. Should the nucleoplasmic bridges remain intact this would also explain the cells containing greater than 4N DNA content due to the subsequent failure of cytokinesis. All of these anomalies in the N1-KO cells link the SMC5/6 complex with playing an important role in chromosome segregation (Harvey et al., 2002) but suggests NSMCE1-RING domain plays an functional l role in this segregation given the N1-RING cells do not entirely recover like their N1-W4 and N1-W14 counterparts. However based on the small sample size

of this experiment and the variability of the results obtained through the different staining preparations, further analysis would be required to determine if the physiological defects detected in the N1-KO and N1-RING cells as a result of NSMCE1 disruption is canonical.

It is surprising that within our cell cycle progression analysis, N1-RING mutant cells do not show the same evidence of DNA damage or checkpoint activation as the N1-KO cells despite sharing similar proliferation and cell cycle defects as the N1-KO cells. This suggests that the loss of the function caused by the double Cys to Ala changes in the RING domain, although impacting on cell cycle progression, does not result in exactly the same effect as in the N1-KO cells where the whole complex is disrupted, which points towards NSMCE1's enzymatic activity potentially being important in G2/M. This analysis of the physiological effects of NSMCE1 mutation yields further insight into the difference between the N1-RING and N1-KO and suggests that NSMCE1 and/or the larger SMC5/6 complex plays a important role in regulating the cell cycle either before or during the G2/M transition.

4.4 NSMCE1 and ubiquitin modification

Following the work of Doyle et al. our attempt to demonstrate the ubiquitin modifying abilities of NSMCE1 in conjunction with the other SMC5/6 complex components yielded mixed results, partly due to difficulties obtaining a consistently functioning protocol (Doyle et al., 2010). Our preliminary results were promising with either FLAG-N1 and FLAG-N1-RING, co-transfected with Bio-Ub, demonstrating complete functionality in a wild-type background, though further experiments were less fruitful. Due to unknown factors the FLAG-N1-Bio-Ub transfection failed to maintain equivalent levels comparable to the FLAG-N1-RING even with varying transfection quantities. We were able to detect some potential ubiquitination of the overexpressed FLAG-N1-RING but not of the overexpressed FLAG-N1 or endogenous NSMCE1, though without comparable levels in the FLAG-N1 these results are inconclusive. This experiment ultimately would require a degree of fine tuning as it has potential to yield insight into ubiquitin modification of NSMCE1 itself, and ultimately its effects on ubiquitination of other proteins, but due to time constraints we were ultimately unable to complete this troubleshooting.

Establishing a reliably working protocol for this experiment would be ideal for analysing *in vivo* ubiquitin modification of the endogenous SMC5/6 complex components under variable conditions. It could potentially yield insight through comparisons between wild-type, N1-KO and N1-RING cells as to the enzymatic activity of NSMCE1 by looking for the differences in protein modification in the presence of NSMCE1 and identifying specific targets of this activity for the first time. As we have hypothesized NSMCE1 could be primarily active around G2/M, modifying a functioning protocol for this experiment to analyse the activity of a fixed G2/M cell population could potentially answer this

hypothesis and additionally provide a conclusive insight into NSMCE1's enzymatic activity should any be detected during these phases.

5.0 Concluding remarks

The data obtained in this study supports previous works into the importance of SMC5/6 complex components being reliant on each other for complete complex stability. Our results also demonstrate how NSMCE1 plays a critical role in maintaining the complex, without which a multitude of cellular consequences arise most probably during DNA replication and mitosis. While we were unable to obtain clear results of NSMCE1's ubiquitin related activities, evidence using our N1-RING cell line indicates the enzymatic function of NSMCE1 is a key contributor to cell cycle progression in addition to the structural role NSMCE1 has in maintaining the SMC5/6 complex. Future investigations should focus on attempting to understand the enzymatic role NSMCE1 plays through experiments like the Bio-Ub pull down, perhaps focusing at mitosis where the greatest number of complications appear to arise in the N1-KO and N1-RING cells.

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