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Correlation between posttranslational modifications and dynamics of microtubules and DNA damage response signalling

Correlation between posttranslational modifications and dynamics of microtubules and DNA damage response signalling

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Abstract

Microtubules (MTs) are tubulin polymers that play vital roles in cells, such as chromosome segregation or transport of cargo. The imbalance in MT dynamics might lead to neurodegenerative diseases or cancers, which can also be a result of excessive DNA damage. Hence, Lottersberger, *et al.,* 2015 and Lawrimore, *et al.,* 2017 started studying that connection. Both groups showed that dynamic MTs are required for DNA damage repair. The presented work in this thesis aims to reveal the link between posttranslational modifications (PTMs) and dynamics of MTs and DNA damage response signalling using cellular assays. Initially, non-synchronised and G1 synchronised RPE-1 cells cultured without serum for 48 hours as well as G1 synchronised RPE-1 and then re-stimulated with 10 % serum were treated with DNA damaging agents producing double strand breaks: neocarzinostatin (NCS) (radiomimetic,acts via oxidative stress), phleomycin (acts via oxidative stress) or etoposide (integrates with a DNA strand and topoisomerase II preventing DNA strands re-ligation) to establish the most effective DNA damaging drug that allowed to observe any alterations in the PTMs of α-tubulin overtime. Immunoblotting and immunofluorescence results demonstrated that the NCS acts most rapidly and efficiently among tested DNA damaging drugs. Thus, time course experiments were performed, determining whether DNA damage can affect PTMs and dynamics of MTs. The immunoblotting and immunofluorescence analyses indicated a significant decrease in PTMs of acetylated, tyrosinated and polyglutamylated α-tubulin and simultaneously MT motion assay demonstrated an increase in MT dynamics in response to DNA damage induced by NCS at a 30 minute time point, suggesting that DNA damage affects MTs. Therefore, the next set of experiments was performed to establish whether perturbation of MTs could affect DNA damage response (DDR) signalling. Hence, the MTs were hyperstabilised or destabilised using taxol (time course 1 hour to 7 hours) or nocodazole (time course 1 hour to 7 hours), respectively, followed by NCS-induced DNA damage. After hyperstabilisation of MTs and DNA damage by neocarzinostatin, immunoblotting of G1 synchronised cells that were stimulated with 10 % serum shows that the level of DNA damage marker, KAP1 phosphorylated on serine 824 decreased compared to control. Interestingly, the level of another DNA damage marker- γH2AX was not altered. These preliminary results might imply that MTs affect DNA damage in a non-γH2AX-dependent manner. Nevertheless, these results should be further validated in order to unequivocally claim the correlation between PTMs and dynamics of MTs and DDR signalling.

Acknowledgements

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Also, I would like to thank Dr Andrew Herman and Lorena Sueiro Ballesteros at the Flow Cytometry Department for their technical help.

Finally, I dedicate this thesis to my parents and my fiancé for their constant support, understanding and faith in me.

Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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MT- microtubule

GTP- guanosine triphosphate

GDP- guanosine diphosphate

MTOC- MT organising centre

MAP- microtubule-associated protein

PTM- posttranslational modification

HDAC6- histone deacetylase 6

TTL- tubulin tyrosine ligase

SDS- Shwachman-Diamond Syndrome

FAP- Familial Adenomatous Polyposis

AML- acute myeloid leukaemia

APC- adenomatous polyposis coli

ATM-ataxia telangiectasia mutated

HD- Huntington's disease

AD- Alzheimer's disease

MCM2-mini-chromosome maintenance complex component 2

LINC- linker of nucleoskeleton and cytoskeleton

NHEJ- non-homologous end joining

DDR- DNA damage response

DSB- double-strand break

MDC1- mediator of DNA damage checkpoint protein 1

KAP1- KRAB-associated protein 1

MRN- Mre11-Rad50-Nbs1

HR- homologous recombination

DNA-PKcs- DNA-dependent protein kinase catalytic subunit

RPE-1- Retinal pigment epithelial-1

NCS- neocarzinostatin

SS- serum stimulation

SF- serum free

NT- non-treated

DMSO- Dimethyl sulfoxide

53BP1- p53-binding protein 1

Chapter 1: Introduction

1.1. Microtubules at a glance.

Microtubules (MTs) were first identified by Ledbetter and Porter in plant cells of *Phleum pratense, Juniperus chinensis, and Spirodela oligorrhiza* using electron microscopy in 1963. The authors defined a longitudinal section of MTs as 'two parallel lines', while the cross-section of MTs was observed as a 'hollow-like structure'. Since then, their presence has been confirmed in the cytoplasm of eukaryotic cells and recently also in some *Prosthecobacter* species of bacteria (Pilhofel, *et al*., 2011). The discovery of MTs in prokaryotes was a striking finding as no tubulin genes had been identified in any other bacterial genome before. These MT-positive bacteria species have similar hollow-like architecture of MTs to MTs present in eukaryotic cells.

Structural studies have revealed that MTs consist of α- and β- tubulin units bound together via longitudinal and lateral noncovalent bonds forming a tubulin dimer and stabilising the structure. Dimerisation of spherical tubulin monomers results in a break of symmetry (Kononova, *et al*., 2014). Studies have shown that lateral bonds are stronger compared to longitudinal, which are present between α-tubulin and β-tubulin (Ayoub, *et a*l. 2014). This results in the fragility of microtubule structure, which is a well-known feature of these polymers. The dimers bind to each other in a characteristic head-to-tail manner to create a protofilament. Chains of protofilaments bind together with hydrogen bonds, forming a microtubule (Barsegov, *et al.*, 2017). The length of MTs varies widely from a few micrometres to a hundred micrometres.

Pilhofel and co-workers (2011) have observed the significant difference between *Prosthecobacter* species and eukaryote in a number of MT protofilaments. These bacteria have merely five MT protofilaments binding adjacently, whereas eukaryotic MTs consist of thirteen protofilaments. According to Pilhofel, these observations support the idea of the process of divergent evolution occurring in relation to MTs.

Although the mammalian alpha and beta tubulin isotypes come from numerous genes, it is thought that they were separated from a single gene in a process of evolution due to 50 % of similarity at the amino acid level and identical molecular weight of approximately 50 kDa. So far, 10 α-tubulin and 9 β-tubulin functional genes have been identified (https://www.genenames.org/cgi-bin/genefamilies/set/778 accessed on 11/10/2018). The type of MT produced depends on the products of different α- or β-tubulin genes.

Other isotypes of tubulin also exist such as gamma tubulin. The main function of gamma tubulin is determination of the MT polarity through binding to β- tubulin which plays an important role in MT nucleation. In live cells, MT nucleation, which is a *de novo* formation of MTs, is initiated in MT organising centres (MTOCs), also known as the centrosomes. These structures are responsible for organising mitotic spindle apparatus for chromosome segregation during cell division (Roostalu and Surrey, 2017). Interestingly, studies have shown that a high concentration of free tubulin monomers can also lead to *de novo* microtubule nucleation under *in vitro* conditions (Caudron, *et al*., 2002). This is achieved in the presence of GTP, appropriate buffer and temperature. Centrosomes comprise γ-tubulin ring complexes that contain γ-tubulin, forming a platform for MT nucleation (Roostalu and Surrey, 2017).

MTs perform vital functions within a cell, such as determining cell polarity, enabling cargo transport, or facilitating chromosome segregation (Porter and Lee, 2001). These roles are consequences of their structure and features, which have been discovered mainly through loss-of-function experiments (Lottersberger, *et al.,* 2015).

1.1.1. Microtubules as structures defining cell polarity.

Asymmetric organisation of structure, function and shape within a cell is referred to as cell polarity. The cell polarity can occur spontaneously or is determined by intrinsic or extrinsic signals (Wedlich-Soldner and Li, 2003). MTs have plus ends and minus ends, giving rise to directionality, which is a cause of cell polarity that mediates vesicle transport. The source of energy for tubulin dynamics derives from guanosine triphosphate (GTP) hydrolysis into guanosine diphosphate (GDP) and inorganic phosphate. This process allows rapid polymerisation of microtubules. An increased assembly of tubulin dimers occurs on exposed β-tubulin of plus ends, whereas a site of both decreased assembly and disassembly of tubulin dimers is characteristic to minus end that exposes α-tubulin (Nogales, *et al.*, 1999). Dimers of tubulin bind to the plus end until there is a lag in addition of new tubulin subunits, when a GTP cap forms. The GTP cap is considered as a marker of stabilised MTs (Akhmanova, *et al.*, 2015). The lag is short-lived and according to Akhmanova, *et al.* (2015) due to the force exerted by obstacles encountered during the formation of a MT, the GTP cap is lost and consequently, tubulin dimers dissociate. This process of a sudden switch from polymerisation to depolymerisation is known as catastrophe. This is thought to be a

single-step process (Howard and Hyman, 2009), but more and more evidence suggests that for a catastrophe to occur, a multi- step mechanism is required, involving microtubule-associated proteins (MAPs) (Gardner *et al*., 2013; Foethke *et al*., 2010). Conversely, a sudden change in dynamics from shrinking to growth is known as a rescue. The rescue of MTs is driven by GTP-tubulin islands and tension (Gardner *et al*., 2013). This constant alteration of the state of tubulin dimers leads to a lack of equilibrium, illustrated in Figure 1.1. Also, these constant dynamic changes can be affected by posttranslational modifications (PTMs) and microtubule-associated proteins (MAPs) (Westermann and Weber, 2003). Interestingly, shorter, newly created MTs are less likely to decay compared to longer ones, which indicates that age of MTs is a factor (Odde e*t al*., 1995). Also, the shorter MTs are, the more dynamic they become.

Altogether, the essential feature of MTs, which is a dynamic instability, determines the vital role of MTs in cell polarity. This is important for determining the direction of cargo transport within a cell.

1.1.2. Microtubules trace roads for a transport of cargos.

Another important intracellular role of MTs is to mediate transport of cargo, such as proteins, neurotransmitters, enzymes or even organelles. The material is carried by motor proteins using ATP as an energy source. There are two types of motor proteins binding to MTs: kinesins and dyneins. They share a general structure, which includes two motor domains and the tail. The direction of the movement along the MTs defines the type of motor proteins used. Kinesins are a type of motor protein moving anterogradely (towards the plus end of MTs), whereas dyneins move retrogradely along the MTs (towards the minus end of MTs) (Franker and Hoogenraad, 2013).

Before kinesin dissociates from MTs, it takes tens of unidirectional 8 nm steps, equivalent to the distance between successive tubulin subunits. A number of models of kinesin movement have been proposed. However, a hand-over-hand walking manner appears to be broadly accepted (Asbury, *et al*., 2003). It states that during

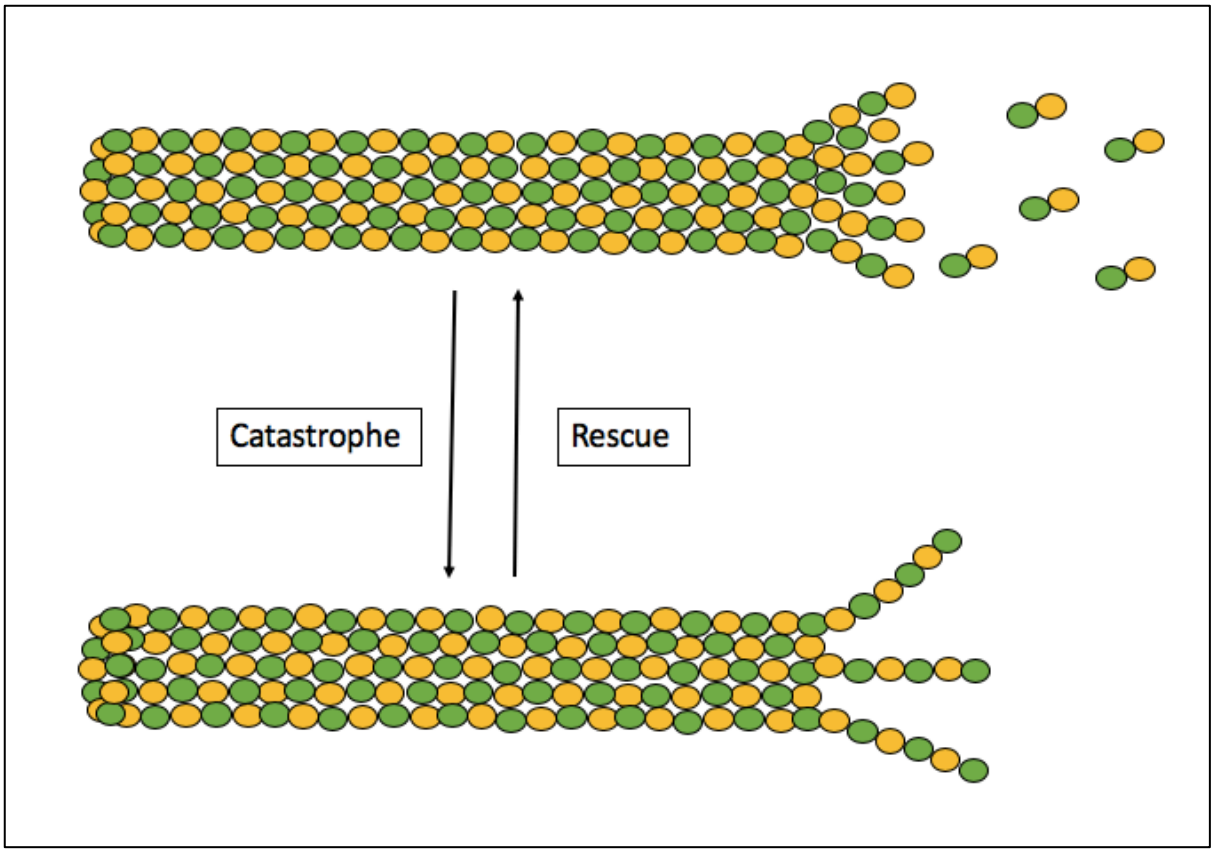


Figure 1.1. Microtubules are highly dynamic structures.

The sudden switch from polymerisation to depolymerisation known as catastrophe involves the loss of GTP-cap. This process involves depolymerisation of the MT structures and disassembly, which is followed by a reverse rescue event that results in polymerisation and assembly of tubulin dimers. In general, MTs are highly dynamic structures that do not achieve equilibrium in typical conditions due to the role of MT dynamics in cell functioning.

the movement, one domain must be attached to the MT. Simultaneously, the other domain is on the move to the subsequent binding site. The other component of kinesin, the tail, is responsible for binding to the cargo. However, the exact way of binding is not fully understood due to a limited number of identified receptors (Asbury, *et al*., 2003).

Dynein is a large protein complex. In contrast to kinesins, the tail of dynein is the site of interaction with a MT (Burgess *et al.,* 2003). Reck-Peterson and colleagues (2006) showed in single-molecule studies a similar hand-over-hand walking manner of dyneins. Other features of dyneins, such as the step size and directionality have not been clearly defined due to a significant differences in the reported findings (Reck-Peterson, 2006).

1.1.3. Importance of microtubules in cell division.

MTs are responsible for spindle formation during the course of cell division and chromosome segregation in metaphase. At metaphase, MTs create a mitotic apparatus, consisting of a central mitotic spindle (made of symmetrically positioned MTs) and astral MTs forming the aster at opposite spindle poles. Microtubules attach to kinetochores of chromosomes or sister chromatids during the metaphase and then they moved them away towards the opposite spindle poles, separating the chromosomes or sister chromatids (Forth and Kapoor, 2017). In this process, three types of MTs have been identified, such as astral MTs, polar MTs and kinetochore MTs, illustrated on Figure 1.2.

The first astral MTs compose the aster, a structure facilitating the proper position of the mitotic apparatus and after the anaphase determining the cleavage plane. Both polar and kinetochore MTs form the mitotic spindle. The polar MTs interact with the polar MTs from the opposite centrosome in order to hold the spindle fields together. The last set of MTs participating in cell division are kinetochore MTs. They attach to kinetochores of the chromosomes and their pull and push forces allow the movement of sister chromatids or chromosomes towards the opposite MTOCs (Maiato *et al.,* 2004). Loss-of-function study revealed that each chromosome must be attached to the MTs so that the cell division can occur (Maresca and Salmon, 2010).

Altogether, MTs constitute an important factor in the process of the cell division. Impairments in the MT function could result in a defective mitosis or meiosis.

All the above mentioned functions of MTs, which are results of dynamic instability and fragility of MT structure, demonstrate an importance of MTs in the cells. However, the exact mechanism of MT dynamicity is still not fully understood. Filling this gap in knowledge could reveal other uncharacterised functions of MTs. Thus, one way of the uncovering the mystery of MT dynamicity is researching the factors affecting it, such as posttranslational modifications of tubulin.

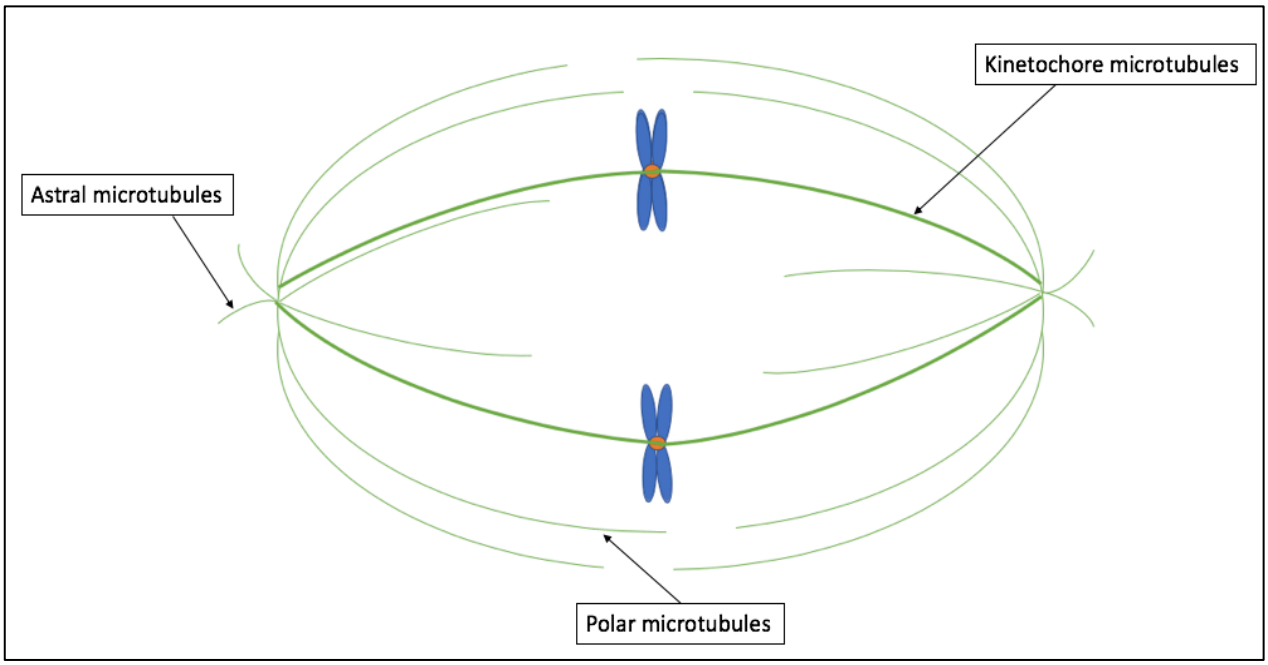


Figure 1.2. Types of microtubules involved in cell division.

Illustration depicts three types of MTs involved in the process of cell division. Kinetochore MTs attach to the kinetochore of the chromosomes and facilitate the movement of sister chromatids or chromosomes towards the centrosomes. Polar MTs interact with the polar MTs on the opposite side determining the area of cell division. Astral MTs ensure the proper spindle position and subsequently establish the cleavage site.

1.2. Importance of post-translational modifications of microtubules.

Interestingly, there is currently no well-defined correlation between posttranslational modifications and their roles in dynamicity. Posttranslational modifications (PTMs) of tubulin usually occur on the C-termini of both isotypes of tubulin. They occur post-polymerisation and are highly dynamic. The overview of some of the PTMs of tubulin subunits is illustrated in Figure 1.3 and more detailed comparison of the PTMs is depicted in Table 1.1. However, the work reported mainly in the thesis focuses on three modifications: acetylation, tyrosination and polyglutamylation, occurring on α-tubulin. Thus, only they will be described here in more details.

1.2.1. Acetylation

The best understood post-translational modification of tubulin is acetylation of lysine 40. That modification is a consequence of the activity of the acetyl transferase enzyme αATAT1, which is encoded by the *ATAT1* gene in mammals (Coombes *et al*., 2016). The reverse process, deacetylation, is possible through the activity of histone deacetylase 6 (HDAC6) (Hubbert *et al*., 2002) or sirtuin2. Coombes *et al*, (2016) and Soppina *et al*, (2012) have proved that acetylation can occur not only in the outer surface of protofilament but also, in the lumen of MTs.

Acetylation occurs on α-tubulin only and is found on stable microtubules and recently also on dynamic microtubules but to a lesser degree (Belmadani, *et al*., 2004). This observation led Hubbert *et al*., (2002) to conclude that MT stability is caused by acetylation, which was undermined by Palazzo and colleagues (2003), who reported that this proposed marker of age is resistant to nocodazole, a drug that causes the destabilisation of MTs, suggesting that tubulin acetylation itself does not result in stabilisation of MTs and other factors are involved. Furthermore, acetylation does not influence the rates of polymerisation and depolymerisation (Maruta *et al*., 1986). Other studies have suggested a role for tubulin acetylation in MT transport and binding of MAPs (Hammond *et al*., 2010) and in neuronal growth and development (Creppe *et al*., 2009).

1.2.2. Tyrosination

Tyrosination is another reversible post-translational modification of tubulin, catalysed by tubulin tyrosine ligase (TTL), leading to the addition of tyrosine to the C-terminus of α-tubulin. C-terminal tyrosine residue is removed in a process of detyrosination, which is the result of the activity of an unidentified enzyme from the cytosolic carboxypeptidase 1 family (Wolga and Gaertig, 2010). The removal of additional amino acid residues from detyrosinated tubulin results in the formation of Δ2- and Δ3-tubulin. It is mediated by the catalysis of enzymes from the cytosolic carboxypeptidase family. Recent studies suggest a role for tyrosination/detyrosination in the stabilisation of MTs and regulation of MAPs. Due to the lack of tyrosinated tubulin on long-lived MTs as well as an observed increase in detyrosination in response to the MTs stabilising drug, taxol, it is thought that detyrosination mediates stabilisation of MT, although does not stabilise them alone; other factors are involved (Wolga and Gaertig, 2010; Khawaja, *et al.*, 1988). In agreement with this, Cassimeris and co-workers (2013) confirmed the presence of tyrosinated tubulin, but not detyrosinated tubulin on dynamic MTs.

1.2.3. Polyglutamination

Polyglutamylation occurs on MTs forming mitotic spindles, neuronal projections, centrioles, basal bodies and axonemes. It involves the addition of glutamate residues to the peptide chains of the tubulin. It is found on C-terminal tails of both α- and β-tubulin. This process is catalysed by glutamylases that are members of the TTL family (Ikegami, *et al.,* 2006). Recently, carboxypeptidases 1,4,5, and 6 have been identified as deglutamylases, reversing polyglutamylation (Kimura *et.al.*, 2010; Rogowski *et al.*, 2010). This modification is important for binding of MAPs, suggesting a role in coordinating the diversity of microtubule functions (Ikegami *et al*., 2007; Boucher *et al*., 1994). Moreover, polyglutamylation is thought to stimulate MT severing, implying a role in the regulation of MT stability (Lacroix *et al*., 2010).

Post-translational modifications of MTs are imperative for the correct functioning of MTs. They affect both their structures and functions, thus it is important to consider the modifications while researching the association of MTs with other proteins. Importantly, the awareness of the impact of MT changes and disregulation on various

Type of modification	Tubulin subunit	Forward Enzymes	Reverse Enzymes	Function	References
Acetylation	α-tubulin	ATAT, MEC-17	HDAC6, SIRT2	Stabilise MTs	Choudhary, *et al.,* 2009 Akella, *et al.,* 2010
Tyrosination	α-tubulin	TTL	TTL	Destabilise MTs	Ersfeld, *et al.,* 1993
Detyronisation	α-tubulin	Unknown	TTL	Stabilise MTs	Ersfeld, *et al.,* 1993
Polyglutamylation	α-tubulin β-tubulin	TTLL 1,4,5,6,7, 9,11,13	CCP1,4,5,6 CCPP-1,6	Regulate binding of motor proteins to MTs	Ikegami, *et al.,* 2006
Polyglycylation	α-tubulin β-tubulin	TTLL 3,8,10	gDIP1, gDIP2	Regulate binding of motor proteins to the MTs	Lalle, *et al.,* 2011
Phosphorylation	α-tubulin β-tubulin	Cdk1, cyclin B, PSK, Jak2	Unknown	Facilitates the rescue of MTs	Fourest-Lieuvin, *et al.,* 2006
Ubiquitination	α-tubulin β-tubulin	Parkin, BRCA1	Unknown	Facilitates the catastrophe of MTs	Xu, *et al*., 2010

Table 1.1. Overview of posttranslational modifications of tubulin.

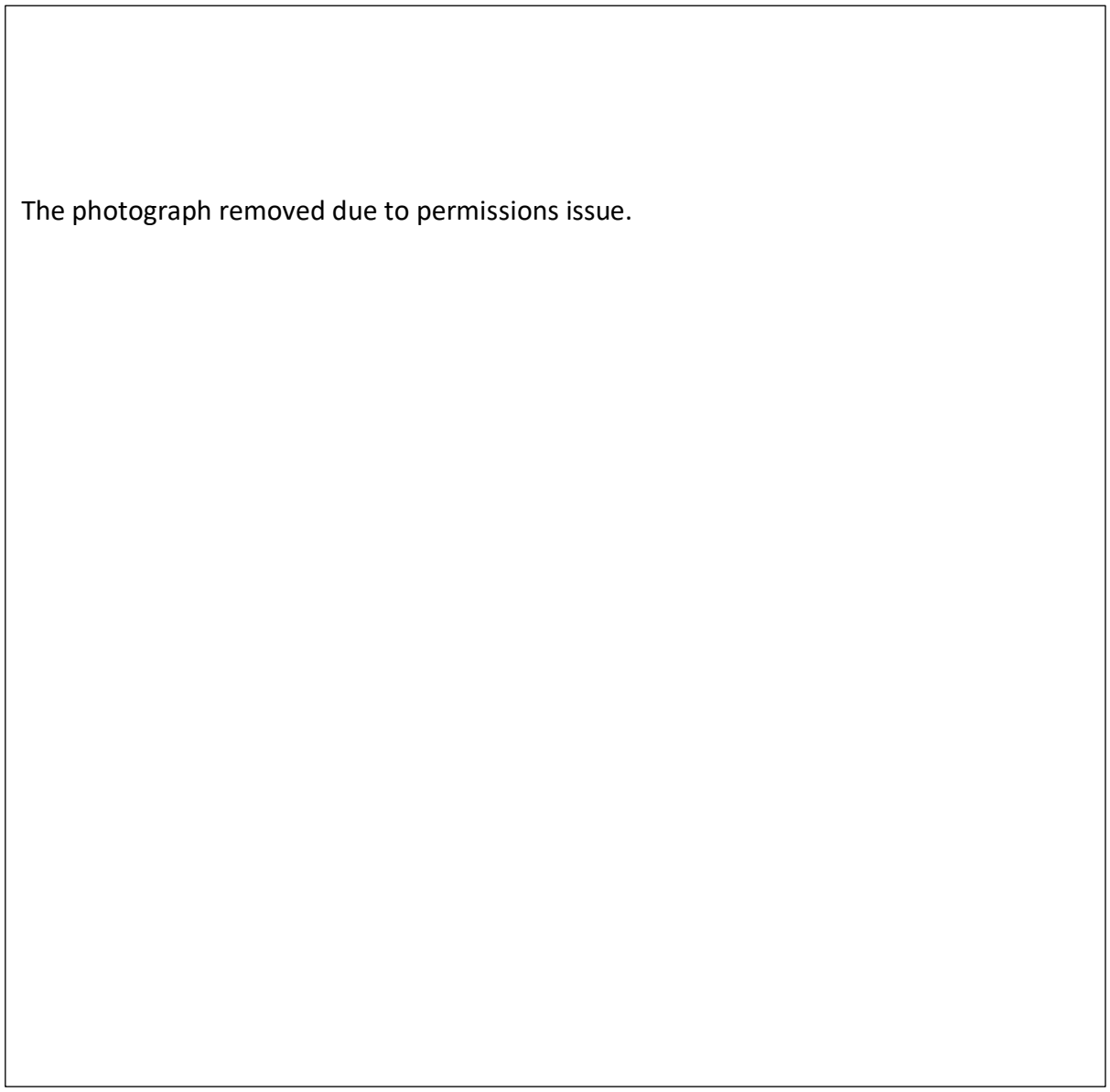


Figure 1.3. Overview of posttranslational modifications of tubulin.

This picture represents PTMs of tubulin with their mechanisms and enzymes. While detyrosination is limited to tubulin, polyglutamylation and polyglycylation occur on both tubulin subunits.

Also, drugs causing perturbation of MT dynamicity such as taxol are used in anti-cancer chemotherapy. Because of this association with cancer, there is a need to investigate possible links between MTs and DNA damage.

1.3. Consequences of abnormal MT functions

Accurate chromosome segregation facilitated by MTs is essential for maintaining genome integrity. MT destabilisation during that process is associated with the formation of aneuploidy and consequently chromosome instability, which is a feature of neoplasm (Yang, *et al.,* 2003). A number of studies suggest the relationship between aneuploidy and tumorigenesis (Nicholson and Cimini, 2013; Hanks, *et al.,* 2004). Additionally, production of some proteins regulating MT dynamics in mitosis is altered in many unstable cancerous cells (Li, *et al.,* 2009). This relationship implies the importance of improper spindle formation in preventing tumour development. Some inherited diseases influence the stabilisation of mitotic spindles, such as Shwachman-Diamond Syndrome (SDS) or familial adenomatous polyposis (FAP) (Austin, *et al.,* 2008; Li, *et al.* 2011).

1.3.1. Shwachman—Bodian-Diamond Syndrome

SDS is caused by downregulation of the Shwachman—Bodian-Diamond Syndrome (*SBDS*) gene. This autosomal recessive disorder results in bone marrow defects and predisposition to acute myeloid leukaemia (AML). Interestingly, AML in these patients forms complex genetic abnormalities and aneuploidy. Loss-of-function studies of SBDS protein on human lymphoblasts and skin fibroblasts showed abnormal multipolar mitotic spindles during cell division, leading to chromosomal abnormalities and the formation of aneuploidy (Austin, *et al.,* 2008). While the precise mechanism of MT spindle stabilisation by SBDS as well as predisposition to AML and bone marrow defects are not understood, from the consequences of protein loss one can conclude that SBDS are regulators of MTs involved in mitotic spindle organisation.

1.3.2. Familial Adenomatous Polyposis

Another inherited disease that predisposes to cancer and is related to MTs is familial adenomatous polyposis (FAP). It is caused by downregulation of the adenomatous polyposis coli (*APC*) gene. This autosomal dominant mutation results initially in the formation of colonial polyps, which become invasive with age. Recently, Li *et al.* (2011) published promising studies, in which heterozygous *APC* mouse model treated with EM011, a synthetic component that reduces MT dynamics, showed a significant stabilisation of MTs using immunofluorescence. That resulted in a decrease in adenomas and a loss of proliferation feature of the tumor. This outcome is a supportive argument for studying and understanding MT dynamics in order to prevent some DNA damage-associated diseases.

Approximately 80% of *APC* mutations in FAP have a truncated C-terminus of the APC protein. This characteristic change in the protein structure is responsible for altered interaction with MTs as well as the assembly of mitotic spindles (Tighe *et al.,* 2004).

Whilst both SBDS and APC proteins regulate stabilisation of MT spindles, and truncated proteins could predispose to cancer development (Stumpff *et al.,* 2014), the consequences on downstream effects of these mutations are not completely understood. Altogether, these diseases demonstrate the importance of SBDS and APC proteins in healthy cells and the need to study MT dynamics to reveal all the factors responsible for the control of MT polymerisation/depolymerisation events.

1.3.3. Stathmin/ Oncoprotein 18

It is not only inherited mutations that perturb MT activity that are associated with disease. There are also some regulatory proteins controlling MT dynamics and changes in their expression can also be deleterious. One example is stathmin, also called oncoprotein 18. Stathmin is a ubiquitously expressed protein, destabilising MTs once phosphorylated. It binds to free tubulin heterodimers at pH 6.8 or mediates an occurrence of catastrophe at pH 7.5 (Honnappa, et al., 2003). It has been shown that stathmin facilitates GTP hydrolysis by forming a bond with plus end of MTs (Amayed, Pataloni, and Carlier, 2002). The structure of the protein indicated that its NH2-terminus promotes catastrophe and its C-terminus is involved in tubulin sequestration.

Phosphorylation of some sites in stathmin, such as serine 16 and serine 63, result in impairment of the stathmin MT-destabilising function (Lu, *et al*., 2014). Overexpression of this MT destabiliser has been found in many malignancies, such as breast cancer (Baquero, 2012), leukaemia (Melhem, *et al*., 1997; Sellin *et al*., 2008), prostate cancer (Ghosh R. *et al*., 2007), cervical cancer (Xi *et al*., 2009), and lung cancer (Singer, *et al.,* 2009). Supporting these observations, a study revealed that stathmin is more abundant in proliferative cells than in healthy cells. Xi and co-workers (2009) have demonstrated the correlation between increased stathmin production with the diameter of a cervical tumour and its clinical classification and survival rate. As a result of high stathmin level, nucleation decreases. This implies that destabilised MTs could also be associated with cervical tumorigenesis and cancer progression.

1.3.4. Neurodegenerative diseases

Furthermore, loss of MT function has been observed to lead to neurodegenerative diseases. Uniform MT polarity is essential in axons for retrograde and anterograde transport functions. Defects in the MT pattern can lead to sending cargos in a different direction or destination.

1.3.4.1. Alzheimer's disease

One of the proteins that can affect MT is tau. There are six isoforms of the protein that have been associated with propagation of the Alzheimer's disease. The tau isoforms are different in a number of tubulin binding sites (Alonso, *et al.,* 2001). This MAP protein attaches the MTs and both stabilise them and hence promote MT stabilisation. Defects in a tau protein leads to Alzheimer's disease, in which hyperphosphorylation of tau protein occurs, resulting in the dissociation of tau from MTs (Shin, *et al.*, 2018). Although the exact mechanism is unknown, there are some hypotheses. The most popular claims that once tau protein is removed, MT depolymerisation rate increases, resulting in abnormal amyloid-β accumulation, leading to degeneration of neuronal cells. Another states that MTs are more sensitive to microtubule-severing enzymes, such as katanin following tau detachment, leading to their degradation. The third theory declares that tangles of tau protein facilitate MT catastrophe (Lu, *et al.,* 2014). Moreover, recent Shin and colleagues' (2018) findings shed a light into tau-tubulin interaction in Alzheimer's disease. The researchers generated a bifluorescence complementation method, in which both alpha tubulin and tau proteins were tagged.

The tagged proteins were visible under the confocal microscope only when aggregates were formed. Interestingly, the results showed that tau aggregates together with tubulin. This finding might be crucial for tracking the pathogenesis of Alzheimer's disease.

1.3.4.2. Huntington's disease

Moreover, a failure in appropriate MT dynamics is associated with Huntington's disease, an autosomal dominant disorder. Studies have showed that a truncated huntingtin protein, which causes Huntington's disease, also attaches to MTs decorating them and blocking the transport of cargo along them (Hoffner, Kahlem and Djian, 2002). Small molecule analysis showed that drugs destabilising MTs resulted in the apoptosis prevention in mutant huntingtin-expressing neurons and mouse models (Varma, *et al.*, 2010). Thus, the question arises whether MT stabilising drugs, such as taxol, could help in reversing the effects of the disease, which is the aim of ongoing research.

Overall, although neurodegenerative disorders are not fully understood, they demonstrate that there must be a mechanism coordinating the cell polarity regulation that once failed results in the above mentioned diseases.

All these examples show the importance of proper MT function and link with DNA damage. More precisely, intense depolymerisation of MTs leads to many diseases, including cancers and neurodegenerative diseases. Hence, deepening the knowledge about MTs is imperative for understanding the above mentioned diseases, and may lead to new treatment strategies. Moreover, revealing the comprehensive regulation of MT dynamics could aid in understanding DNA damage response and repair.

1.4. DNA damage at a glance.

Thousands of DNA damaging events occur every day in every human cell. DNA damaging events are divided into endogenous and exogenous causes. The first one, endogenous, encompasses reactive oxygen species production as a by-product of metabolism. Whilst exogenous events include radiations such as ultraviolet, gamma or x-ray. Also to this group belong to mutagenic chemicals, certain toxins, or the actions of some viruses. As a result of these harmful events, mutations leading to neoplasms, apoptosis or senescence might occur (Blackford and Jackson, 2017).

Mutation is a heritable alteration in a nucleotide sequence, which might change the expression or structure of a protein, and consequently its function. As the mutant protein acts differently from the wild-type version or if it is produced at larger or smaller than normal amounts, it could change interactions with other proteins. For example loss of BRCA1 expression that is a tumour suppressor protein, can lead to increased risk of cancer as the protein is responsible for initiating signalling for one of DNA damage repair pathways (Hall, *et al*., 1990).

The activation of p53 in response to stress signals might lead to the cell arresting in the G1 phase of the cell cycle, the programmed cell death response (apoptosis) or senescence, a state in which cells stop performing physiological functions, such as mitosis. All of them are different ways of preventing tumour promotion by promoting the removal of the mutated cell (Haupt *et al.,* 2003) (Figure 1.4.). During the G1 phase, a cell prepares for DNA synthesis and growth. Arresting the cell at the G1 checkpoint is a protective mechanism, preventing a duplication of damaged DNA.

Due to these deleterious consequences, DNA repair is tightly regulated. Recent findings from loss-of-function experiments suggest that MTs might play a significant role in repairing DNA (Lottersberger, *et al.,* 2015).

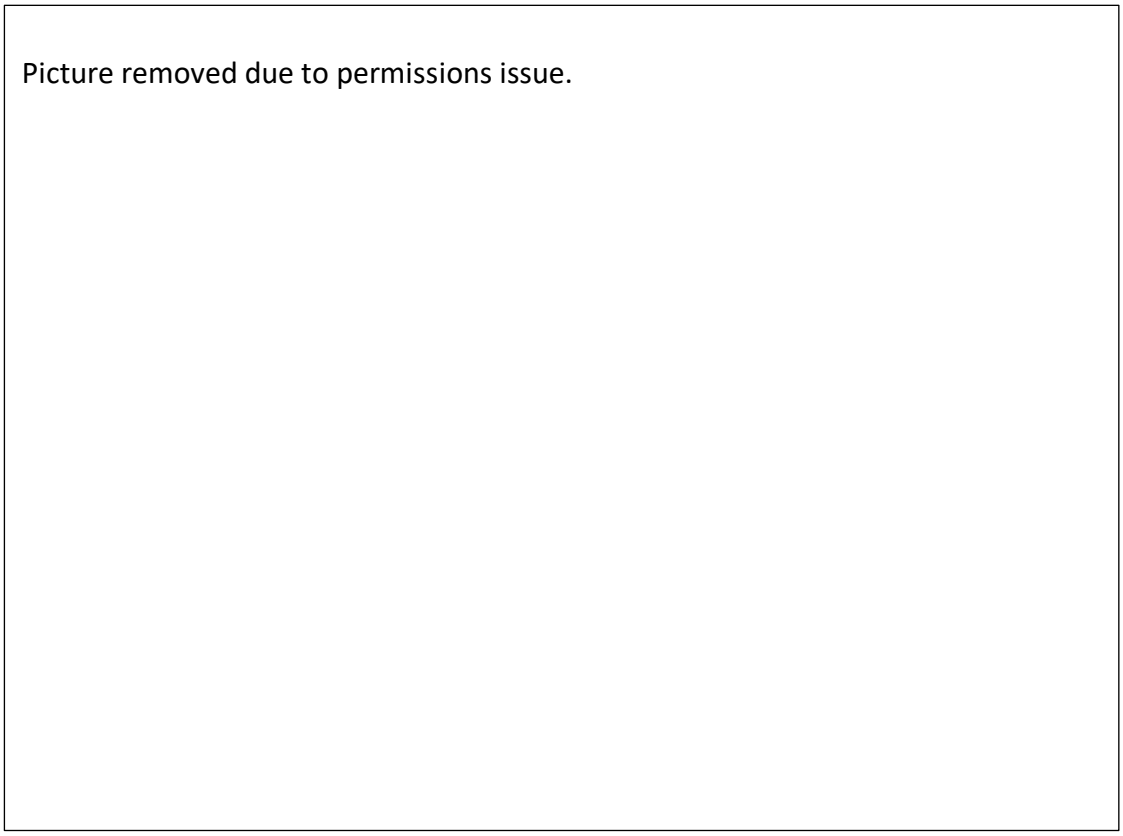


Figure 1.4. Cell cycle and checkpoints.

The picture depicts cell cycle phases with associated checkpoints for every stage.

1.5. The connection between MTs and DNA damage.

MTs are indirectly connected to chromatin via the linker of nucleoskeleton and cytoskeleton (LINC) complex illustrated in Figure 1.5. The LINC complex consists of lamin A/C, emerin, SUN domain proteins and KASH domain proteins. Lamin A/C serves as a nuclear scaffold and decorates the inner nuclear membrane. Another component of the LINC complex, emerin, is a protein located in the inner nuclear membrane but the exact function is unknown. The SUN domain proteins, SUN-1 and SUN-2, are located in the inner nuclear membrane and are linked to the KASH domain proteins, nesprin-1 and nesprin-2, elongating to MTs and constituting the platform for motor proteins on MTs (Janin *et al.,* 2017).

Another connection between MTs to chromosomes is via the kinetochore during cell division. Thus, there is a clear link with DNA segregation. Moreover, increasing evidence implies that proper functioning of MTs facilitates DNA repair.

Recently, Lottersberger *et al.*, (2015) proposed the role of MTs in DNA repair. They knocked down SUN-1 and SUN-2 in mice, and used taxol and nocodazole, drugs over-stabilising and destabilising MTs respectively, which was followed by damaging the cells using ionising radiation. Loss of SUN domain proteins resulted in a reduction of MT dynamics. In the parallel experiment, the mCherry-tagged 53BP1 neuronal cells showed a decreased chromatin mobility in response to treatment with the MT poisons and then DNA damage drugs. Further, Lottersberger showed that nesprin-4 and kinesins were responsible in the signal directly affecting MTs. The overall observations demonstrated that dynamic MTs are required for DNA repair as the knockout cells showed an increased time for DNA repair compared to control. Hence, they concluded that 53BP1, the LINC complex, and dynamic MTs are important in DNA repair. In their further experiments, the researchers demonstrated that 53BP1 promotes NHEJ as the DNA repair pathway.

Similarly, Lawrimore *et al*., (2017) supported the importance of the correlation between MT mobility and chromatin in response to DNA damage. The researchers induced DNA damage using drugs, such as Phleomycin or Zeocin, or a single site-specific double-strand break endonucleases and then they assessed the chromatin mobility. Whilst the first drug chelates metal ions and binds to DNA, cleaving the DNA helix structure, Zeocin intercalates into DNA strand and destroys it. Single-protein trafficking GFP assay showed an increased chromatin mobility compared to control. Finally, their

results also supports the Lottersberger's findings that dynamic MTs positively enhance chromatin decompaction and increased DNA repair.

On the other hand, Lawrence and co-workers (2016) working with loss of function mutations in UNC-84 from *Caenorhabditis elegans,* which is a homolog of the SUN protein, as well as HeLa cells with depleted Sun-1 protein, showed impairment in loading and disassembly of RAD51, a protein responsible for stabilisation of DNA strands and recruitment of DNA repair proteins in homologous recombination. Also, FAN-1 protein recruitment was disturbed, an event that occurs during homologous recombination. All together, these data suggest that the LINC complex promotes homologous recombination, inhibiting non homologous end joining (NHEJ). The authors proposed that SUN domain proteins suppress the NHEJ pathway by interaction with the DNA-PK/Ku70/Ku80 complex.

The difference in the observations of various DNA repair pathways (non-homologous end joining or homologous recombination) promoted by MTs in the studies could be explained by way the DNA damage was generated and/or the species or type of cells being studies. Although in both studies, the cells were irradiated, Lottersberg et al used mitotic cells, whereas Lawrence, *et al.* (2017) used cells in interphase.

Another explanation is a quicker response in mouse cells and inhibited response human cells, which was previously documented in Adamo, *et al.* (2010) and Bunting *et al.* (2012).

Despite these discrepancies, all studies are congruent regarding the importance of MTs in DNA damage response of one type or another and an increased chromatin motion at the periphery of the nucleus. However, how the LINC complex facilitates the DNA damage response, as well as the exact role of MTs in this response, remains elusive.

To prevent the accumulation of an enormous number of inactivated or mutant cells, cells are equipped with DNA repair mechanisms known as DNA damage response (DDR) pathways. Firstly, histone H2AX can be phosphorylated on serine 139 by ATM, ATR, and DNA PKcs at the damaged sites in a form of γH2AX foci and recruits other proteins to DNA damage sites. Moreover, γH2AX controls cell cycle progression, allowing DNA repair before progression to the next cell cycle phase. After DNA repair, γH2AX is immediately removed most likely through phosphatases (Downs *et al*., 2004).

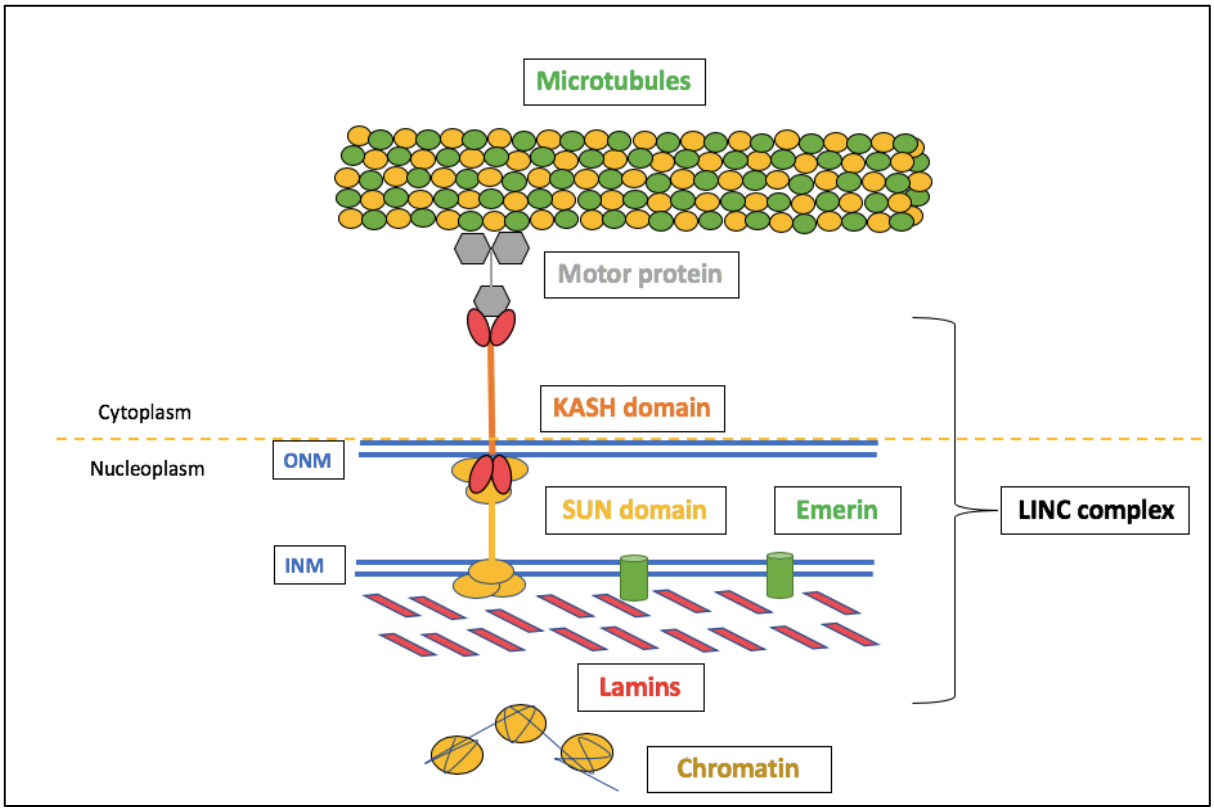


Figure 1.5. Microtubules are indirectly connected to chromatin via the LINC complex in interphase.

The interaction platform between MTs and the nucleus is a protein complex called the linker of nucleoskeleton and cytoskeleton complex (the LINC complex). The LINC complex is composed of the SUN domain proteins, the KASH domain proteins as well as lamin A/C and emerin. The SUN domain proteins, SUN-1 and SUN-2, connect chromatin to the nuclear membrane. The KASH proteins, Nesprin-1 and Nesprin-2, link the nuclear membrane with the MTs. Emerin binds the inner nuclear membrane to lamin A/C. Lamin A/C proteins are intermediate filaments providing a scaffold to the nucleus (Janin *et al*., 2017).

1.6. DNA damage response signalling.

Various DNA damaging agents act differently on the genome. They are able to create single-strand breaks or double strand breaks, depending on the severity of the destruction. The most deleterious damages are double-strand breaks (DSBs) because they affect both strands of helical DNA. The DNA damage response signalling of DBSs is illustrated on Figure 1.6.

As a result of DSBs, a complex of three proteins, Mre11-Rad50-Nbs1 (MRN) senses and arrives at the damage site. This complex also attracts other proteins necessary for DNA signalling and repair. After autophosphorylation of ATM , a cascade of reactions is initiated. ATM phosphorylates H2AX on serine 139 also known as γH2AX. Due to generation of γH2AX just after DBSs formation and ubiquitylation of γH2AX after the damage is repaired, it is a commonly used indicator of DBSs. Furthermore, downstream from ATM, γH2AX recruits and activates a mediator of DNA damage checkpoint protein 1 (MDC1). After that, the recruitment of RNF8 and RNF168-dependent chromatin ubiquitination occurs, which is followed by the activation of these E3 ligases. This complex enables a high-affinity accumulation of DNA damage proteins, including 53BP1 at the double-break strand sites (Panier and Boulton, *et al*., 2013). Interestingly, some studies suggest that 53BP1 can decorate DBSs independently upon γH2AX (Celeste, *et al*., 2003). Celeste and co-workers showed an accumulation of 53BP1, BRCA1 and Nbs1 proteins upon DNA damage in γH2AX knockdown human fibroblast cells. Furthermore, 53BP1 foci colocalise with γH2AX foci in response to DSBs, therefore it is used as a marker of DNA damage. Also, depletion of either 53BP1 or RIF1 or both resulted in significant defects in NHEJ. These observations imply that both proteins promote this signalling pathway. Moreover, studies have proved that 53BP1 acting together with RIF1 prevent DNA resection. Although the exact mechanism of how 53BP1 accumulates at DSBs is unknown, Panier and Baulton (2014) proposed that 53BP1 and RIF1 work together so that they stabilise the chromatin state, impeding double-strand break resection.

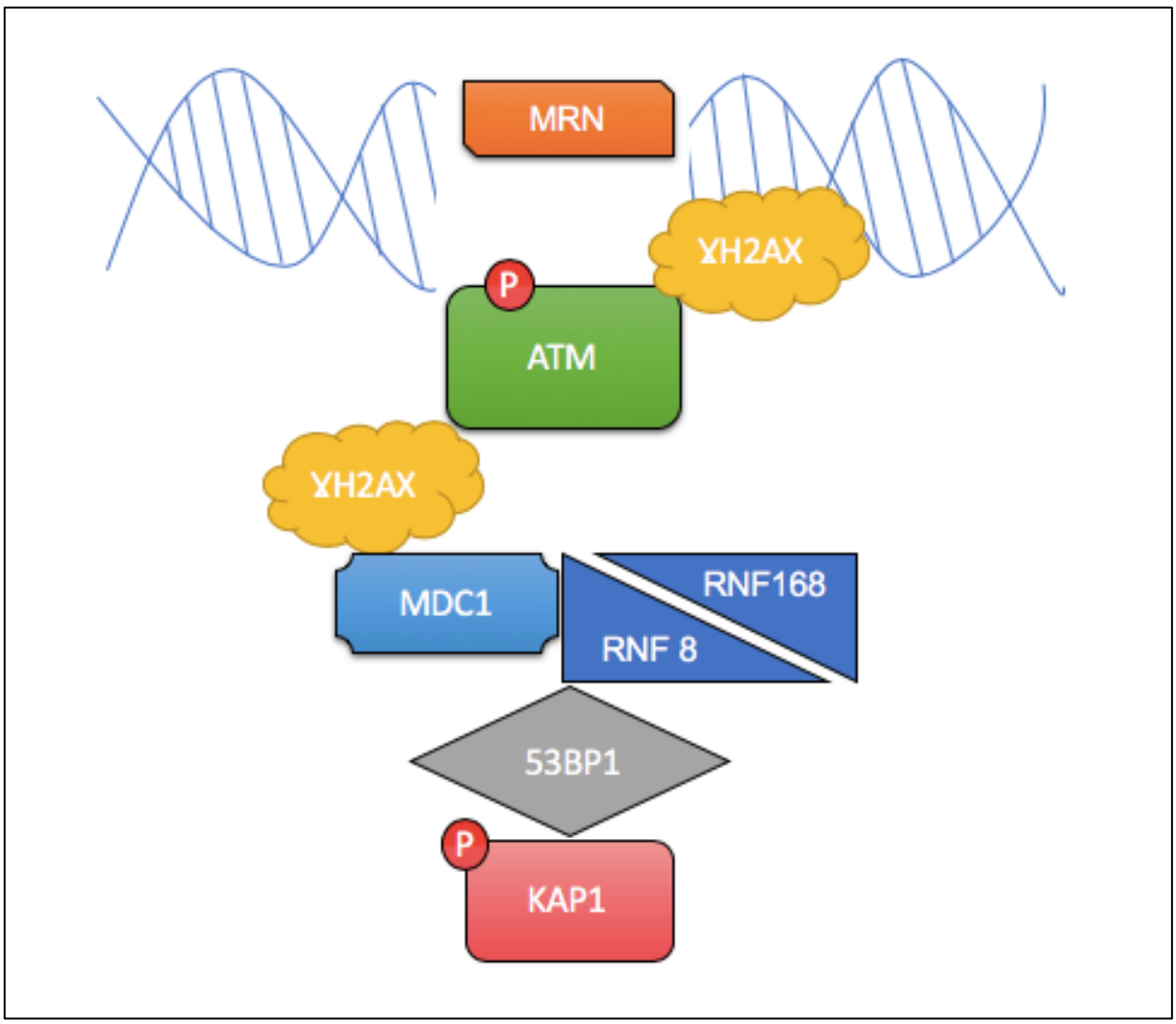


Figure 1.6. DNA damage response signalling.

The picture depicts the cascade of reaction in response to double strand breaks. The damage is sensed by the Mre11-Rad50-Nbs1 complex. Within a few seconds ATM is recruited and phosphorylated on serine 1981. This activation of ATM results in accumulation of γH2AX specifically at the DNA damage site and the recruitment of MDC1. Then RNF8 and RNF168 arrive to the damaged site and facilitate the recruitment of 53BP1, which subsequently attracts KAP1. Once KAP1 becomes phosphorylated, the chromatin around the damage site is sufficiently opened, allowing DNA repair machinery get to the damaged site.

Another important player in DDR signalling is KRAB-associated protein 1 (KAP1). This genome modulator has been demonstrated to become phosphorylated in response to DNA damage. It is thought to be recruited by 53BP1 or ATM and phosphorylated on serine 824 or 473. KAP1 regulates chromatin states around the damaged site (Iyengar and Farnham, 2011). This process allows DNA repair proteins to enter the affected site and repair it.

This cascade of events, presented in Figure 1.5., forms a platform for the recruitment of DNA repair proteins to DNA damage site.

1.7. DNA damage repair.

There are two major DDR pathways for double-strand breaks: non-homologous end joining (NHEJ) and homologous recombination (HR). DDR is cell-cycle dependent. The most prevalent signalling pathway is NHEJ, which occurs primarily in the G1 phase and can happen in G2 phase as well. Whilst the HR pathway is favoured in S phase, wherein a sister chromatid is available. HR is more frequent compared to the NHEJ pathway. The differences between the pathways are listed in Table 1.2. Nevertheless, the work reported in this thesis focuses on NHEJ, and so a detailed description of this process is presented below.

In response to a double-strand break in the G1 phase, Ku heterodimers (Ku70 and Ku80) become activated and arrive at DNA damage sites. Roberts and Ramsden (2007) proved using *in vitro* studies that Ku dimer is responsible for slight end processing of broken strands by peeling up to 50 base pairs from a histone octamer. Apart from sensing the sites, these proteins activate cell cycle checkpoint. The carboxy-terminal domain of Ku80 recruits and binds to DNA-dependent protein kinase catalytic subunit (DNA PKcs) (Hammel M., *et al.,* 2010b). DNA PKcs attracts endonuclease Artemis and dephosphorylates it (Ma *et al.,* 2002, Rooney *et al.,* 2003). Artemis trims the single-stranded tails at the presence of the break (Jiang *et al.,* 2015). DNA Ligase IV, is responsible for DNA ligation by ATP-dependent catalysis of phosphate bonds (Ellenberger and Tomkinson, 2008) and it can perform its function independently on another strand (Ma *et al.,* 2004). Moreover, XRCC4, a nonenzymatic ligase partner, interacts with BRCA1 and Ligase IV. XRCC4 has been found to regulate the level of Ligase IV at the damaged sites.

Non-homologous end joining	Homologous recombination	References
Occurs through the cell cycle but predominantly in G1 phase	Occurs in S and G2 phases	Moore and Haber, 1996 Rothkamm, *et al.,* 2003 Saintigny, *et al.,* 2007
Promoted by 53BP1	Promoted by BRCA1	Bunting, *et al*., 2010 Moynahen, *et al.*, 1999
No requirement for DNA template	Requirement for sister chromatid	Szostak, *et al.,* 1983
Minimal end processing through exonuclease 1	Intense end processing	Bahmed, *et al.,* 2011 Bunting, *et al*., 2010
DSB repair by ligation of DNA strands	DSB repair by crossing-over	Robins and Lindahl 1996; Sonoda *et al*., 1999
Risk of deletions or insertions leading to mutations	Reconstruction of the original DNA strand	Guirouilh-Barbat *et al*., 2004 Sonoda *et al*., 1999
Fast process (takes 30 minutes to complete)	Slow process (takes 7 hours or more to complete)	Mao *et al.,* 2008
Repair of 85 % of DSBs across the cell cycle	Repair of 15 % of DSBs across the cell cycle	Mao *et al.,* 2008
High IR sensitivity	Low IR sensitivity	Thompson and Schild, 1999; Essers *et al.,* 1997

Table 1.2. Comparison of the two main DSBR DNA damage response signalling pathways.

Table above summarises the most important differences between HR and NHEJ pathways.

XRCC4-like factor (XLF) binds to Ligase IV-XRCC4 via its head domain (Ahnesorg, Smith and Jackson, 2006). XLF stimulates Ligase IV activity and its re-adenylation (Riballo, *et al*., 2009). Also, XLF results in proper DNA strand alignment (Tsai, *et al.,* 2007). Ligase IV, XRCC4, and XLF form a complex that joins together the strands of DNA without the renewal of lost nucleotides. The scaffold of these DNA repair factors around damaged chromatin is formed by PAXX protein (Ochi, *et al.,* 2015). Once the DNA strands are ligated, Ku80 is ubiquitylated by the E3 ligase RNF8, promoting dissociation of the complex from the DNA damage and blocking the signal to attract DNA repair protein (Postow *et al.,* 2008; Feng and Chen 2012). NHEJ is error-prone because it can result in shifting the codon frame by deletion or insertion of a number of nucleotides, not divisible by three, or by deletion of insertion of triplets, which might lead to mutations in proteins. Nevertheless, the pathway is imperative for maintaining genome integrity (Knight, *et al*., 2017).

1.8. Consequences of DDR failure.

Genetic information is stored in DNA and it is imperative to maintain genome integrity to prevent loss of genetic information. Failure to repair DNA results in a number of disorders, such as cancer, immunodeficiency, neurodegenerative diseases and cardiovascular disorders (Blackford and Jackson, 2017).
Some impairments in the production or function of DDR proteins results in an increased predisposition to cancers (Bristow and Hill, 2008).

1.8.1. Cancer

One of the DDR proteins whose loss leads to cancer is ataxia telangiectasia mutated (ATM). Phosphorylation of the serine 15 residue results in stabilisation and activation of p53 on multiple sites. Also, ATM is involved in an interaction of p53 with MCM2, the ubiquitin ligase, during double-strand break repair and recruitment of DNA repair proteins to the site of DNA damage (Blackford and Jackson, 2017). A mutation in the ATM gene contributes to defects in coordination known as ataxia and formation of small dilated blood vessels, reducing the oxygen supply, referred to as telangiectasia, which both are hallmarks of the disease. Hence, the disorder is called an ataxia-telangiectasia syndrome. Moreover, further cycled impairments in oxygen supply in

ATM-deficient patients have been associated with genomic instability promotion and tumorigenesis (Bristow and Hill, 2008). Loss of ATM leads to downregulation of a tumour suppressor protein p53, resulting in the impairment in DNA repair as well as genome instability that consequently results in leukaemia and lymphomas development. Evidence has shown that as well as having a role in cancer, impairment in the NHEJ pathway is associated with immunodeficiency.

1.8.2. Immunodeficiency

Loss of ATM or NBS1 corresponds to T-cell and B-cell dysfunctions (Vacchio, *et al.,* 2007). Studies have shown that in patients with ATM or Nijmegen breakage syndrome diseases, these immune cells have impaired V(D)J rearrangement (Dai, *et al.,* 2003). This feature predisposes affected cells to genomic instability and tumour development and progression.

1.8.3. Spermatogenesis

Moreover, DDR has been found to be crucial in a course of spermatogenesis, meaning that impaired DDR functions is associated with infertility (Bartkova, *et al.,* 2005). Accurate repair of germline cells is essential so that possible mutations are not passed on to future generations. Therefore, the HR pathway rather than NHEJ is prevalent in order to accurately repair DNA.

1.8.4. Neurodegenerative disorders

Unrepaired DNA lesions in neurons progress to neurodegenerative disorders, such as Alzheimer's, Huntington's or Parkinson's. Whilst neurons display a high level of mitochondrial respiration, they are vulnerable to reactive oxygen species, damaging mitochondrial and nuclear DNA. The removal of impaired neurons leads to a permanent loss of these terminally-differentiated cells. The apoptosis of irreplaceable neuronal cells is characteristic in neurodegenerative disorders (Rass, Ahel and West, 2007).

1.8.5. Ageing

An accumulation of DNA damage occurs through life and is believed to accelerate ageing. Over time the build-up of lesions could decrease the function of DNA repair proteins. Of note, some patients with inherited DDR defects demonstrate characteristics of premature ageing, such as growth retardation. Both insulin-like growth factor 1 and growth hormone control longevity. Thus, their downregulation as a result of active DDR signalling is an argument supporting the link between DNA damage and ageing.
Interestingly, these examples show that active DDR signalling is beneficial for the proper functioning of the cell by preventing tumorigenesis and reproduction, whereas later in life the activation of this pathway leads to stem-cell removal that accelerates the process of ageing (Yang, *et al*., 2008).

1.8.6. Atherosclerosis

Atherosclerosis is a disorder characterised by build-up of plaques inside the arteries. An extensive activation of a p53 protein in response to oxidative stress might eventually result in increased DDR signalling and atherosclerosis. This link between DNA damage and the cardiovascular disease further supports the study establishing a senescence of vascular smooth muscle cells and apoptosis, leading to atherosclerotic damages in response to DNA lesions (Mercer, Mahmoudi, and Bennett, 2007).

Both MT dynamics as well as DDR signalling are still not fully understood. It remains elusive how exactly MAP proteins and PTMs of tubulin modify MT dynamics, which have showed to be imperative in the cell division, cell polarity and transport of cargo. Similarly, there is ongoing worldwide research aiming to discover the correlations between various DDR signalling proteins. In particular, 53BP1 and KAP1 are currently main proteins of interest due to their variety of mysterious roles in DDR signalling. Altogether, the impairment of the components of MT dynamics and DDR signalling demonstrate how important the appropriate regulation of both of them is. Perturbation of these mechanisms might lead to a deregulation of the cell functioning and eventually

lead to a number of diseases, such as cancers and neurodegenerative disorders that are similar in case of MTs and DDR. Therefore, it is vital to fully understand the connection between MTs and DDR proposed by Lottersberger (2015).

The current research in this context is focused on chromatin regulation states (Lottersberger *et al.,* 2015; Lawrimore *el at*., 2017). Due to the fact that the factors responsible for the decompaction state of chromatin observed in these studies are still unknown, that is one of the major questions in the field.

These pioneering works were mainly focused on DDR, overlooking the roles of posttranslational modifications (PTMs) that is known to have a significant effect on MT motion, and that could explain how the alterations of MT dynamics affect DNA damage.

Thus, the work presented in this Master's thesis aims to understand the correlation between PTMs of tubulin, MT dynamics and DNA damage response signalling.

1.9. Aims and potential benefits from this research

Establishing the link could deepen our understanding of the extended DDR signalling pathway as well as further explain the role of PTMs in MTs dynamics.

In practice, greater knowledge of these links may lead to better understanding of diseases where DDR or MT dysfunction are involved, such as cancer or neurodegenerative disorders and could aid in the creation of effective drug treatments or genetic therapies.

1.10. Aims of the project

- Identifying the effect of DNA damage on posttranslational modifications and dynamics of microtubules;

- Establishing the impact of perturbation of microtubule dynamics on DNA damage signalling.

Chapter 2: Materials and methods

2.1. Cell culture

Retinal pigment epithelial 1 (RPE-1) cells were maintained in 25 cm^2 or 75 cm^2 tissue culture flasks and incubated at 37°C with 5 % CO_2. The cells were cultured in Dulbecco's Modified Eagle's Medium/ Nutrient F-12 Ham (Gibco) supplemented with 10 % foetal bovine serum (Gibco), 2 mM L-glutamine and 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco).

2.2. Serum starvation

The media of settled cells was changed twice with serum-free media Dulbecco's Modified Eagle's Medium/ Nutrient F-12 Ham (Gibco) with 2mM L-glutamine and 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco). Cells were kept in serum-free media for 48 hours.

2.3. Serum stimulation

Cells that had been starved for 48 hours were stimulated with 10% foetal bovine serum (Gibco) one hour before any drug treatment.

2.4. Flow cytometry

To cells in 6 cm^2 flasks 1 ml of tyrosine was added for 5 minutes and then 3 ml 1 % BSA (Bovine Serum Albumin) in PBS (phosphate-buffered saline) was added. The cells were centrifuged at 2500 RPM for 2.5 minutes and the supernatant was aspirated. Then, 100 µl 4 % PFA in PBS was added and mixed gently but firmly. The incubation was in the dark room for 15 minutes at room temperature. Cells were washed with 3 ml 1 % BSA in PSB and then pelleted. After 10 µl of 1:10 000 Hoechst dye was added, tubes were incubated overnight at 4 °C. The Novocyte machine was used for analysis, which was performed by The Flow Cytometry department at the University of Bristol.

2.5. IncuCyte

Cells were seeded in 96-well plate at 10% confluence. Then drugs (DMSO, NCS, Phleomycin, Etoposide, Taxol and Nocodazole) were added and placed in the IncuCyte machine. Images were taken every 2 hours for 68 hours. After the last time point, the plate was removed from the IncuCyte machine and supernatant was aspirated. In order to count the nuclei, the cells were stained with IncuCyte NucLight Rapid Red Reagent (EssenBioscience). The IncuCyte programme was trained for identification of the nuclei.

2.6. Immunoblotting

2.6.1. Cell lysis

Cells were grown to 80-95 % confluence in either 6 or 10 cm dishes. Media was removed, and the cells were washed with phosphate-buffered saline (PBS), using the same volume, as medium in which cells were cultured. Then, 80 µl of 2x Laemmli (4 % sodium dodecyl sulphate and 120mM Tris, pH 6.8) was added and cells were scraped from the dishes and pipetted into labelled 1.5 ml Eppendorfs.

2.6.2. Sample preparation

The Eppendorfs were heated to 95 °C for 5 minutes and subjected to centrifugation in a mini centrifuge. Afterward, the suspension with cells was passed through a 25G needle attached to a syringe, and the lysate centrifuged at 5000 RPM for 2 minutes. To determine the concentration of proteins in the samples, a NanoDrop Lite machine was used and then 2x laemmli buffer was added to samples to make equal concentration in all samples. All samples were diluted to be equal of the lowest concentration sample.

2.6.2. 10 % SDS-Polyacrylamide Gel preparation

A running gel mixture was made using 6.3 ml ddH_2O, 5.4 ml 30% Acrylamide 80µl 10 % APS and 8 µl TEMED and 4 ml Resolving buffer (1.5 M Tris and 0.1 % SDS, pH 8.8). The solution was poured between glass plates and covered with 70 % ethanol and left for 30 minutes. Then, a stacking 4 % stacking gel was pipetted into the cassette and a comb applied. The stacking gel was prepared by mixing 3 ml ddH_2O, 670 µl 30 % Acrylamide, 1.3 ml stacking buffer (0.5 M Tris HCl 0.1 and SDS, pH 6.8), 60 µl 10 % APS, 6 µl TEMED. 4 % stacking gel was left in a cassette for around 20 minutes.

2.6.3. Loading and running gel

50 μg of protein was loaded into a pre-made 4- 12 % Bis-Tris Plus cassette (Invitrogen) or 10 % gel prepared as described above. The gels run in 1x 2-(*N*-morpholino) ethane sulfonic acid (MES) buffer or 1 x MOPS SDS Running buffer (50mM MOPS,1mM EDTA, 50 mM Tris Base, 0.1 % SDS, pH 7.7) . After that western blots were made at 200 W for 35 minutes.

2.6.4. Western blotting

The gel from the cassette was removed and placed into water. Then a sandwich in a Power Blot cassette was made, including the following layers immersed in 1- step transfer buffer (Invitrogen) prior to use: 2 thick filter papers, nitrocellulose, gel and 2 thick filter papers. The transfer was run at 25 V in the selected on the machine programme ‘mixed range of molecular weight of protein’ for 7 minutes. Then, the nitrocellulose blot was rinsed with water and Ponceau S was added to visualise whether proteins have been successfully transferred. Then, the blot was washed with TBST (Tris-buffered saline [50 mM Tris, 150mM NaCl] and 0.5% Tween 20). Afterwards, the blot was blocked with 5 % milk solution or 1 % bovine serum albumin in TBST for 1 hour.

2.6.5. Antibody staining

The blocked blot was rinsed with TBST, cut into pieces and placed in tubes with primary antibodies overnight in a cold room. The dilution of specific antibodies is shown in Table 2.1. Next day, the pieces of blot were washed with TBST three times for 5 minutes. Secondary antibodies: the Anti-rabbit IgG (H+C) Dylight 800 and the Anti-mouse IgG (H+C) Dylight 680 (CellSignalling) were then added in TBST containing 1 % BSA for 1 hour hidden from the light. The dilution of the secondary antibodies was constant 1:10 000. After that, the blot was washed in TBST three times for 5 minutes and then left in the water ready for imaging. To image the western blot, a LiCore machine was used with wavelength 600 nm for mouse antibodies and 800 nm for rabbit antibodies.

ImageJ software was used to visualise the fluorescent bands.

2.7. Immunofluorescence (IF)

Cells were seeded in 8-well ibidi plate or 96- well plate. RPE-1 cells were visualised at 80-95 % confluence. Media was removed, and dishes were washed with PBS. The cells were fixed with 2 % Paraformaldehyde (PFA) [PFA, PBS, sodium azide, NaOH/HCl] for 10 minutes and washed with PBS three times. The permeabilisation step was carried out by addition of 0.1 % Triton x100 [1 % Triton, PBS, BSA, sodium azide]. After 10 minutes, 1 % BSA in PBS was added to block the cells for 30 minutes, which was followed by a single wash with PBS. Primary antibodies were added to the dishes with the dilutions listed in Table 2.1. and left overnight at 4ºC. Next day dishes were washed three times with PBS and then secondary antibodies Alexa Fluor 594 Goat anti-rabbit, Alexa Fluor 488 Goat anti-mouse (Invitrogen) were diluted in 1 % BSA in PBS with the constant 1:1000 were put into the dishes for one hour in room temperature covered from the light exposure. To stain the nucleus, DAPI was added for 10 minutes and the dishes were washed three times with PBS. Last wash with PBS was not removed and the cells were ready to image.

Images were taken with a Delta Vision widefield microscope with 60 X lens with 1.522 viscosity oil immersion. Alternatively, the cells were visualised using a CellVoyager

confocal microscope with a 40 X lens. Maximum intensity projection images were analysed with CellProfiler (DNA damage) or ImageJ (Tubulin and modifications).

2.8. Live-cell imaging

Cells were imaged and recorded in 35 mm dishes when their confluence reached 20 %. Then, a microtubule dye, SIRTubulin (SpiroChrome) with a concentration of 500 nM , was added to the dishes together with 1 μM of verapramil (SpiroChrome). Verapramil suppresses the efflux pumps of the cells, resulting in a decreased background and consequently more specific signal. After addition of the reagents, cells were kept in the incubator.

After 30 minutes, media was replaced with the fresh media four times to remove nonspecific binding of dye.

Then, the dishes were imaged using a Delta Vision widefield microscope with 60 X lens with 1.522 viscosity oil immersion at 37 °C. Microtubules were observed using the far red CYT-5 setting with the excitation of 50 % and 0.6000 %T. A series of 51 pictures were taken in a space of 2 seconds, making a short video. The videos were analysed using the MATLAB programme.

Target	Host	Dilution factor WB	Dilution factor IF	Supplier
α- tubulin	Rabbit	-	1:500	Invitrogen
α- tubulin	Mouse	1:1000	-	Cell Signalling Technology
Polyglutamylated α- tubulin	Mouse	1:1000	1:500	Sigma-Aldrich
Acetylated α-tubulin	Mouse	1:1000	1:500	Invitrogen
Tyrosinated α-tubulin	Mouse	1:1000	1:500	Millipore
H2AX	Rabbit	1:1000	1:500	Abcam
γH2AX	Mouse	1:500	1:500	Millipore
KAP1	Rabbit	1:1000	1:500	Abcam
pKAP1S824	Rabbit	1:1000	1:250	Abcam
53BP1	Mouse	-	1:500	
H3S10p	Mouse	1:1000	-	Abcam

Table 2.1. Primary antibody details.

2.1. Analysis of microtubule dynamicity.

Adjustments of the MATLab programme for the determination of microtubule motion was created by the Wolfson Bioimaging Facility in the University of Bristol and optimised for RPE-1 cells by Maria Beatriz Villagomes Torres.
The analysis involves the withdrawal of the original data from ordered images so that the mean velocity can be presented as described below.

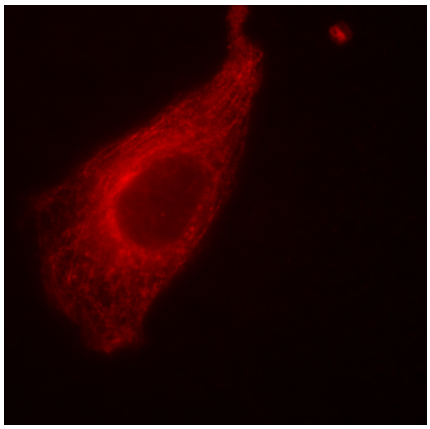
2.9.1. Extraction of data with the two-dimensional movement- pre-processing.

Initially, the videos (Figure 2.1. A.) were loaded in the ImageJ software and photobleached and the first two to five images were removed. Then, each image in a TIFF format was saved as it is recognised the microtubule motion programme. Then the microtubule motion software was opened and the TIFF images were loaded. A 2D Gaussian function was used to reduce image noise and details by smoothing effect (Figure 2.1. B.). Then, the movie was checked and adjusted using a process of manual thresholding (Figure 2.1. C.).

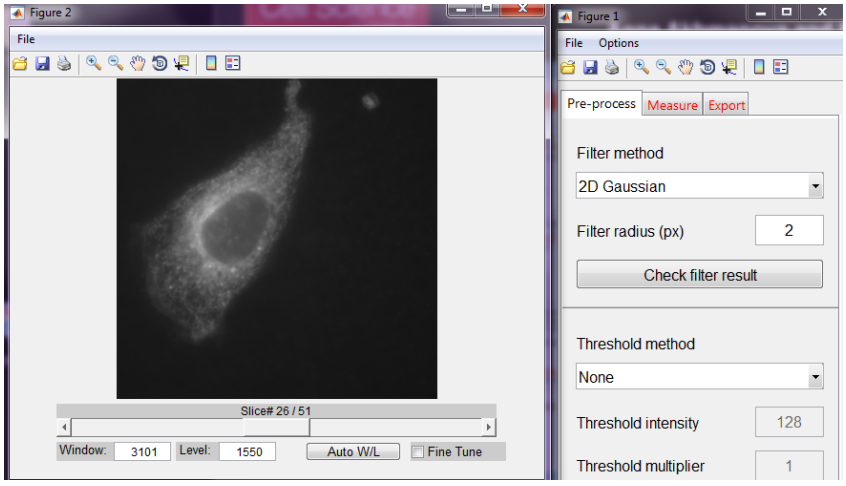
2.9.2. Extraction of data with the two-dimensional movement- measurement.

In the Measure tab, different ways of running an optical flow could be chosen. For microtubule dynamics, a Farneback algorithm was used as it allows calculation of the movements by tracking the displacements of the moving points (Figure 2.2. A.). It compares the points in an image to the previous image. This feature provides information about the speed and direction of the movement.
Afterwards, in the Export tab the displayed data was adjusted to read the Mean velocity. Importantly, as the values between the images could vary a lot, the metric in all analyses were scaled with 0.0 as the minimum movement and 1.0 as the maximum movement (Figure 2.2. B.).

A.



B.



C.

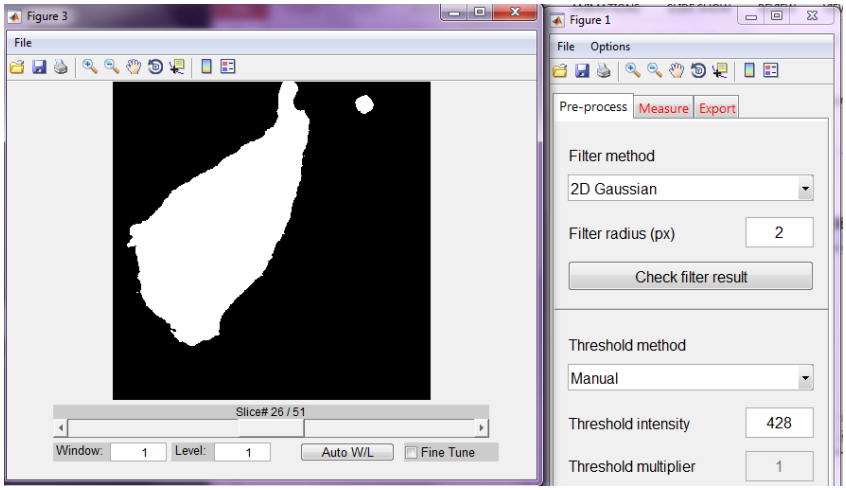
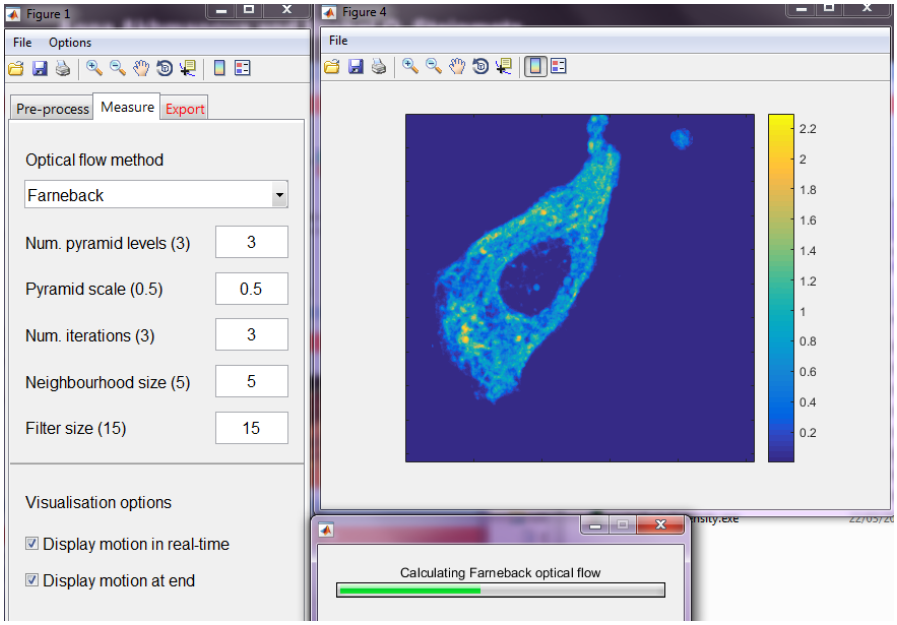


Figure 2.1. Pre-processing of the extracted data using the microtubule motion software.

A.



B.

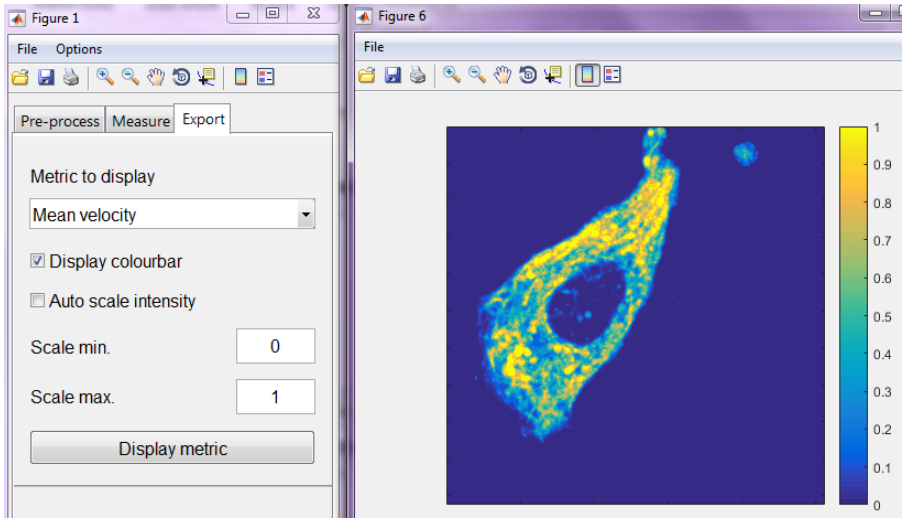


Figure 2.2. Measurement of microtubule velocity using the microtubule motion software.

Chapter 3: Results and Discussion

The effect of DNA damage on posttranslational modifications and dynamics of microtubules.

3.1. Validation of experimental conditions for RPE-1 cells.

Posttranslational modifications of tubulin influence dynamicity of microtubules and are dependent on the cell cycle phase. In order to replicate and develop Lottersberger and co-workers' (2015) findings that increased microtubule dynamics is required to repair DNA damage in G1 phase, the aim of the work reported in this chapter was to study the influence of DNA damage on posttranslational modifications of tubulin and dynamicity of MTs.

Firstly, experimental conditions were validated using flow cytometry. This technique was performed in order to confirm the distribution of the cell cycle phases in serum starved, re-stimulated and non-starved population of RPE-1. The main purpose of using serum starvation for 48 hours in this research was to synchronise cells in G1 (Pirkmajer and Chibalin, 2011) to imitate research done by Lottersberger and colleagues (2015). In the G1 phase, the cell grows and prepares for DNA replication. The G1 synchronisation allows to reduce a number of factors that could potentially influence the dynamic alterations of MTs as the G1 is less abundant in some proteins, such as the transcription factors that are necessary for DNA replication. The direct effect of serum starvation can cause either cell cycle arrest, senescence or apoptosis depending on the amount of time that cells are deprived of serum.

In this experiment, 48-hour serum starvation was done based on the well-defined protocol for cell cycle arrest in the G1 phase. The non-synchronised population of RPE-1 cells served as control (Figure 3.1. A). The analysis showed the ratio 1.92 between G1 and G2 in a control, which is an expected distribution for cells in cell cycle phases in typical conditions (Qiao, *et al.,* 1996).

The cell cycle distribution analysis indicated that around 70 % of RPE-1 cells in serum-free media after 48 hours were in G0/G1 (Figure 3.1. B). Importantly, in order to eliminate the effect of senescence (entering G0), cells were re-stimulated with 10 % serum and the analysis showed that after two (Figure 3.1. C) and six (Figure 3.1. D) hours following serum stimulation, the majority of the cells were still progressing through G1.

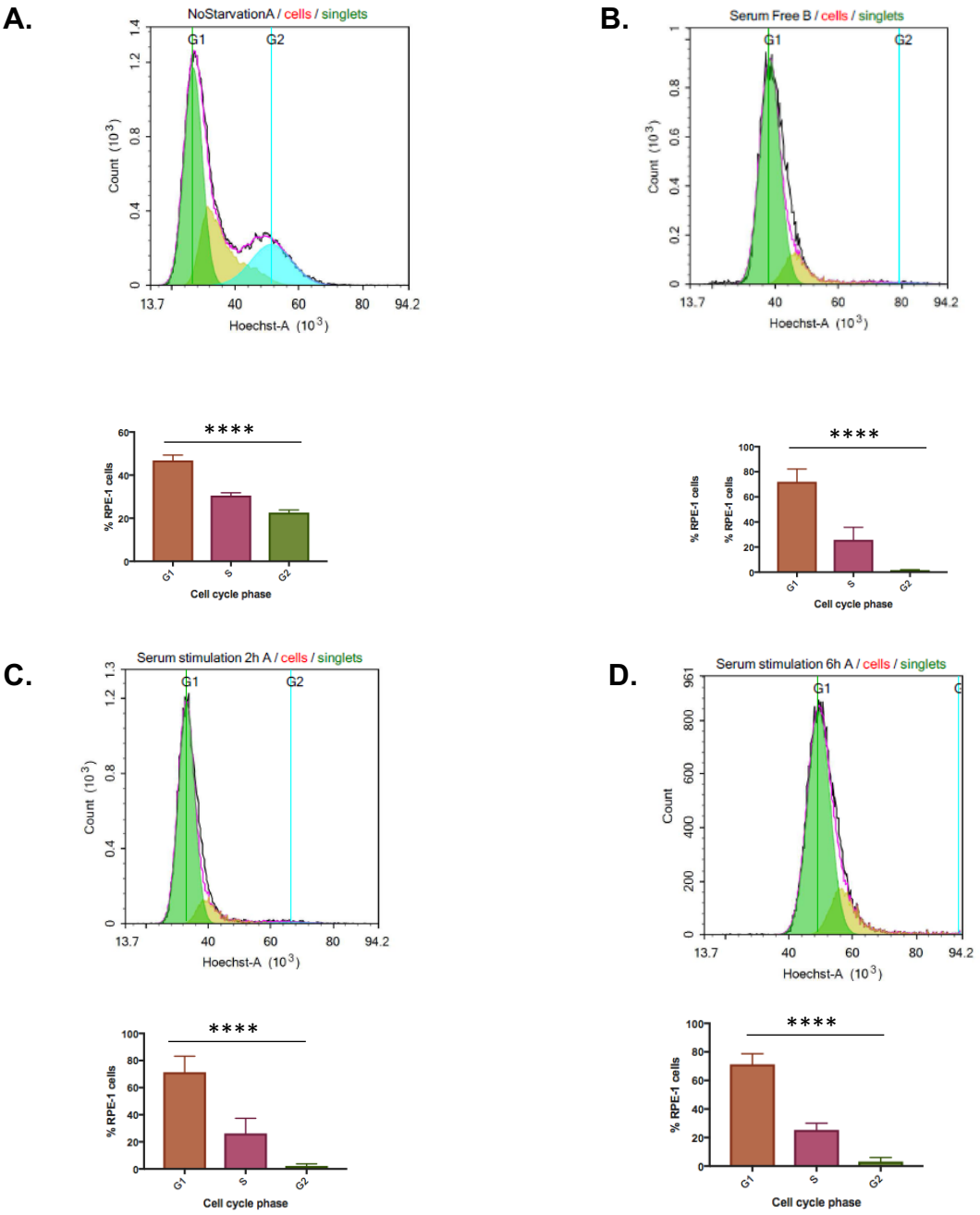


Figure 3.1. Flow cytometry analysis of growing and serum starved RPE-1 cells.

Cells were 80-90 % confluent at the time of collection. **A.** Control cell population in complete media. **B.** Cells serum starved for 48 hours prior to collection. **C.** Cells serum starved for 48 hours and returned to complete media two hours before collection. **D.** Cells deprived serum for 48 hours and returned to complete media 6 hours prior to collection. The analysis graphs represent the percentage of cells in each phase from a population phase of cell cycle. This experiment was repeated four times in the way that all the samples from each repeat were collected at the same time. number of technical replicates = 4, ****p<0.0001, One-way ANOVA.

These time points are equivalent to the first and last points used in all experiments with drug treatments for the work described in this chapter, and so it can be concluded that for the whole time of performing these experiments, approximately 70 % of RPE-1 cells were in G1 phase once serum-stimulated. This was an expected result as the lag in the cell cycle progression in the re-stimulated cells have been observed to last about 12 to 15 hours (Scher, Stone, and Stiles*,* 1979).

3.2. Screening DNA damaging agents.

DNA damage can be induced through physical agents such as UV light or gamma radiation, and chemical agents. Examples of the second group include neocarzinostatin (NCS), phleomycin or etoposide. NCS is a radiomimetic drug that consists of an active component, chromophore, and 113 amino acid single chain protein. Whilst the chromophore cleaves DNA, the single chain protein stabilises non-covalently attached chromophore. NCS acts rapidly by entering through the cell membrane and bombarding the nucleus with toxic biradical species generated by the chromophore (Meschwitz and Goldberg, 1991). Phleomycin chelates metal ions that form a pseudoenzyme, which together with oxygen form superoxide and free radicals that cleave DNA. Additionally, phleomycin blocks integration of thymidine to DNA stands (Sleigh, 1976). Etoposide, in turn, forms a ternary structure with topoisomerase II (functions in unwinding DNA) and DNA. The complex prevents re-ligation of DNA after cleavage by topoisomerase II and eventually causes DNA to break (Montecucco, *et al.,* 2015). The exact mechanisms how these drug enter the nucleus is not known.

Initial experiments aimed to determine the most powerful DNA damaging agent that would allow to trace potential changes in PTMs of tubulin over time. Hence, a one-hour time point experiment was performed on RPE-1 cells that were treated with the above described drugs in non-serum starved, serum starved for 48 hours and re-stimulated with 10 % serum prior to 48-hour starvation conditions using a western blot. The western blot is a qualitative technique used to detect proteins of interest. The western blot results demonstrated that NCS is the most effective drug for rapid induction of DNA damage (Figure 3.2.).

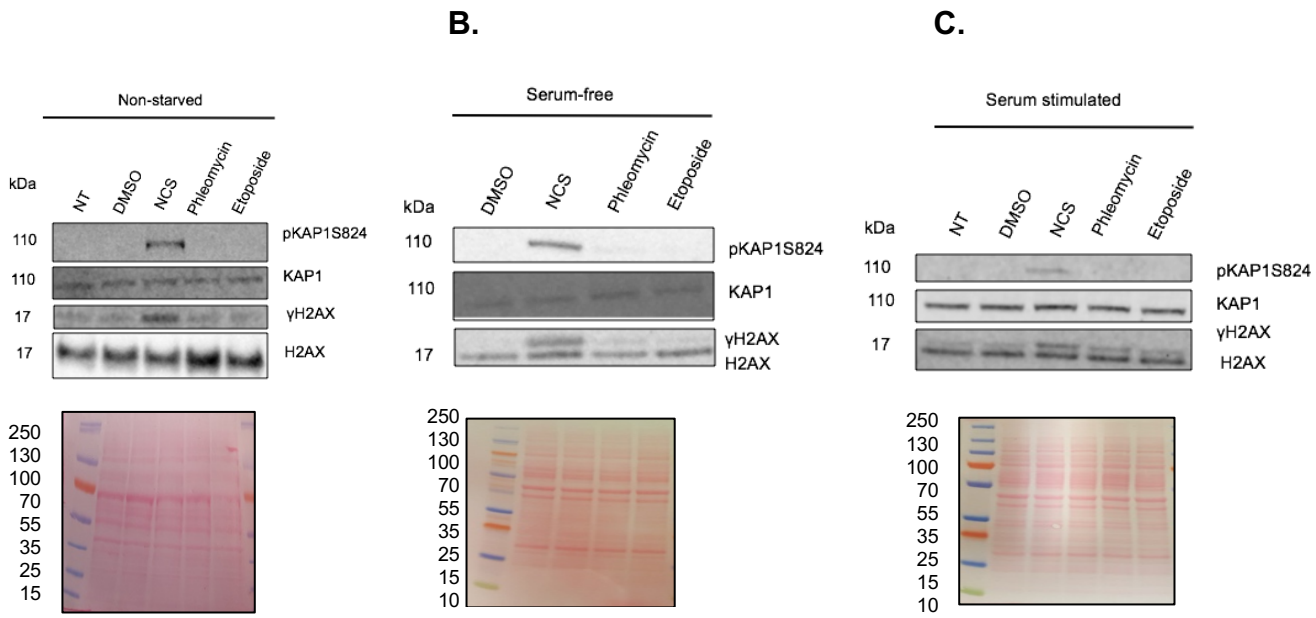
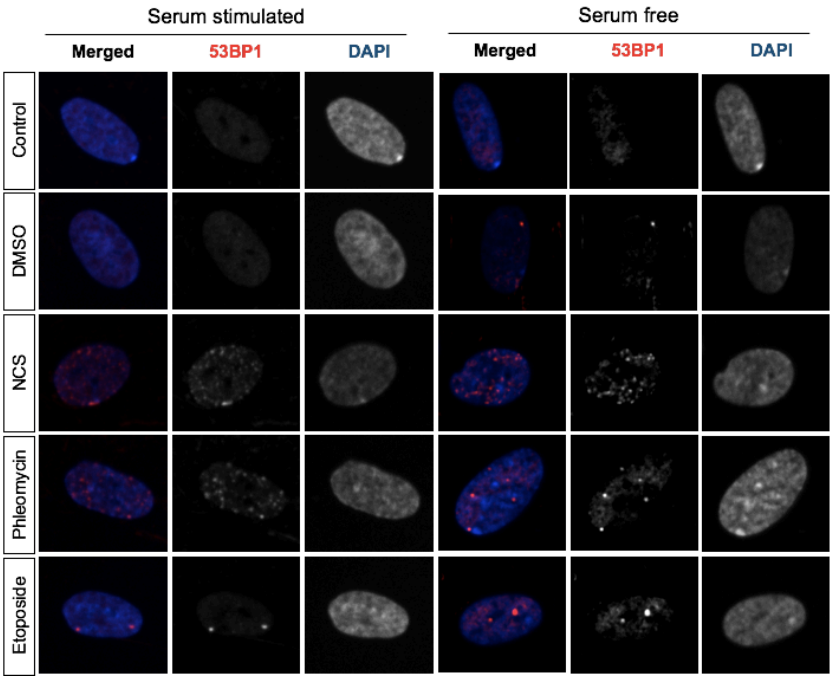


Figure 3.2. Screening different DNA damaging agents for their potency using immunoblotting.

RPE-1 cells were treated with NCS 250 ng/ml, phleomycin 10 µg/ml, and etoposide 1 µg/ml at various cell growth conditions **A.** non starved **B.** serum free for 48 hours **C.** serum re-stimulated after 48 hour starvation. One hour after treatment, the cells were collected. Total KAP1, α-tubulin and H2AX were used as loading control. The ponceau images were taken to demonstrate equal loading. Number of technical replicates= 3.

A.



B.

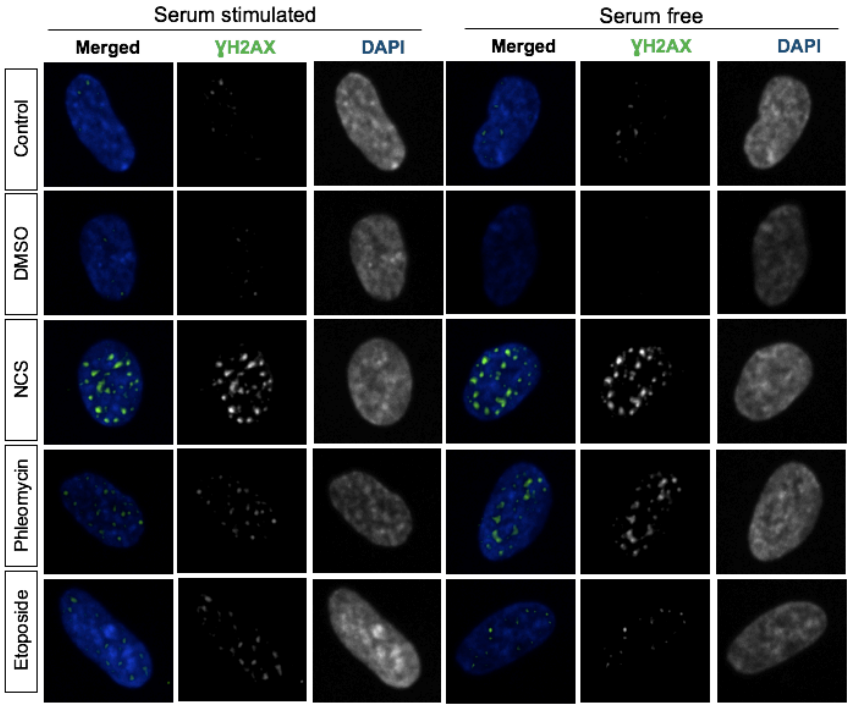
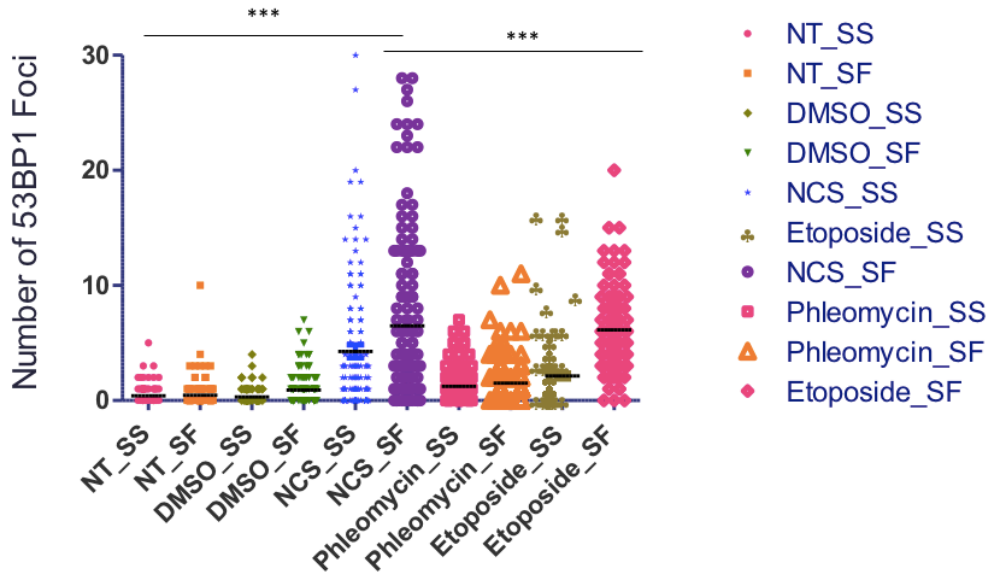


Figure 3.3. Comparison DNA damaging agents in relation to their potency. RPE-1 cells were treated with NCS 250 ng/ml, phleomycin 10 µg/ml, and etoposide 1 µg/ml at various cell growth conditions. 40 X images were taken with Cell Voyager. Number of technical replicates= 3, total cell count= 243.

A.



B.

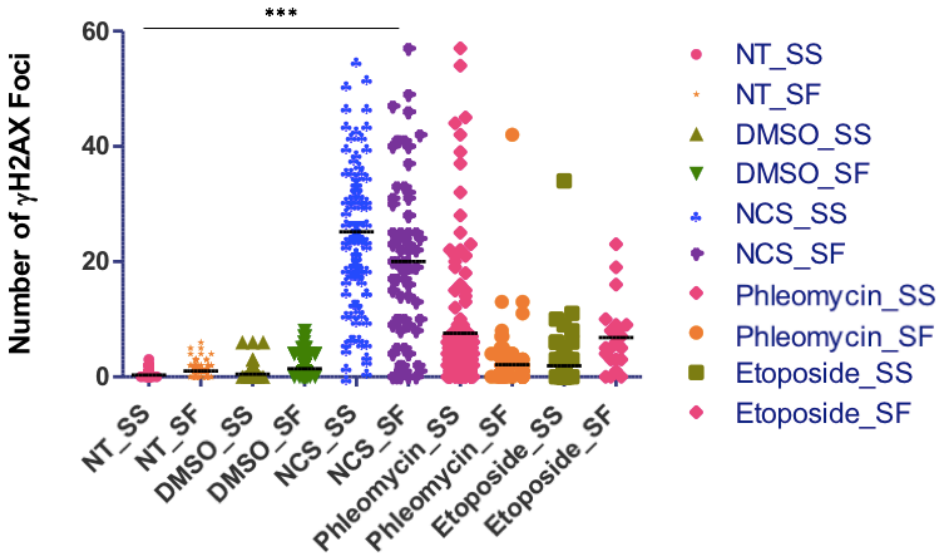


Figure 3.4. Analysis of immunofluorescence results of RPE-1 cells determining the efficiency of DNA damaging agents.

A. NCS induced the highest number of 53BP1 foci **B.** and induced the formation of twice as many γH2AX foci as 53BP1. SS, Serum Stimulation; SF, Serum Free. One-way ANOVA test was used for the image analysis. *** P value < 0.0001. number of technical replicates= 3.

Figure 3.2. A B C also showed that NCS caused phosphorylation of KAP1 on serine 824 in all cell growth conditions. That phosphorylation is a marker of DNA damage signalling activation and plays a role in chromatin decondensation. Furthermore, in whole cell analysis, DNA damage markers, 53BP1 and γH2AX were found to be most upregulated in response to NCS treatment (Figure 3.3. and Figure 3.4.). The number of γH2AX foci were twice as many compared to 53BP1, confirming the upstream activation of γH2AX. Overall, therefore, NCS was found to be the most effective DNA damaging agent among the three tested regardless of the conditions of the experiment over a short period of time.

Next, the IncuCyte experiment was performed to observe a long-term effect of the above characterised drugs using well-established concentrations to determine the most potent DNA damaging treatment in non-synchronised population of RPE-1 cells. The IncuCyte is a live-cell analysis system that in this case was used to measure the rate of the cell growth (Figure 3.5.). The cells were seeded in an equal density in each well, reaching 100 % confluency at the 68-hour time point. Non-treated cells and DMSO vehicle were used as negative controls. Whilst all the drugs resulted in a decreased the amount of cell number, NCS and phleomycin caused elongation of cell bodies (Figure 3.5.). In order to precisely determine the number of cells using the IncuCyte programme, the nuclei were labelled with IncuCyte NucLight Rapid Red Reagent for live-cells and the IncuCyte ZOOM live-cell programme was trained to count the number of nuclei in each well, corresponding to different treatments (Figure 3.5.). Additionally, changes in morphology were observed (Figure 3.5.).

After 68 hours, the analysis showed that all drugs demonstrated a deleterious impact on cell division (Figure 3.6.A).

All the used drugs caused a significant reduction in a number of nuclei compared to the control, implying the activation of checkpoints and consequently an inhibition of the cell division (Figure 3.6.B.). The number of cells seen following treatment with NCS was the lowest, at the end of the experiment, so it was concluded that NCS is the most effective drug at these time-points.

Overall, both short-time and long-time effects of the drugs showed that NCS is the most damaging DNA drug among the one tested. Therefore, it was chosen for further experiments so that the potential alterations of PTMs of tubulin could be observed.

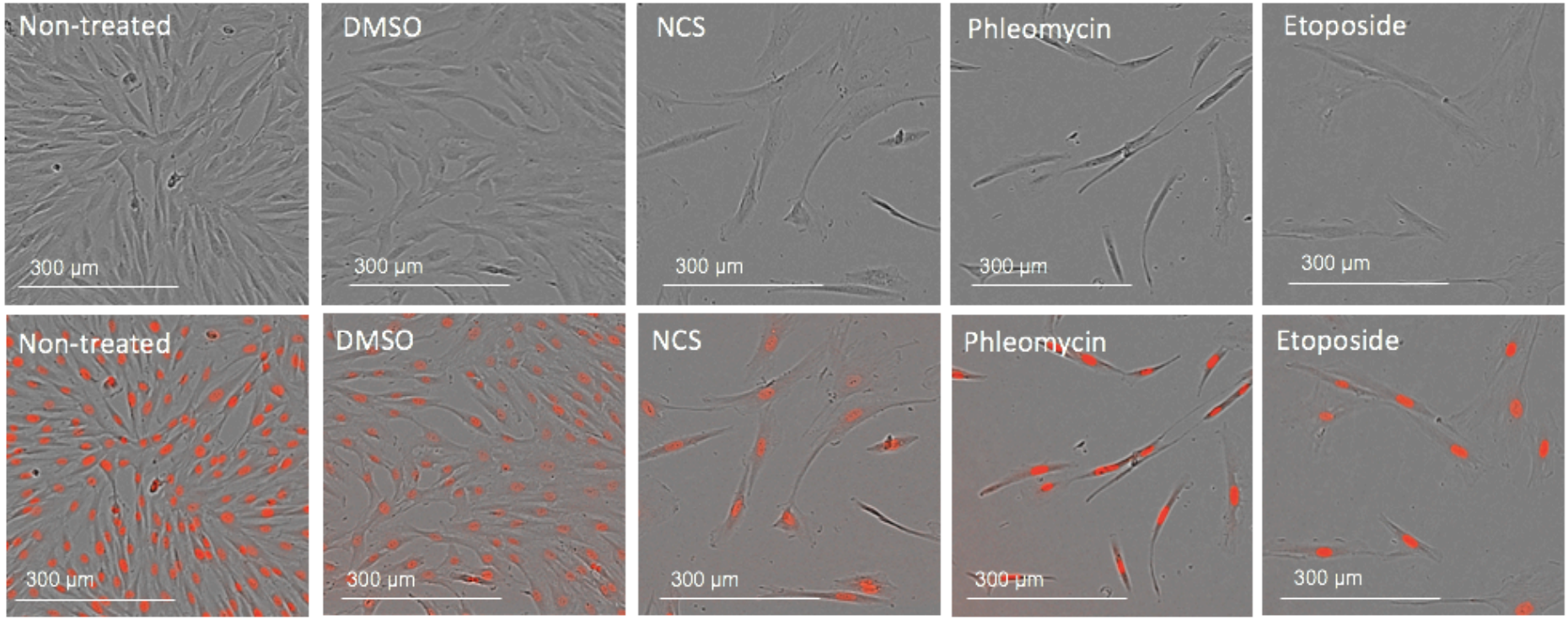
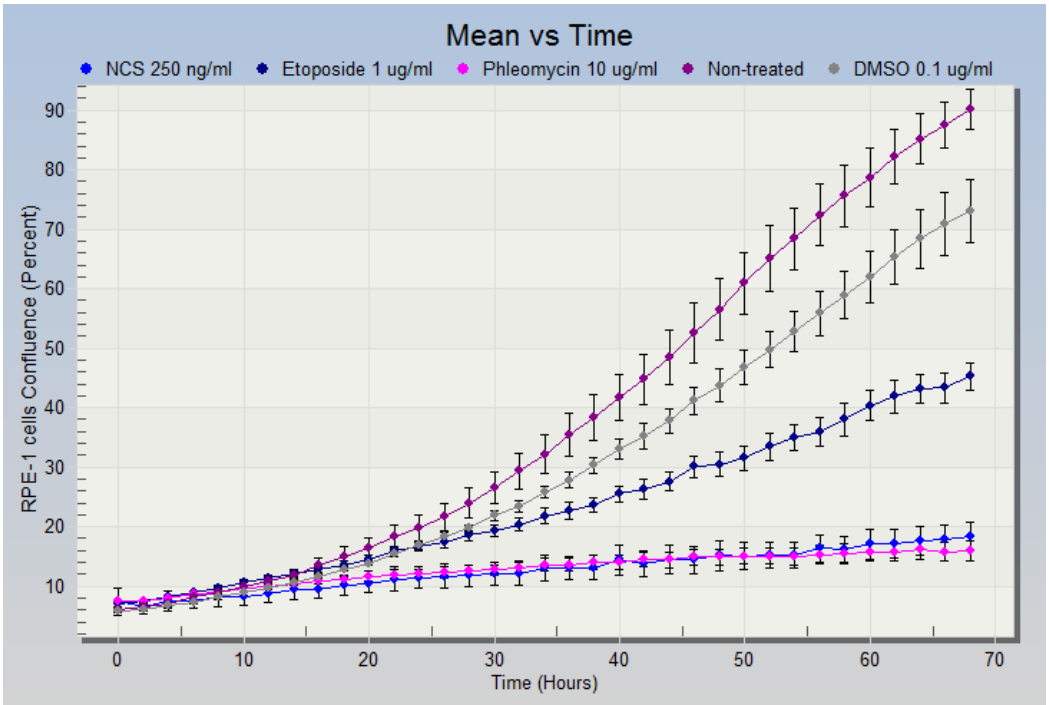


Figure 3.5. Long term effect of chemical DNA damaging agents on RPE-1 cells.

Cells were treated with various DNA damaging agents or DMSO vehicle. The 10x images were taken in an IncuCyte every 2 hours for 68 hours. Images were representative of cells after 68 hours. That experiment was done four times with 8 different samples per condition for each experiment.

A.



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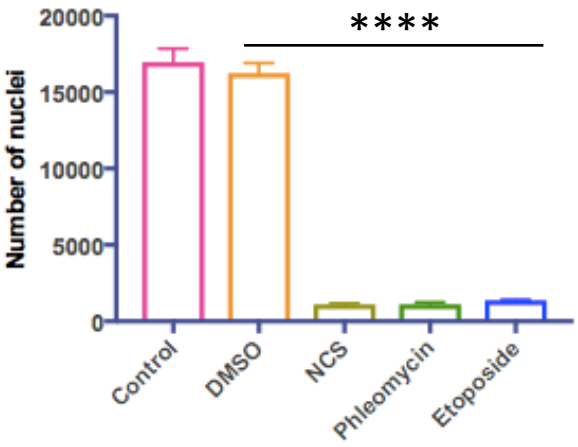


Figure 3.6. IncuCyte analysis of a long-term DNA damaging treatments on RPE-1 cells.

The cells were stained with IncuCyte NucLight Rapid Red Reagent for live-cells and detected using the IncuCyte ZOOM live-cell programme. **A.** Comparison of RPE-1 cell confluency after DNA damaging agents treatment over time **B.** analysis of the average number of nuclei in each well after 68 hour treatments.***P value < 0.0001. Number of technical replicates = 4.

3.3. The effect of DNA damage on posttranslational modifications of α-tubulin and microtubule dynamics.

Lawrimore *et al.,* (2017) showed that dynamic microtubules are required for chromatin motion in response to double-strand breaks. Correspondingly, Lottersberger *et al.,* (2015) showed that dynamic microtubules are needed to repair double-strand breaks and to enable the mobility of damaged chromatin through 53BP1 and the LINC complex. Nevertheless, there is no defined crosstalk between microtubules and DNA damage. Even though it is known that posttranslational modifications (PTMs) are associated with dynamicity of microtubules, no study has so far been performed to investigate its connection with DNA damage. Hence, the next aim was to determine the impact of DNA damage on PTM of α-tubulin and microtubule dynamics.

Firstly, an initial comparison of the effects of DNA damaging agents (NCS, phleomycin, etoposide) on posttranslational modifications of α-tubulin was performed in different conditions: non-starved, serum starved for 48 hour, and serum starved for 48 hour prior to re-stimulation with 10 % serum cells. The cells were treated simultaneously with NCS or phleomycin or etoposide and collected after one hour to test the expression levels of PTMs of α-tubulin and DNA damage markers using immunoblotting. The immunoblotting results showed no significant changes in expression levels of tyrosinated, acetylated or polyglutamylated α-tubulin in all tested growth conditions (Figure 3.7.).

In order to further confirm no change seen in PTMs of α-tubulin, an immunofluorescence was performed. That different approach enables to visualise a distribution of proteins of interest using antibodies and fluorescent dyes. The nonsynchronous cells, serum starved for 48 hours, serum starved for 48 hours and re-stimulated with 10 % serum cells were treated with DNA damaging agents: either NCS, phleomycin or etoposide for one hour and then fixed. The immunofluorescence analysis, performed on the GraphPad showed data normalised to the total α-tubulin, demonstrated no significant difference in the tested conditions, hence there is just one condition representative figure (Figure 3.8.). Immunofluorescence also showed that there was no change in an expression level of acetylated and tyrosinated α-tubulin in response to phleomycin and etoposide and NCS.

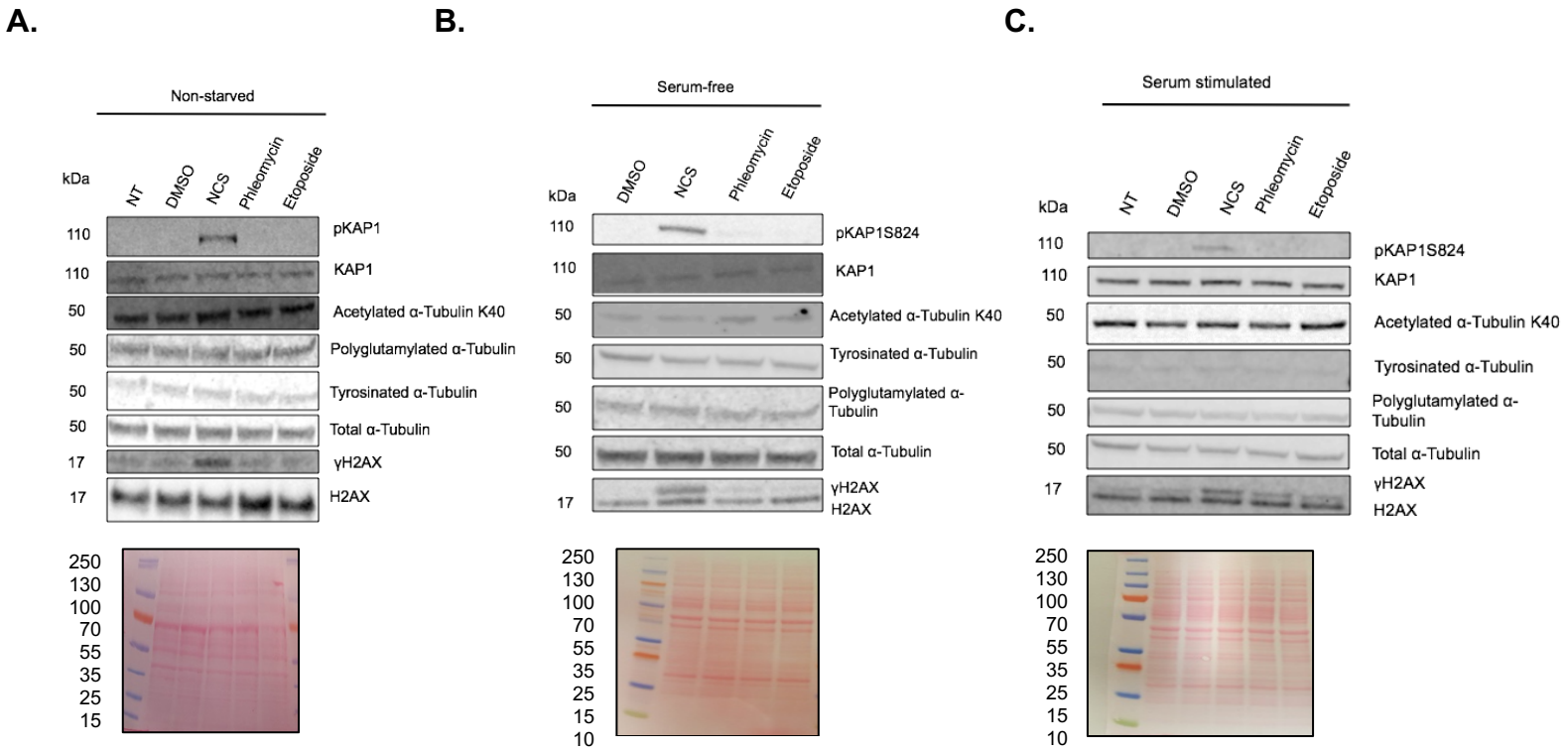
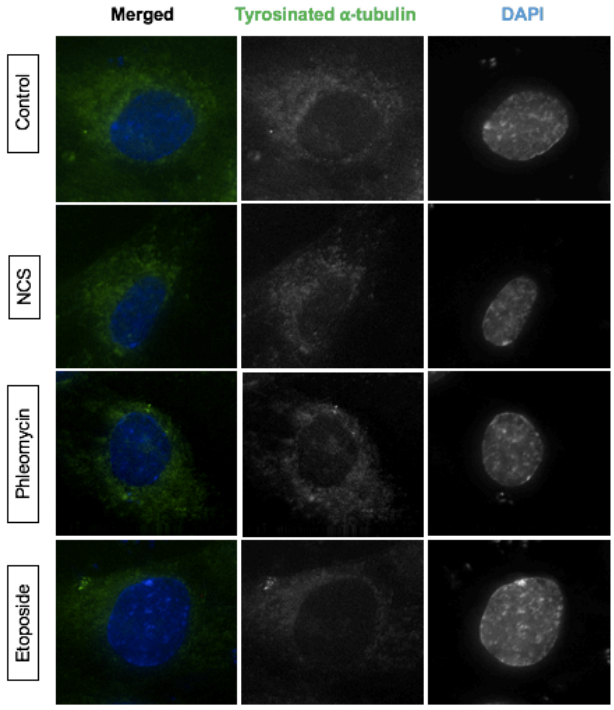
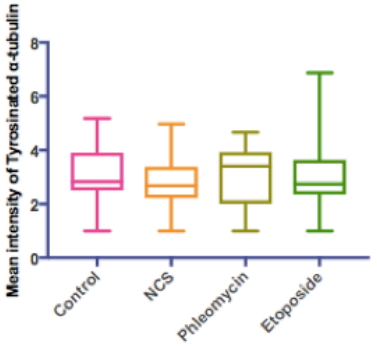


Figure 3.7. The effect of DNA damage on posttranslational modifications of alpha tubulin using immunoblotting.

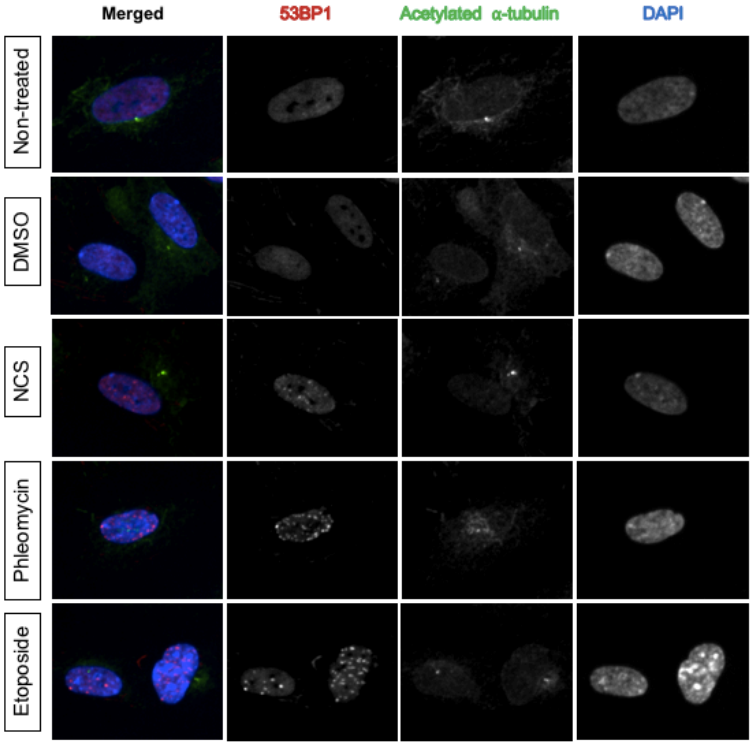
RPE-1 cells were treated with NCS 250 ng/ml, phleomycin 10 µg/ml, and etoposide 1 µg/ml at various cell growth conditions **A.** non starved **B.** serum free for 48 hours **C.** serum re-stimulated after 48 hour starvation. One hour after treatment, the cells were collected. Total KAP1, α-tubulin and H2AX were used as loading control. Number of technical replicates=3.

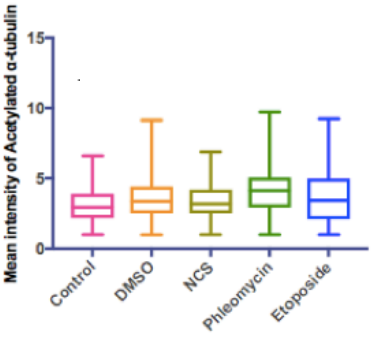
A.


Merged
Tyrosinated α-tubulin
DAPI
Control
NCS
Phleomycin
Etoposide

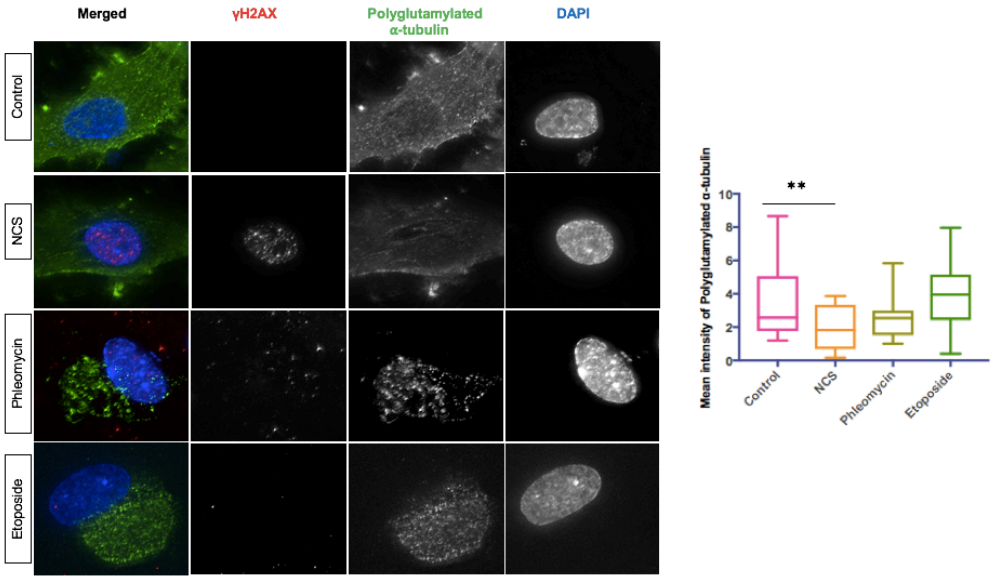

Mean intensity of Tyrosinated α-tubulin
0
2
4
6
8
Control
NCS
Phleomycin
Etoposide

B.


Merged
53BP1
Acetylated α-tubulin
DAPI
Non-treated
DMSO
NCS
Phleomycin
Etoposide


Mean intensity of Acetylated α-tubulin
0
5
10
15
Control
DMSO
NCS
Phleomycin
Etoposide

C.



D.

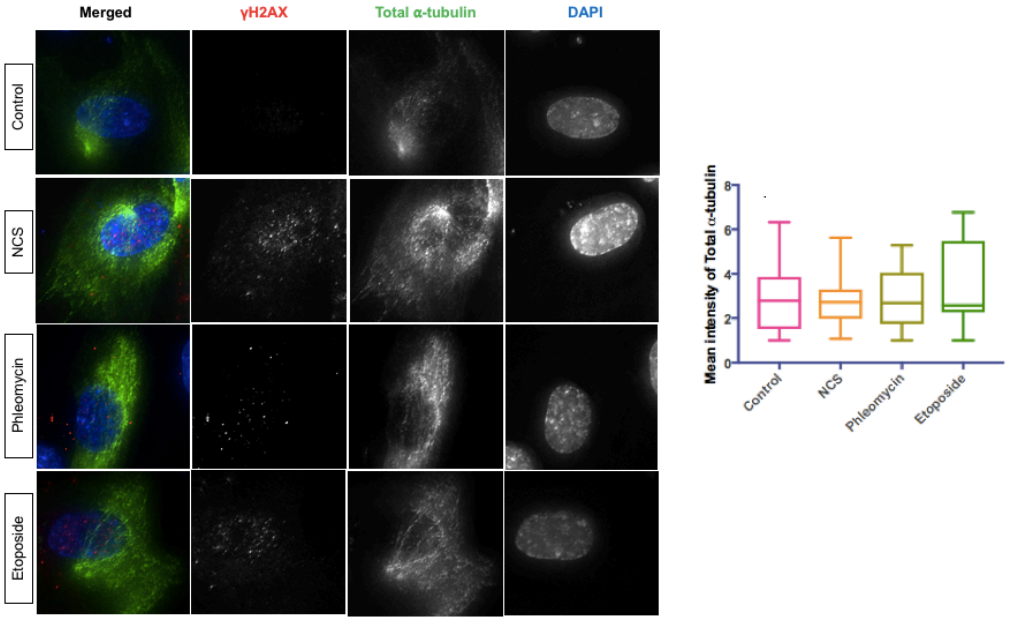


Figure 3.8. Comparison the effect of DNA damaging agents on posttranslational modification of alpha tubulin.

Measurements in **A.** (tyrosinated), **C.** (polyglutamylated) and **D.** (total alpha tubulin) were taken with Delta Vision with 60 X lens, whereas **B.** (acetylated) were taken with Cell Voyager with 40 X lens. All images were analysed using Cell Profiler. Drug treatments are as shown in Figures 3.4. One-way ANOVA test was used for image analysis in the GraphPad Prism. **p=0.0049 number of technical replicates = 3, total cell count= 198.

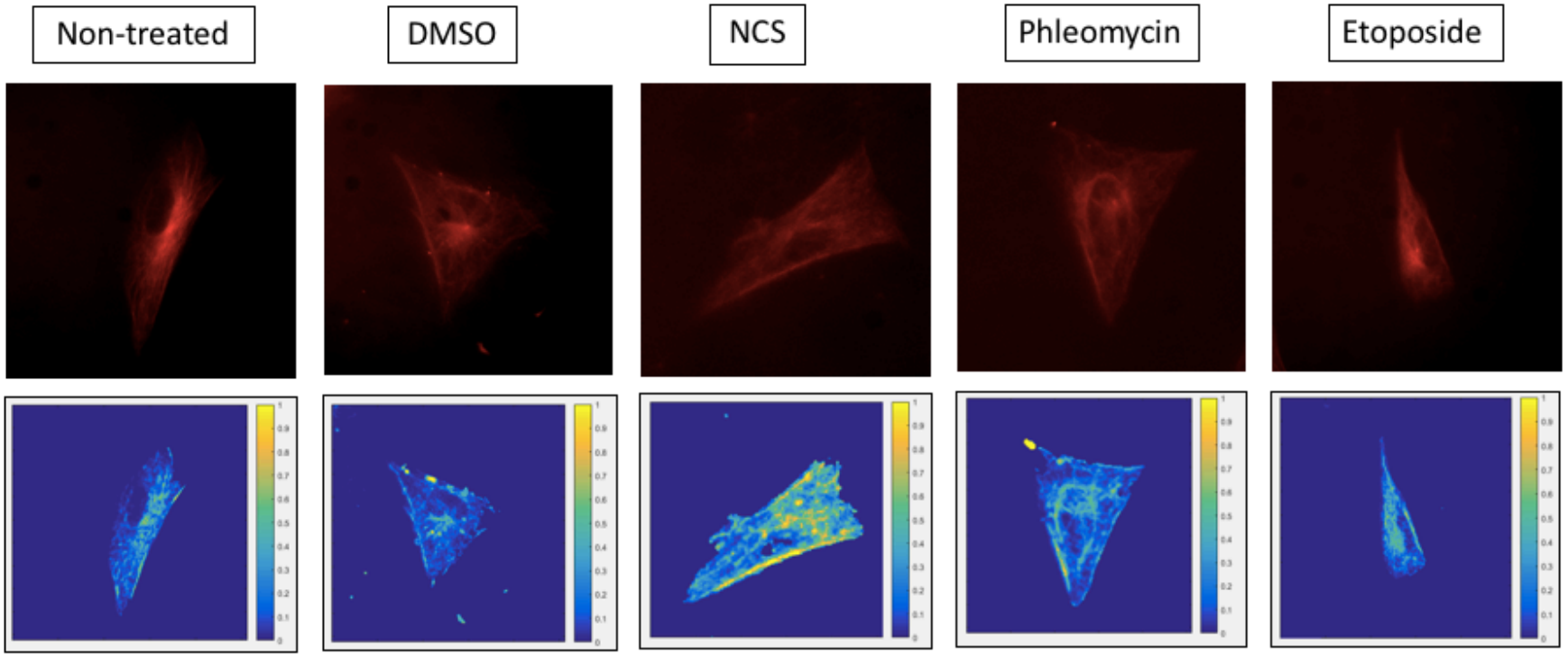


Figure 3.9. Analysis of the influence of DNA damage agents on microtubule dynamics using microtubule motion assay.

Images show the change in MT motion over the time of the videos as a single image with the colour reflecting the signal in the pixels over time. The 60 X representations were taken from videos of microtubules stained with sirTubulin. Yellow areas represents an increase in microtubular motion. Number of technical replicates= 3, total cell count=70.

However, NCS, but not phleomycin or etoposide treatment caused a significant decrease in the level of polyglutamylated α-tubulin (Figure 3.8.). Due to the correlation of PTMs of α-tubulin with MT dynamics, the decrease in polyglutamylated α-tubulin might indicate changes in MT dynamics, therefore, the MT motion assay was performed.

For this live-cell assay, cells were stained with MT dye, sirTubulin, for 30 minutes before imaging. To reduce the background, 30 minutes before the imaging verapramil was introduced to the cells, which inhibited efflux pump of the cells, and consequently reduced the background. The media was replaced four times before the imaging on DeltaVision widefield microscopy. The images were taken every 5 second for 51 times making short videos of MTs. Figure 3.9. illustrates images of MT motion in response to DNA damaging agents. An increase in MT dynamics after NSC treatment relative to other drugs or the control was observed.

Together, these immunoblotting, immunofluorescence and live-cell imaging data confirmed that NCS was the most effective drug among the ones tested and therefore it was chosen for further experiments to track PTMs of α-tubulin in response to DNA damage over time.

Furthermore, a short time course experiment was performed with NCS treatment to observe potential changes in PTMs of α-tubulin over time on the non-starved population of RPE-1 cells. The immunoblotting results demonstrated no significant alterations in PTM of MTs, though the level of γH2AX increased as expected (Figure 3.10. A). Therefore, the time course was extended and tested in serum free for 48 hours cells and re-stimulated with 10 % serum prior to 48 hour serum deprivation cells. At 30 minute after NCS treatment, in serum re-stimulated RPE-1 cells, there was a decrease in the levels of all probed modifications of α-tubulin in re-stimulated RPE-1 cells without changes in total α-tubulin (Figure 3.10. C). Interestingly, the effect was not observed in serum-free cells (Figure 3.10. B). Immunofluorescence was performed and confirmed the results seen in the immunoblots: tyrosinated, polyglutamylated and acetylated α-tubulin reduced upon NCS treatment of re-stimulated cells (Figure 3.11.). A decrease in acetylated and polyglutamylated α-tubulin levels are associated with an increase in microtubule dynamicity. Therefore, a microtubule motion assay was performed to establish whether the observed effects of NCS treatment on α-tubulin

PTM correspond to observed alterations in MT dynamicity. MTs were stained using sirTubulin dye and verapramil was administered to reduce the background. DNA was damaged using 250 ng/ml NCS treatment time course. The videos were taken using DeltaVision widefield microscopy and analysed using a MatLab program. As indicated in Figure 3.12., NCS increased dynamics of MTs compared to control. The effect of the drug was the most significant at the 30 minutes time point. This result confirms that the decreases observed in expression levels of both acetylated α-tubulin and polyglutamylated α-tubulin are correlated to an increase in microtubule motion.

Taking immunoblotting, immunofluorescence and live-cell imaging results into consideration, NCS, a DNA damaging agent, decreases levels of both acetylated α-tubulin and polyglutamylated α-tubulin. These alterations, in turn, cause an increase in the dynamicity of microtubules. This preliminary evidence might suggest that microtubules are important or at least involved in DNA damage signalling. However, to further confirm that link, more MT dynamic regulating players should be tested here. For instance, HDAC6, TTL or deglutamylases to confirm the changes in acetylation, tyrosination and polyglutamylation of α-tubulin, respectively. Moreover, siRNA of the proteins of interest would be valuable to observe whether the lack of these proteins could result in different results indicating importance in the link between DDR signalling and MT dynamics.

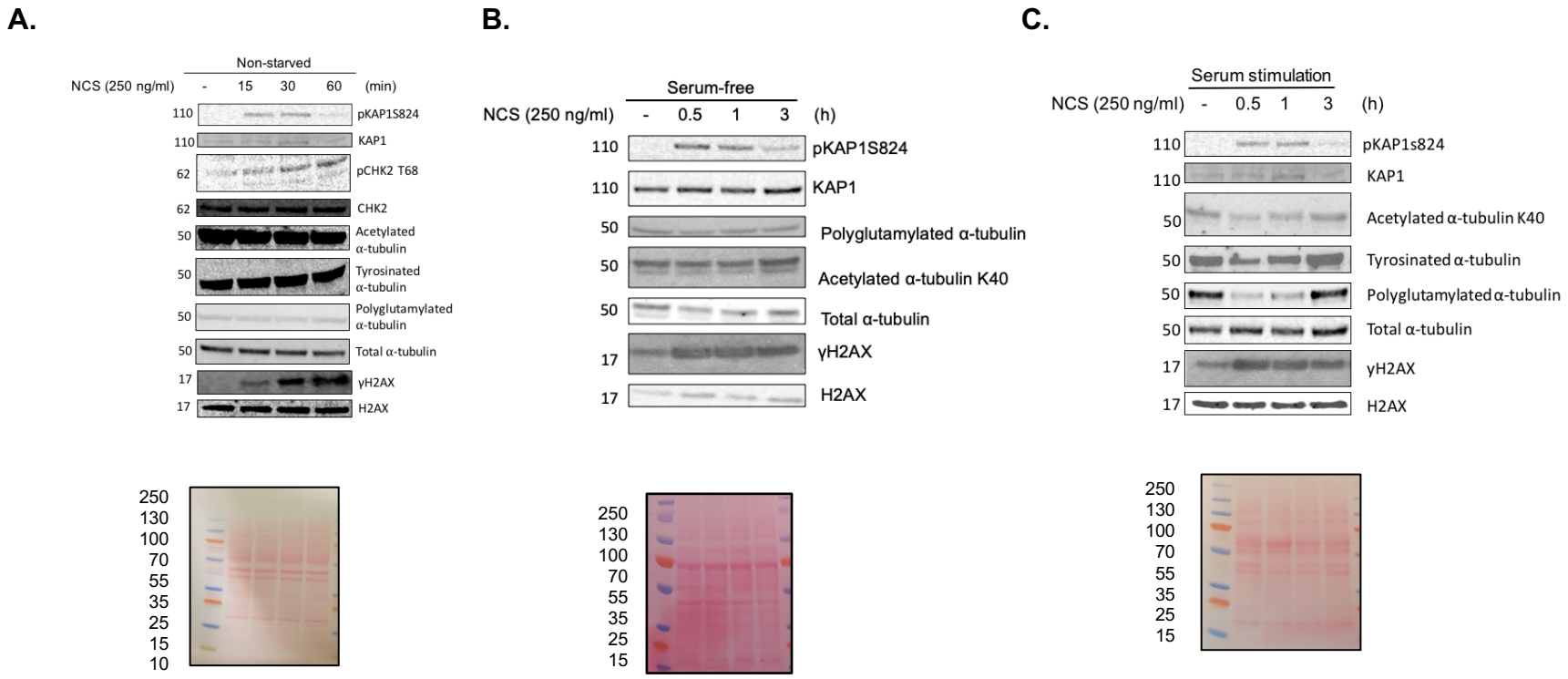
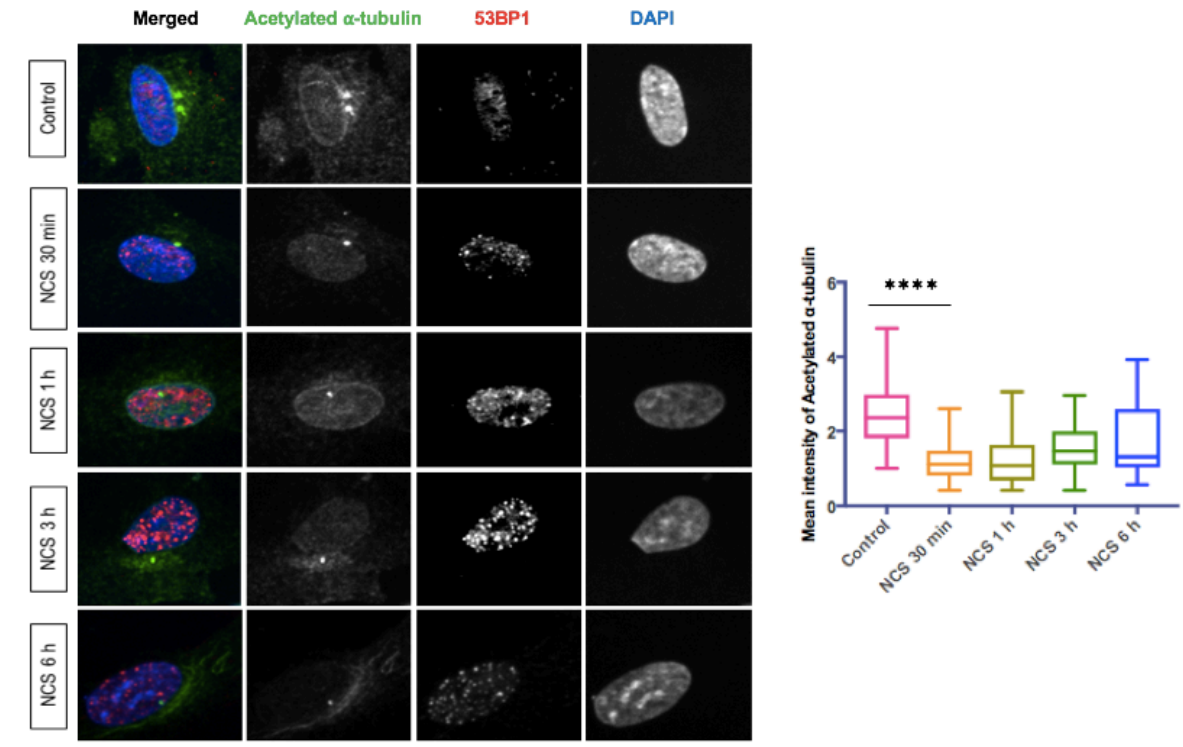


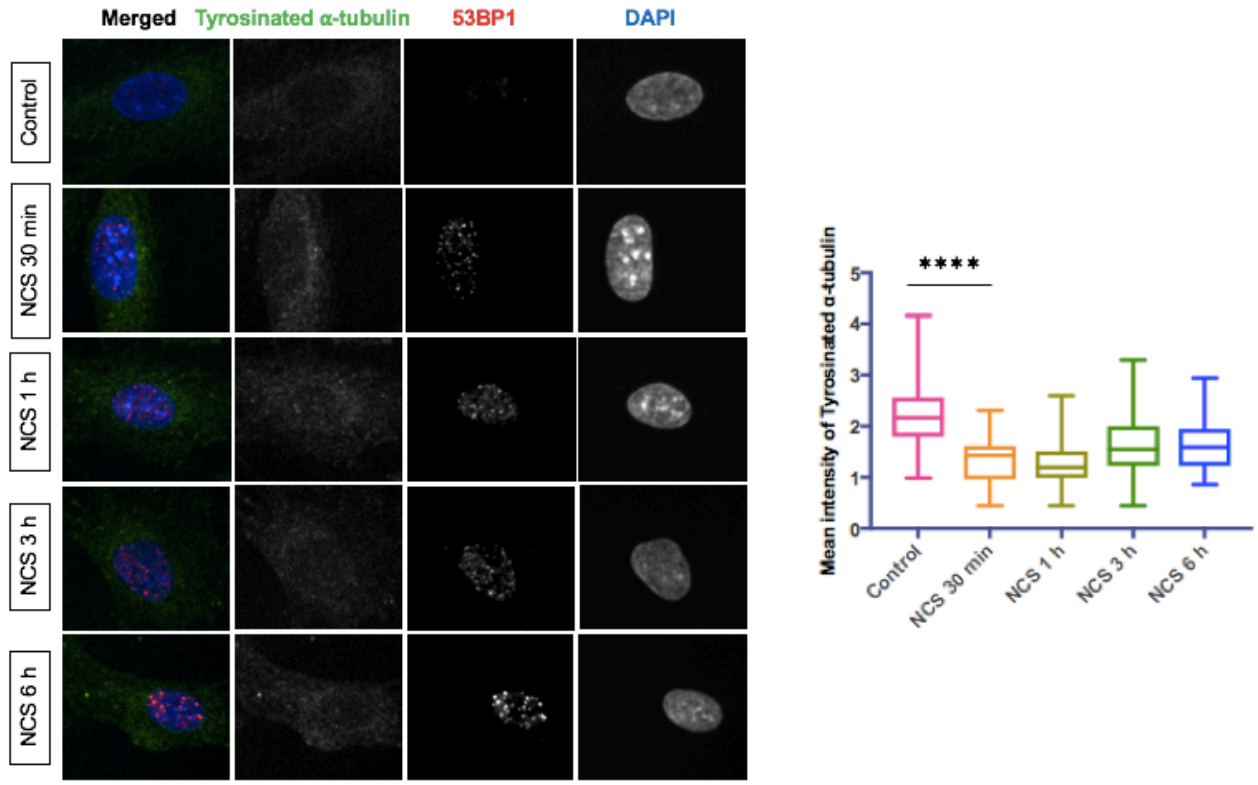
Figure 3.10. Observing the alterations in levels of posttranslational modifications of α-tubulin in response to DNA damage over time on RPE-1 cells using immunoblotting.

These representatives of three independent experiments blots were performed in different culturing conditions. The ponceau images below are additional loading controls. The ponceau blots below represent the blots above them. Number of technical replicates =3.

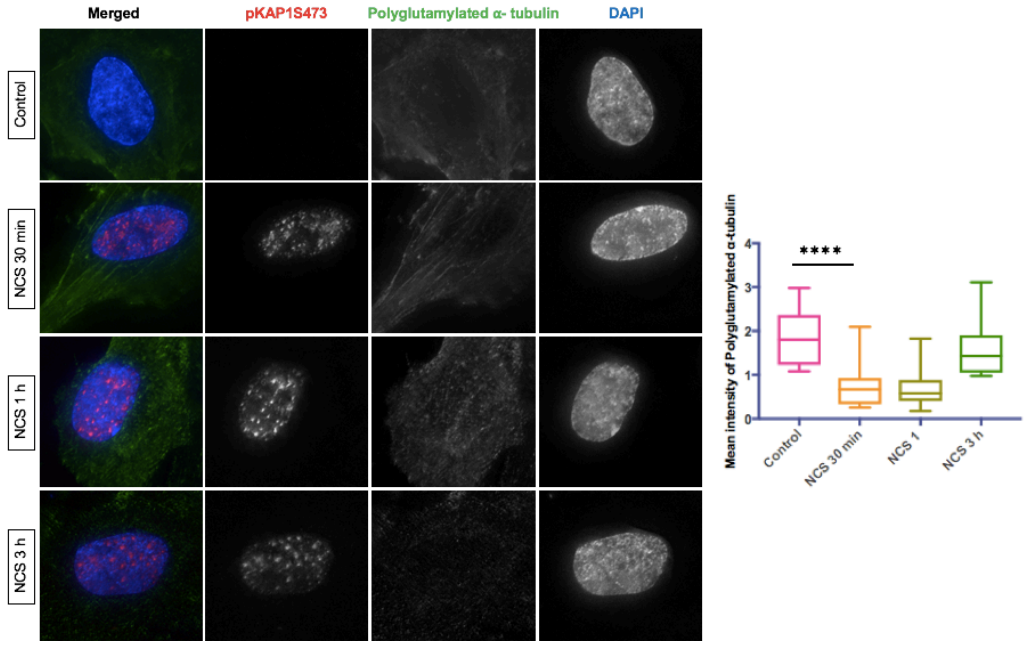
A.


Merged
Acetylated α-tubulin
53BP1
DAPI
Control
NCS 30 min
NCS 1 h
NCS 3 h
NCS 6 h
Mean intensity of Acetylated α-tubulin
****
0
2
4
6
Control
NCS 30 min
NCS 1 h
NCS 3 h
NCS 6 h

B.


Merged
Tyrosinated α-tubulin
53BP1
DAPI
Control
NCS 30 min
NCS 1 h
NCS 3 h
NCS 6 h
Mean intensity of Tyrosinated α-tubulin
****
0
1
2
3
4
5
Control
NCS 30 min
NCS 1 h
NCS 3 h
NCS 6 h

C.



D.

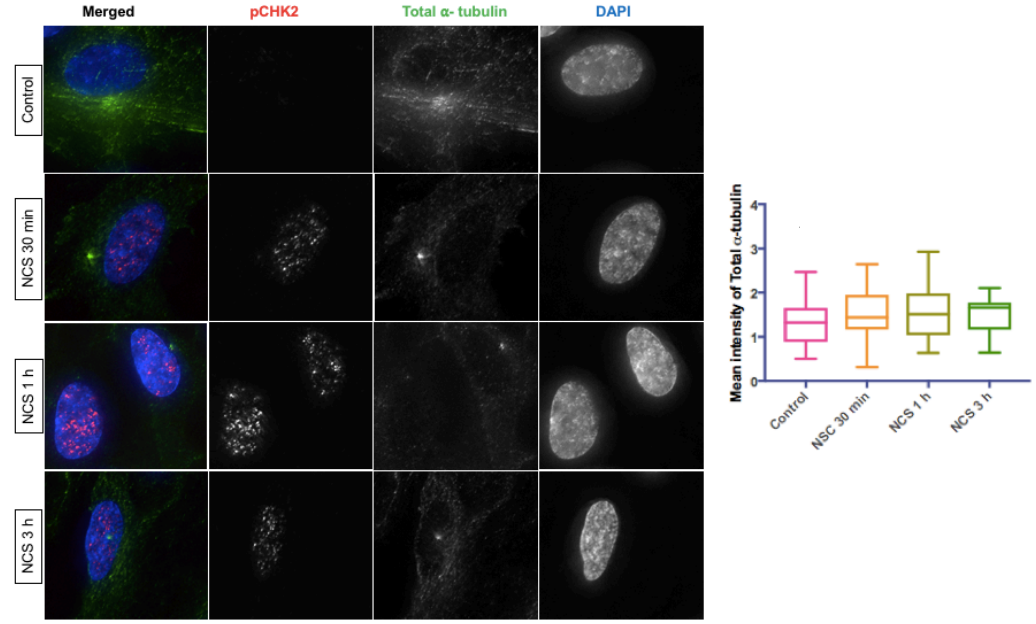


Figure 3.11. Analysis of posttranslational modifications of α-tubulin in response to DNA damage over time in re-stimulated RPE-1 cells using immunofluorescence.

Images **A.** and **B.** were taken with 40 X lens on Cell Voyager microscope, whereas images **C.** and **D.** were taken with 60 X lens on Delta Vision. All analysed with Cell Profilier.*** p< 0.0001. P value for total tubulin is 0.04177 in One-way ANOVA test. Number of technical replicates=3, total cell count= 274.

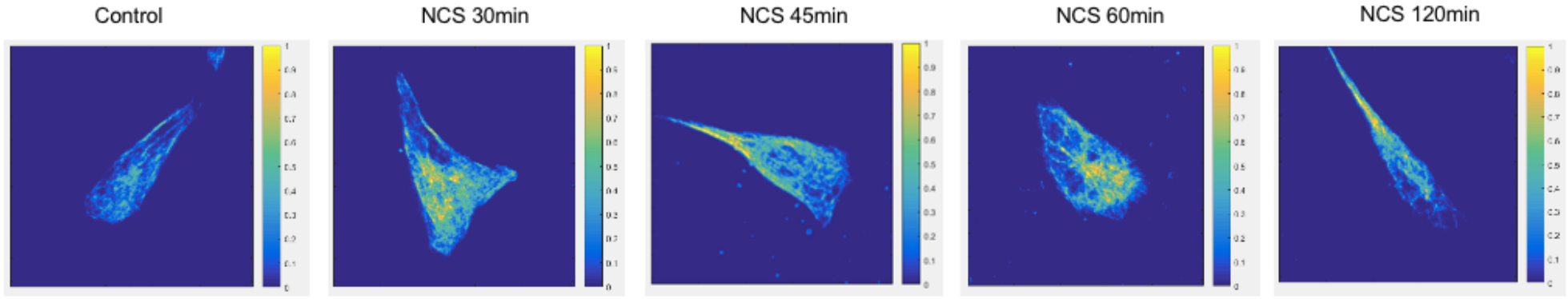


Figure 3.12. Assessment of microtubule movement in response to DNA damage over time.

60 X images taken from videos of microtubules stained with SIRTubulin. Yellow area of the legends of analysed with MatLab program videos represents an increase, whereas blue area of the legend a decrease in pixel intensity movement over time. Number of technical replicates = 4, total cell count= 65.

Discussion

Each day ∽10 000 DNA lesions occur in every human cell in response to drugs, toxins, metabolic events or oxidative stress. A number of mechanisms act to repair the damages. These include DNA damage response signalling (homologous recombination and non-homologous end joining) on the molecular level and when this fails, the immune cell response is triggered. Failure in maintaining genome integrity might lead to various diseases, such as cancers or neurodegenerative disorders. Although there is extensive research on how these DNA damage pathways are regulated, not much is known about how nuclear DNA damage affects structures in the cytoplasm.

Previous studies show that the knockout of the LINC complex components, SUN1 and SUN2, which normally decorate the nuclear envelope, decrease the mobility of telomeres. A similar effect was achieved when 53BP1 was removed. As these results are comparable with a treatment with a stabilising microtubule drug- taxol, hence, it was concluded that 53BP1 and SUN1/2 regulate microtubule movement (Lottersberger, *et al.,* 2015).

Since main characteristic of MTs are rapid polymerisation and depolymerisation, as a consequence, they are responsible for the movement of the telomeres and the whole cell as well as the transport of cargo within the cell. These dynamic events are associated with posttranslational modifications of their molecular unit- tubulin. The examples of PTMs encompass acetylation, polyglutamylation and tyrosination. Addition of acetylated or polyglutamylated groups to tubulin results in a decreased dynamicity of microtubules, whereas the addition of tyrosinated group to the tubulin dimer causes the increase in MT motion. Hence, the modifications are used as markers of the MT dynamic state. Nevertheless, PTMs are not a complete reflection of the MT mobility. For instance, nocodazole treatment that increases the dynamicity of microtubules, does not affect the expression level of acetylated α-tubulin. Thus, the connection between PTM and dynamics of microtubules remains to be further evaluated.

Due to an increasing number of evidence that MTs are affected by DDR signalling, experiments in this thesis aimed to confirm the assumption and also establish whether PTMs of α-tubulin and MT dynamics are altered in response to DNA damage. The

determination of the possible link of PTMs of tubulin and MT dynamics with the genome integrity would be a promising target for treatment of HD, AD, FAP, SBDS, cancers described in the chapter 1.

When cells were treated with three DNA damaging agents: NCS or phleomycin, or etoposide, no changes in the expression levels of PTMs of α-tubulin were observed using western blot (Figure 3.7), however, there was a decrease in polyglutamylated α-tubulin in response to NCS and phleomycin, but not in response to etoposide (Figure 3.8.). Phleomycin and etoposide change the appearance of polyglutamylated α-tubulin compared to the control that might be related to their mechanisms of actions. The common feature of phleomycin and NCS is working through the oxidation, hence, the significant changes in the expression levels of polyglutamylated α-tubulin might be influenced by this mechanism.

Furthermore, the immunoblotting (Figure 3.2.), immunofluorescence (Figure 3.3. and Figure 3.4.), and IncuCyte (Figure 3.5.) data indicated NCS as the most powerful drug. Thus, it was chosen for a time course experiment, which allowed to observe alterations in the expression levels of PTMs of α-tubulin over time.

The immunoblotting (Figure 3.7.) and immunofluorescence (Figure 3.8.) results of the NCS time course on serum re-stimulated RPE-1 cells showed that in response to DNA damage, there is a significant decrease in the expression levels of acetylated, polyglutamylated and surprisingly also in tyrosinated α-tubulin. Interestingly, in response to DNA damage, the acetylated α-tubulin was detected in a form of cilia-like structures (Figure 3.11.A.). Wang and Brautigan (2008) showed the correlation of cilia with the acetylated α-tubulin. However, the exact link remains elusive. In turn, the cilia formation has been previously proved to have roles in cell signalling, for example in the Wnt signalling that is involved in cancer (Wallingford and Mitchell, 2011). Further evaluation of that link could be achieved by probing for proteins involved in cilia formation and possibly Wnt players. Surprisingly, the changes in PTMs of α-tubulin were not observed in the nonsynchronised population of RPE-1 cells, implying that they rely on the cell cycle phase and might be associated with NHEJ pathway. To further establish the link with the NHEJ pathway, the proteins involved in the NHEJ pathway (described in the chapter 1) should be tested using WB and IF techniques. The reduction in the acetylated and polyglutamylated α-tubulin could suggest increase in MT motion, whereas the reduction in tyrosinated α-tubulin implies decrease in MT motion. This contradicted result could be explained by not complete understanding of

the link between PTMs and dynamicity of MTs. In particular, microtubule-associated proteins, which are thought to interplay with posttranslational modifications are directly associated with the regulation of MT dynamicity, could play role in unexplained outcome of the expression level of tyrosinated α-tubulin. In order to deeper understand this outcome, TTL siRNA could be performed as well as WB probed with TTL and MAPs proteins to determine the key player in the observed alterations.

Another way of testing the effect of DNA damage on MTs dynamics is a MT motion assay. The MT motion assay analysis indicated increased MT dynamics in response to DNA damage (Figure 3.12). The most prominent MT dynamics was noted at 30 minute time point, indicating that the observations of the changes in the PTMs of α-tubulin could be related to the increased MT dynamics. In order to confirm that correlation, firstly, it needs to be established whether the changes are due to the enzymes or MAPs and then the cause could be transfected with the siRNA of that protein. Then, the experimental procedure should be conducted as described above. The changes in response to siRNA transfection followed by DNA damage would determine the source of the potential correlation.

In relation to the MT motion assay, it is important to mention that although the assay was performed four times with the similar results and the sirTubulin is a relatively accurate dye, the software has limitations.

First of all, it is only able to present the intensity of the pixel movement without the units. This feature allows to compare the movement between the conditions when there is a significant difference, but it prevents from comparing the dynamicity in the quantitively way. Moreover, without the units it is impossible to compare the results with other studies that use other assays. Also, lack of units means lower accuracy. The analysis merely shows the accumulation of the moving pixels and the intensity of the movement. This element should the further improved.

Another feature of the programme is determination of the direction of the movement. That property was not used due to low accuracy, which misled programme and as a result it pointed various directions of microtubule movement, wherein no pattern was observed.

Another useful feature would be a formation of three-dimensional recordings. So far, the software allows to create two-dimensional videos from the middle of the sample. Although, this data is useful for general comparisons, more accurate data could be collected when three-dimensional features are added.

Importantly, even though the microtubule motion assay misses many properties, it was still able to address the research question about the changes in microtubule dynamics. Addition of the listed above properties would make the programme an incredible structural tool for molecular biologists.

Altogether, the results confirmed that DNA damage affects acetylation and polyglutamylation of α-tubulin, which potentially influence MT dynamics.
The study also revealed the possible importance of acetylated and polyglutamylated α-tubulin expression levels on the DDR signalling. The decrease of expression level of tyrosinated α-tubulin should be further studied in order to discover the role of this alteration in the DDR pathway. Also, it would be interesting to determine the direction of microtubule depolymerisation in response to DNA damage. This information could illustrate the detailed DDR pathway with these cytoplasmic structures and provide clues for discovering therapies against related to DNA damage diseases.

Chapter 4: Results and discussion

The effect of alterations of microtubules on DNA damage response signalling.

4.1. Microtubules as a drug testing platform.

In 1986, Tim Mitchison and Marc Kirschner suggested the role of catastrophe and rescue events of microtubules in a determination of cell shape. When a plus end of microtubules is bound to or 'captured' by kinetochores, it loses its ability to grow. Mitchison and Kirschner called this hypothesis 'search and capture' model. Years of research confirmed that assumption and extended the idea to complexes capturing the MTs, such as formins or EB1. Since then, the importance of MT motion has been intensively research and it was established that dynamic instability of microtubules is tightly regulated through direct interactions with microtubule-associated proteins and indirectly though PTMs of tubulin. This is an emerging field, and much remains to be discovered, but disruption of MT dynamics has been linked with a number of diseases, such as Alzheimer's disease (Lu, *et al.,* 2014) or Huntington's disease (Hoffner, Kahlem and Djian, 2002). It appears from several recent studies that perturbation to the balance within the cell between catastrophe and rescue can give rise to dysfunction and therefore disease (Stumpff, *et al.,* 2014; Godena *et al*., 2014; Marchisella, Coffey and Hollos, 2016)

Because of this link between MT dynamic changes and disease, the study of drugs that affect MTs is important, since it could lead to treatments for, or a greater understanding of these diseases. There are a few groups of drugs known to alter MT dynamicity, and these also aid our understanding of this broad topic. The spindle poisons include taxanes (taxol, docetaxel) known as stabilisers, vincas (rotenone, vinblastine, nocodazole) also called destabilisers and demecolcine (Stanton *et al.,* 2011; Amos, 2011). Studies have shown that rotenone interferes with cellular respiration (Garmier, *et al.,* 2008). Hence, it was rejected for use in this study. Also, demecolcine was discarded due to its ability to cause DNA fragmentation (Yamamoto, *et al.,* 2014), which could interfere with the results in physiological conditions. Similarly, vinblastine was rejected as in higher concentrations it promotes the formation of aggregates (Brito, *et al.,* 2008).

Taxol was discovered in the early 1960's by Wall and Wani, who purified the taxol sample from a bark of the Pacific Yew tree and described as the drug curing ovarian cancer (Wall and Wani, 1995). The first mechanism of action of the drug was published in 1979 and shed light on the correlation between taxol and MTs (Schiff, Fant and Horwitz, 1979). In this study, taxol and tubulin were purified by chromatography, which

was followed by the kinetic assay to establish the polymerisation state and rate of tubulin. The results were further validated using electron microscopy. The findings showed that taxol resulted in increase in the polymerisation rate of tubulin leading to enhanced nucleation and elongation of MTs independent on tubulin concentration or temperature. In support with this study, Nogales and co-workers (1995) discovered a structure of taxol binding site on both alpha and beta tubulin molecules. Further research revealed that taxol affects MTs also regardless of a source of energy or motor proteins (Stanton, *et al*., 2011). Moreover, the more stabilised MTs are, the less dynamic they become. Another effect of taxol is arresting cells in mitosis by activation of the mitotic checkpoint that control the chromosome segregation (Weaver, *et al.,* 2014). From 1992 taxol has been approved for the ovarian cancer treatment by the Food and Drug Administration of the United States. Nowadays, taxol is used clinically to treat Kaposi's sarcoma or breast cancer and in research mostly in relation to MT dynamics.

Nocodazole is a type of destabiliser that binds to tubulin heterodimers and prevents from the formation of disulphide linkages between tubulin chains. While more bonds between tubulin dimers break, the dynamics of MT decreases in smaller concentrations and it promotes disassembly in higher concentrations (Xu, Schwarz and Luduena, 2002). Hence, it was used at the appropriate concentration to be a destabiliser for this research. Nocodazole, similarly to taxol, arrests cells in the G2/mitosis by activating the mitotic checkpoint (Blajeski, *et al.,* 2002). Nowadays, nocodazole is not used clinically.

For the research presented in this thesis taxol and nocodazole were used in order to alter MT dynamicity in opposite ways.

4.2. Assessment of the microtubule poison drugs.

Initially, the activity of the drugs provided for this research was tested. Inactive samples of the drugs would not perturb MT dynamics, but could disrupt the experiments.

Secondly, the effects of the drugs were tested on synchronised in G1 cells. The synchronisation allowed to observe cell cycle-dependent changes as well as limited factors that could result in seen alterations e.g. transcription factors. It is known that both taxol and nocodazole affect cell cycle progression (Weaver, *et al.,* 2014; Blajeski,

et al., 2002). Thus, the initial experiments were conducted to assess the activity and the cell cycle progression using the MT poisons. Once the drugs perform their primary actions and do not affect the cell cycle progression, the key question being addressed here was whether these microtubule poisons could alter the DNA repair pathways.
Next, the long-term effects of the drugs on cell morphology and survival were tested.
Finally, as this research aimed to find the relationship between MT dynamics and DNA damage, the impacts of MT perturbation drugs themselves on DNA damage were assessed.

4.2.1. Confirmation of the function of taxol and nocodazole

The aim was to test whether taxol and nocodazole highly stabilise and destabilise microtubules, respectively. To test this, the same MT motion assay was performed in synchronised in G1 live cells as previously described (Chapter 3). The MTs were stained with sirTubulin dye and the efflux pumps were inhibited using verapramil.
In response to taxol, microtubules became more dense and their dynamicity decreased. That effect increased after 7 hours of treatment (Figure 4.1.). Hence, it can be confirmed that taxol enhanced polymerisation of tubulins in the cells used in this study.
In the presence of nocodazole, the dynamics of MTs increased initially and at a 7 hour time point decreased (Figure 4.2.). That effect could be explained by the initial reduction in motion as a result of depolymerisation of MTs due to decreased amount of tubulin dimers being available. As the assay shows, MTs dynamics decreased after 7 hours of treatment compared to 1.5 hour of treatment, in support of this hypothesis. These results demonstrate that both taxol and nocodazole conferred their expected effects in RPE-1 cells, and can therefore be used in MT dynamics experiments.

4.2.2. The impact of microtubule poisons on synchronised RPE-1 cells

Early studies have shown that even as low doses of taxol such as 0.1 or 1 μM arrest cells in G2/M phases after 6 hours of treatment (Shu, *et al*.,1997).

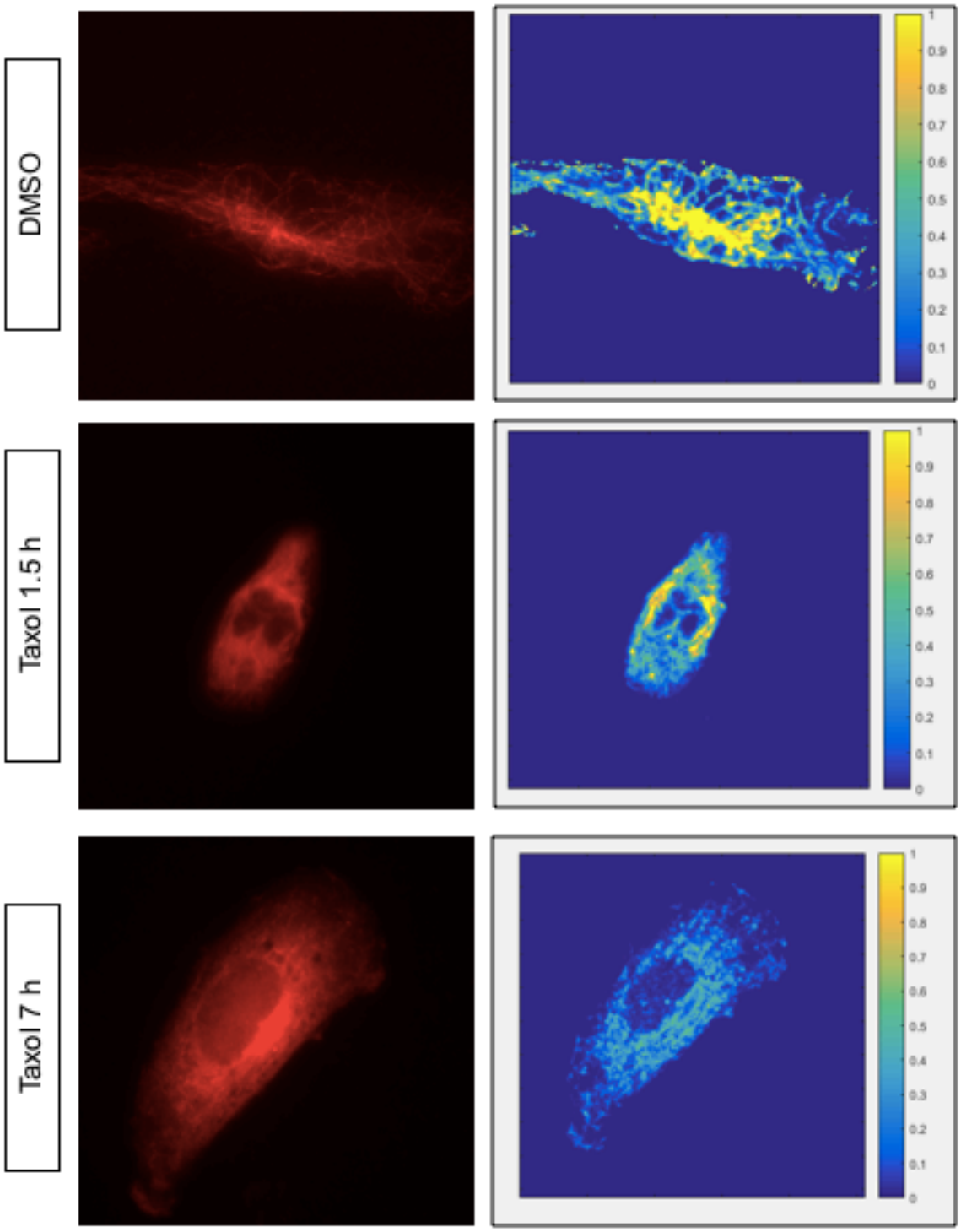


Figure 4.1. Validation of taxol's effects on microtubule dynamics.

Followed by sirTubulin staining of MT, the live-cell images were taken with the DeltaVision widefield microscope with 60 X oil lens at 594 nm wavelength. DMSO was used as a control. The cells remained in the 50 nM Taxol drug for 7 hours. Representative images shown from three replicates, total cell count= 54.

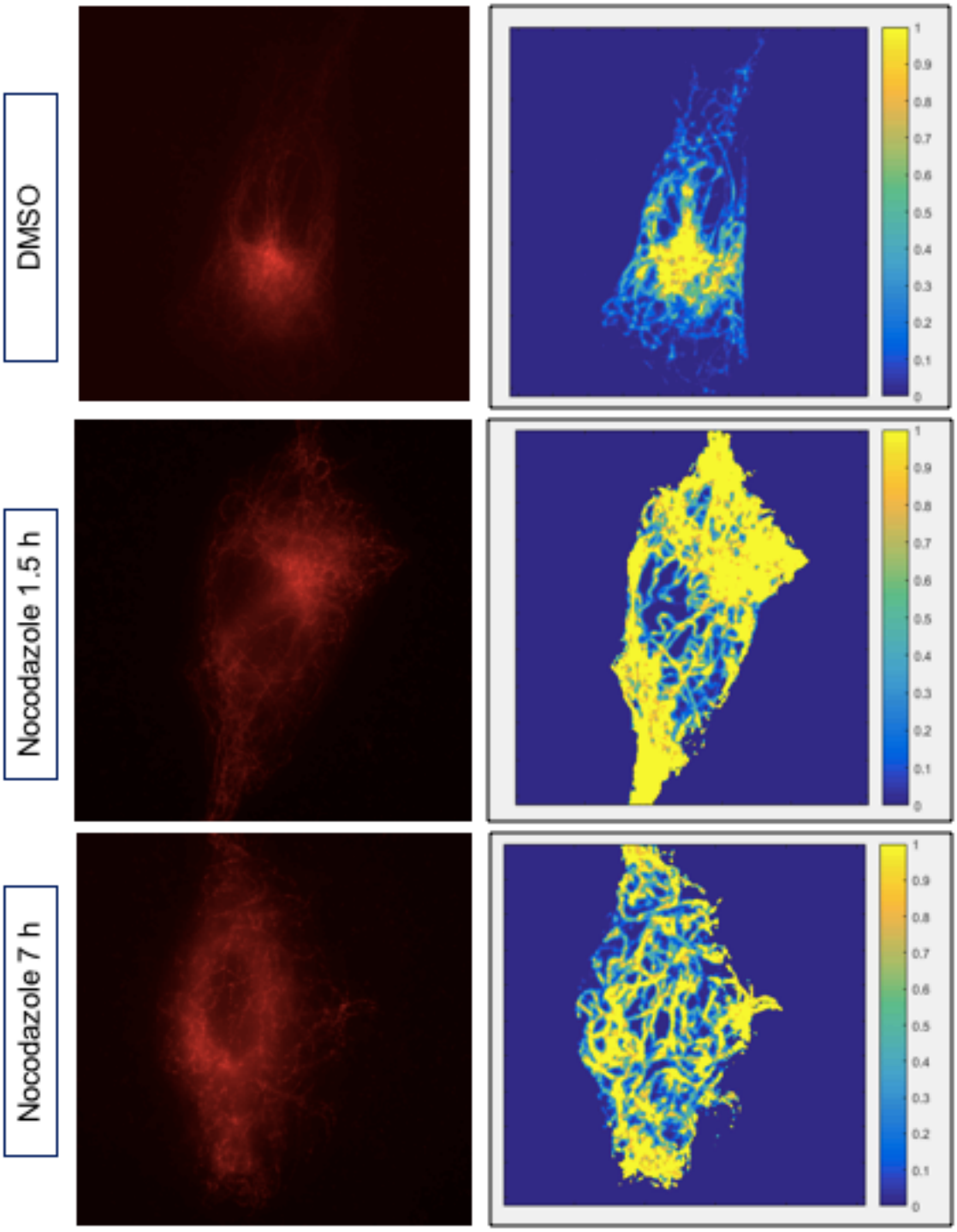


Figure 4.2. Assessment of nocodazole effect on microtubule dynamics.

MTs were stained with sirTubulin and imaged with the DeltaVision widefield microscope with 60 X oil lens at 594 nm wavelength. These live cells remained in the 0.1 ng/ml nocodazole drug for 7 hours. Representative images shown from three replicates, total cell count= 54.

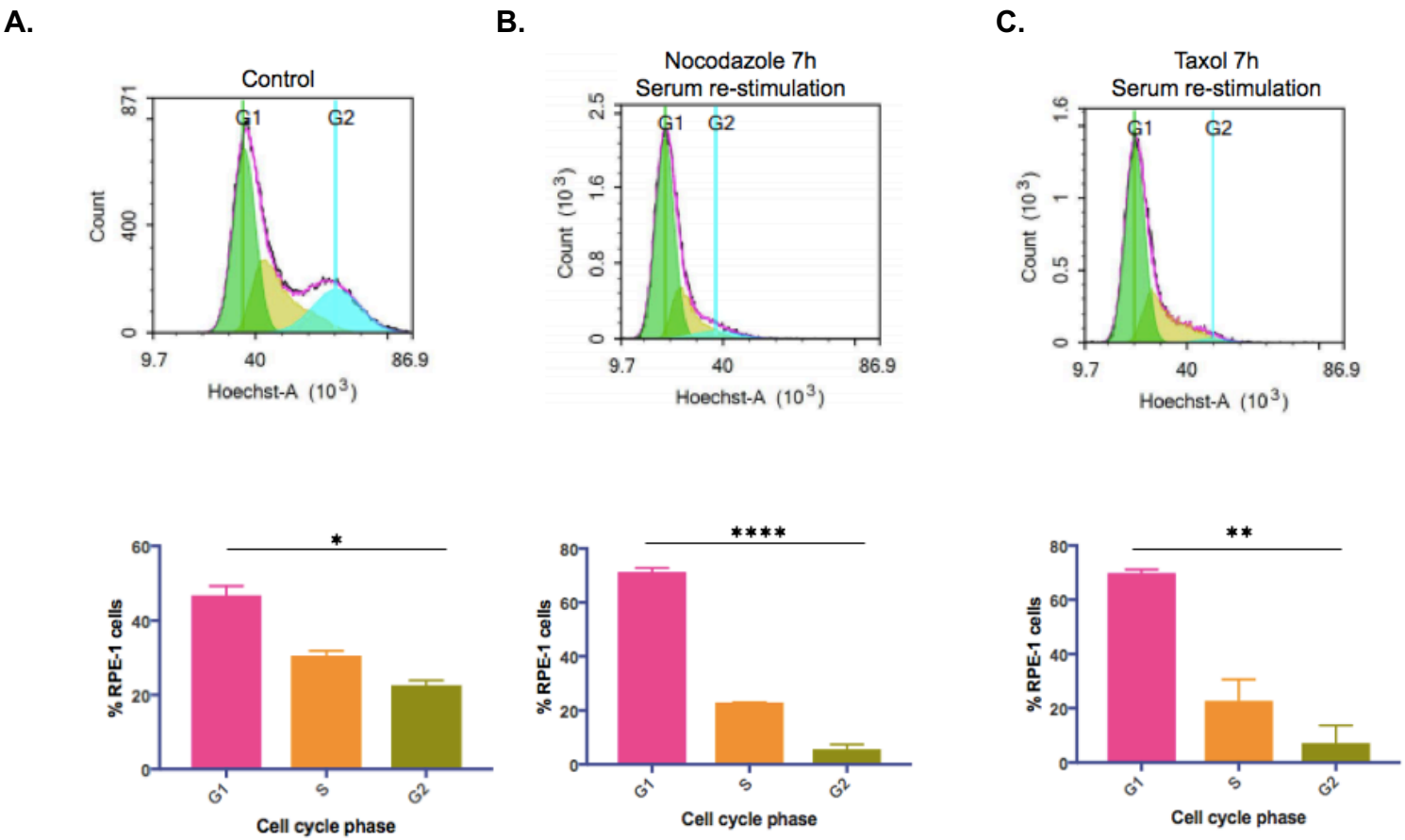


Figure 4.3. Validation of taxol and nocodazole drug effects on cell cycle phases after re-stimulation with 10 % serum.

A. Non-treated cells *p<0.0153 **B.** cells treated with nocodazole (0.1 ng.ml) ****p<0.0001 **C.** taxol (50 nM) **p= 0.0039 remained in the cell culture for 7 hours and studied with flow cytometry machine. The data was analysed using one way ANOVA in the GraphPad Prism software. Number of technical repeats=4.

As G1 phase was the primary interest in this research, it was checked whether 7 hours after the use of taxol in population of cells already synchronised in G1 would facilitate progression to other cell cycle phases, which could alter the DNA repair pathway from non-homologous end joining to homologous recombination.

To test this, flow cytometry was performed on untreated cells or those treated with the MT poison drugs after 7 hours of serum re-stimulation to test the stage of the cell cycle.

The analysis shows that in the absence of MT poison, the average percentage of cells in three repeats of re-stimulated cells was: G1, was 46.77 %, S phase 30.5 % and G2 phase, 22.64 %. In the presence of either poison, around 70 % of the cells were in G1 and 23 % were in S phase after re-stimulation with 10 % serum and treatments with either taxol or nocodazole. Also, the amount of the S phase cells decreased to roughly 23 % (Figure 4.3.).

Overall, the main conclusion from these results is that for the time of performing the experiments taxol and nocodazole both significantly reduced cell cycle progression in re-stimulated cells.

4.2.3. The effect of microtubule poisons on cell morphology and survival

Another aspect of the drugs tested was their long-term effect on morphology and cell survival. Hence, a 68-hour IncuCyte experiments were performed using non-synchronised RPE-1 cells (Figure 4.4.).

In response to both taxol and nocodazole, most cells rounded up, which might be explained by blocking the cells in mitosis that is consistent with the mechanism of action of the drugs but also rounding up of the cells is a feature of cell death. To measure the cell death rate in the IncuCyte, the cells could be stained with the IncuCyte Caspase-3/7 Green reagent. Alternatively, the flow cytometry assay could be performed to assess the cell death rate.

A few days after administering taxol or nocodazole and then NCS, the non-synchronous RPE-1 cells not only sustained this rounded form, but also changed the morphology of some cells, what might suggest that the cells were arrested in mitosis rather than became apoptotic. However, this should be further confirmed. Moreover, the assessment of the cell morphology in taxol+ NCS and nocodazole+ NCS images implies that a significant number of the cells do not progress to mitosis (Figure 4.4.).

Consistent with these observations, the number of cells significantly decreased compared to controls (Figure 4.5. A.). In order to determine whether these results are the actual representative of the altered morphology, the IncuCute Nuclear Rapid Red dye was added to the cells and software was trained by adjusting the detection limit ranges to accurately detect nuclei. The analysis demonstrated a significant reduction in cell divisions in the presence of all tested drugs compared to controls (Figure 4.5. B.).

Together, these data demonstrate that both taxol and nocodazole significantly decreased the number of cell divisions, which might be a result of either cell cycle arrest in mitosis or apoptosis.

4.2.4. The effect of microtubule poisons on DNA damage.

DNA damage can be caused by various drugs. Hence, a number of experiments were conducted to determine whether taxol and nocodazole themselves could lead to DNA lesions. In case of their deleterious effect, the drugs would need to be changed given the aims of this study.

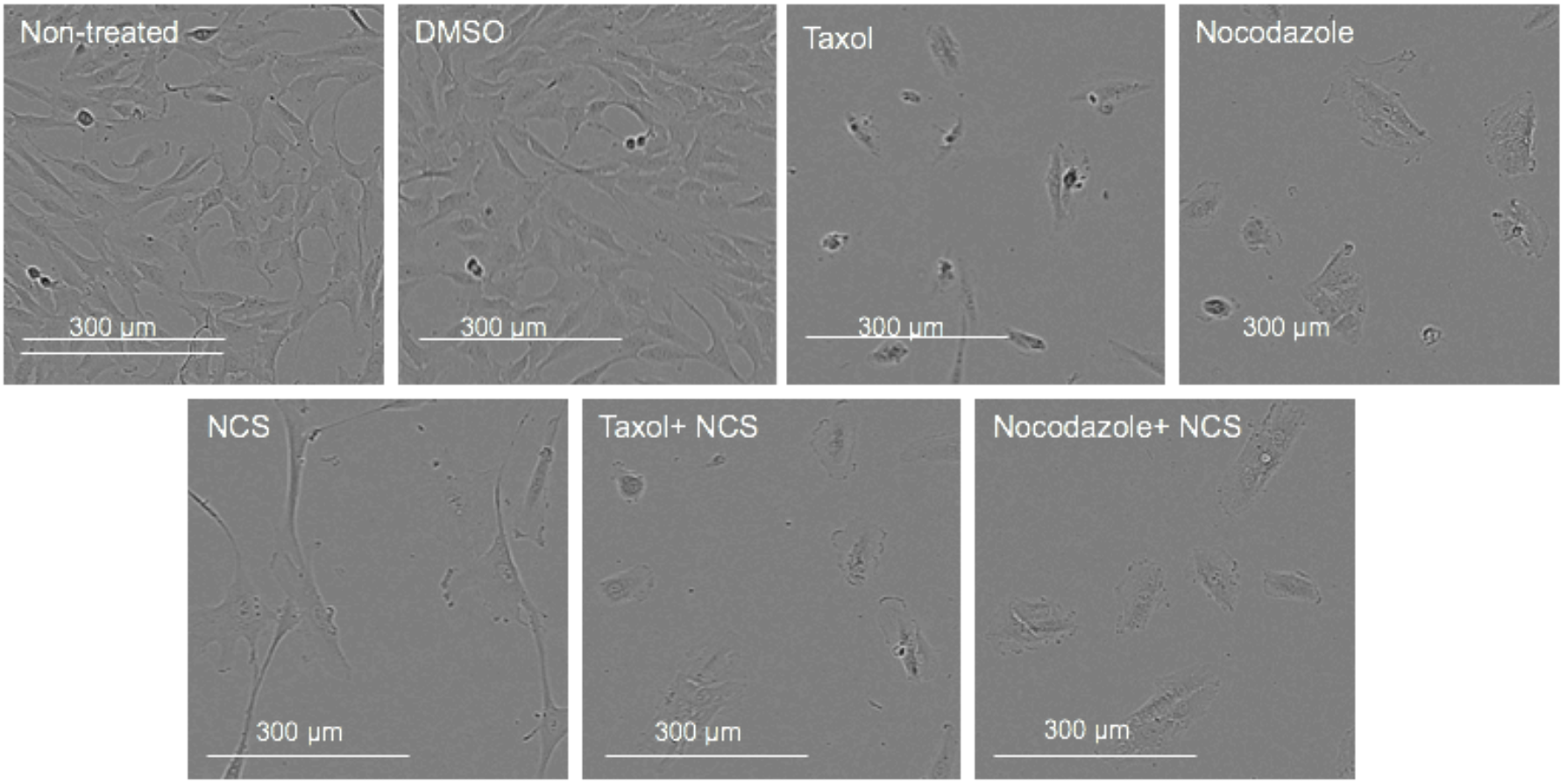
IF results show no γH2AX foci after 7 hours in the presence of taxol compared to control (Figure 4.6. A.). Also, the analysis of a number of γH2AX foci suggests that taxol did not cause damage to DNA. Seven-hour time point was considered as this is the last time point of the core experiments.

In the presence of nocodazole, Figure 4.7. A. DNA remains undamaged, and the number of γH2AX foci are comparable to non-treated cells.

These results prove that taxol and nocodazole do not lead to DNA damage and can be used in the experiments testing the links between dynamics of microtubules and DNA damage.

All tested characteristics prove that both taxol and nocodazole are appropriate examples of stabilisers and destabilisers, respectively. Thus, they were used in the core experiments aiming to establish whether perturbation of microtubules affects DNA damage signalling.

A.


Non-treated
300 µm
DMSO
300 µm
Taxol
300 µm
Nocodazole
300 µm
NCS
300 µm
Taxol+ NCS
300 µm
Nocodazole+ NCS
300 µm

B.

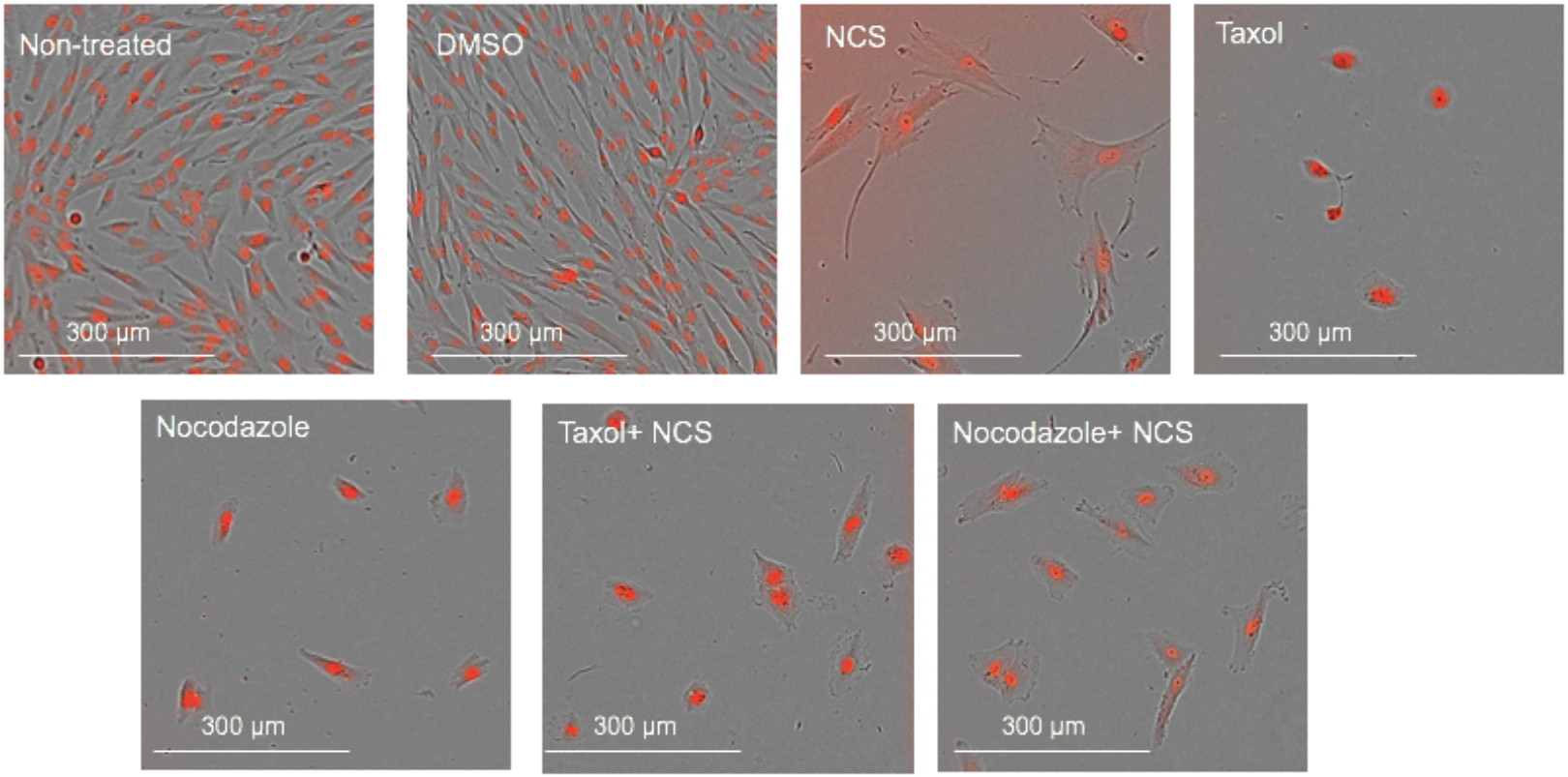
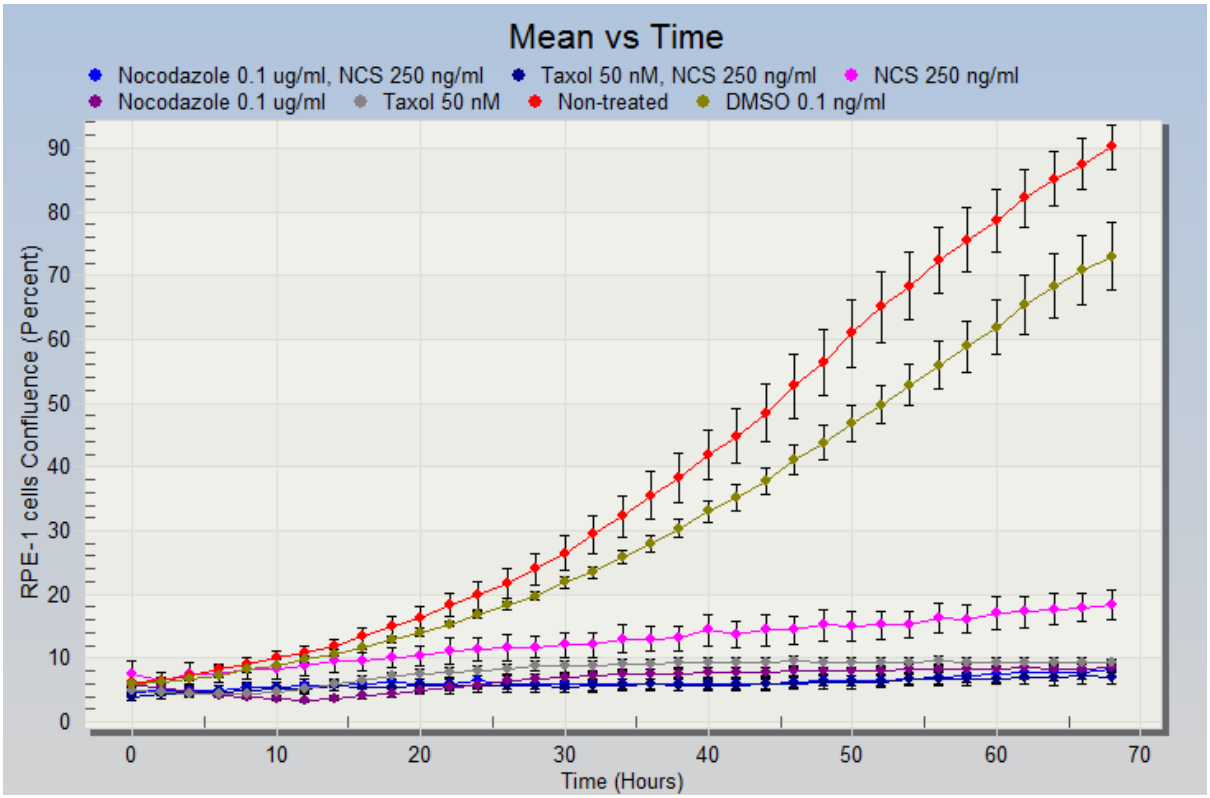


Figure 4.4. Assessment of a long term effect of the drugs on cell survival and morphology.

The 10 x images were taken at 68 hour time point with the IncuCyte®. DMSO was used as a control as taxol and nocodazole were dissolved in DMSO. Drugs were used in the well-established concentrations: NCS 250 ng/ml, Taxol 50nM, Nocodazole 0.1ng/ml. That experiment was conducted four times.

A.



B.

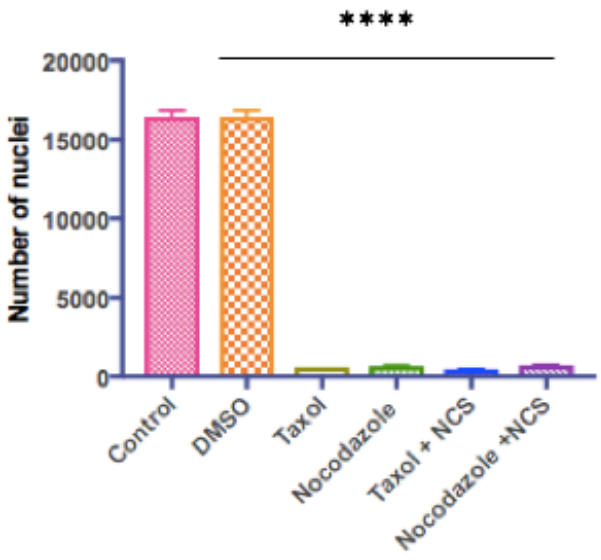
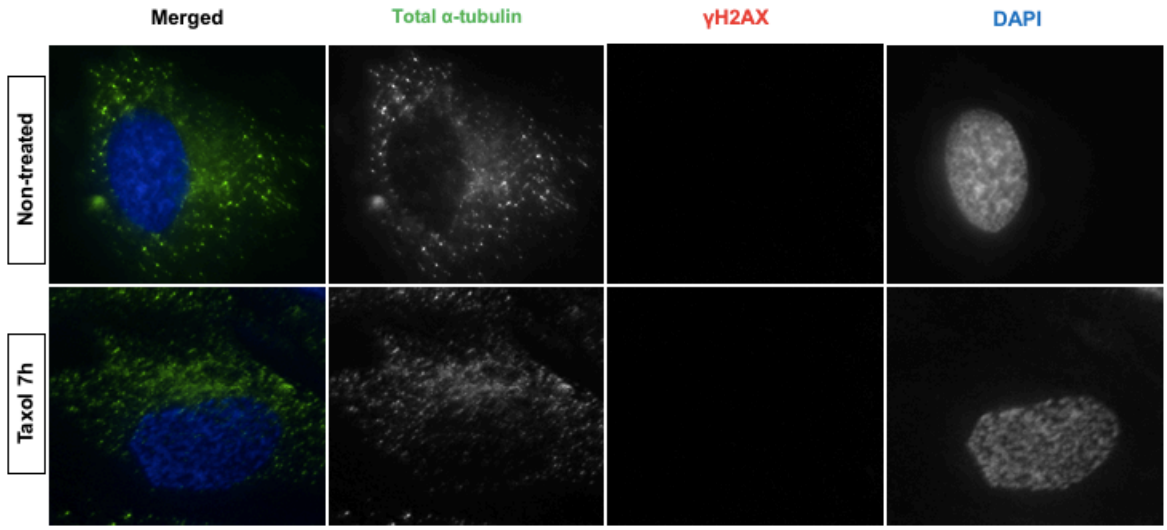


Figure 4.5. Long-term effect of microtubule poison drugs on cell survival.

A. Cell survival assay analysis shows a significantly decreased percentage of cell confluency in response to all drugs tested. **B.** Alike, the number of nuclei is significantly lower in treated with drugs cells. ****p<0.0001, One-way ANOVA, number of technical repeats=3.

A.



B.

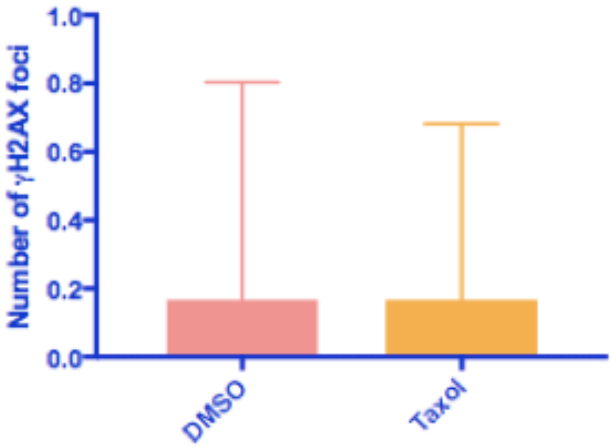
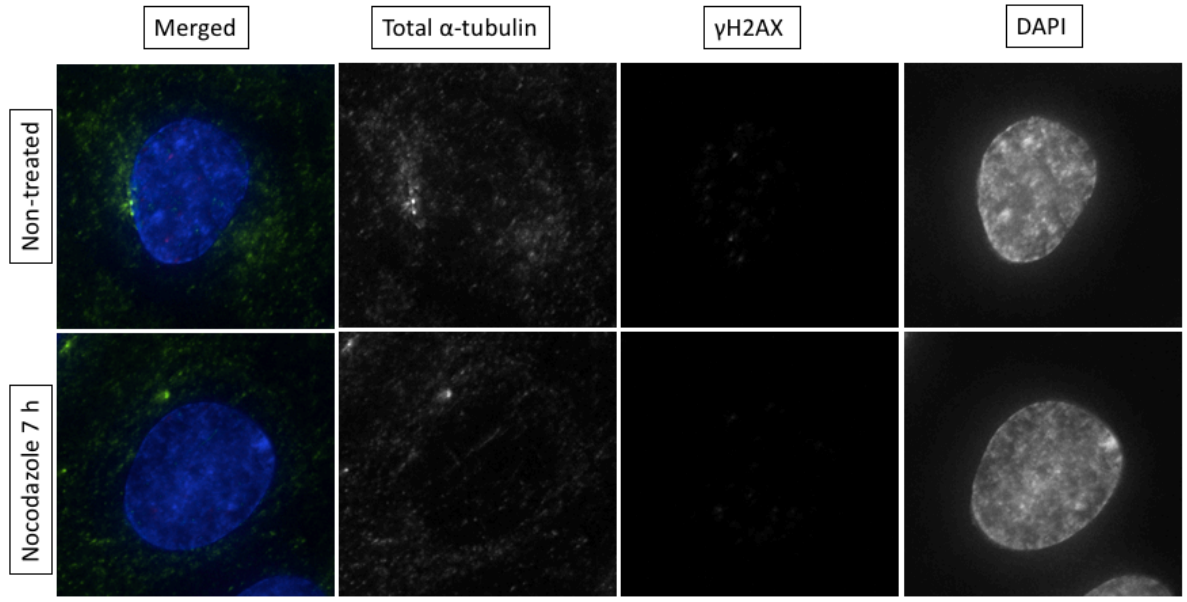


Figure 4.6. Validation of the influence of taxol on microtubules and DNA damage.

Set of 60 X images were taken with DeltaVision widefield microscope **A.** The analysis with CellProfilier and then GraphPad shows no significant difference between DMSO (control) and Taxol (50nM) on γH2AX **B.** MTs were highly stabilised using 50 nM taxol and then the cells were damaged using 250 ng/ml NCS. P>0.9999, t test, number of technical repeats=3, total cell count= 103.

A.



B.

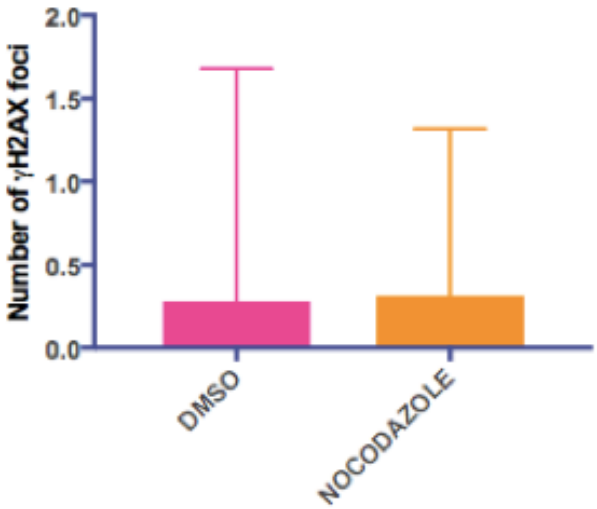


Figure 4.7. Confirmation of the impact of the nocodazole on DNA damage.

Set of 60 X images were taken with the DeltaVision widefield microscope **A.** The analysis with CellProfilier and then GraphPad shows no significant difference between DMSO (control) and Nocodazole on γH2AX **B.** MTs were destabilised using 0.1 ng/ml nocodazole and then cells were damaged using 250 ng/ml NCS. P=0.9252, t test, number of technical repeats=3, total cell count= 132.

4.3. The impact of increased stabilisation of microtubules on DNA damage.

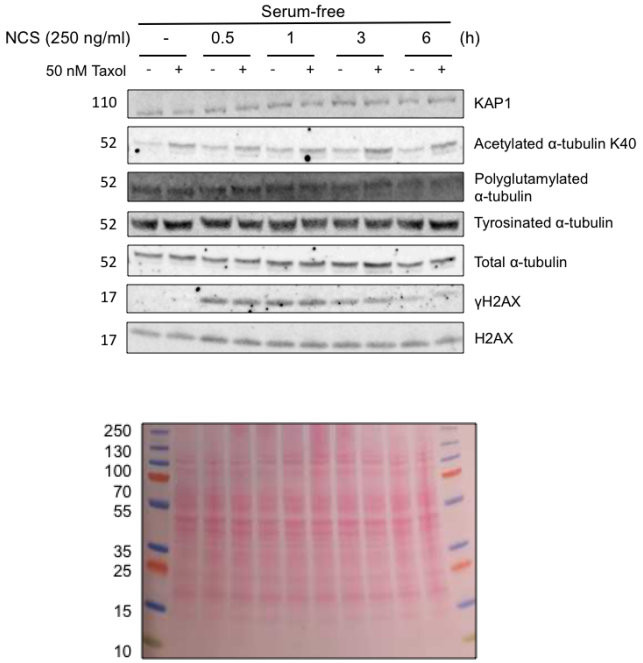
After confirming that taxol and nocodazole are a stabiliser and a destabiliser, respectively, of MT dynamics, and work in an expected way, without damaging DNA allowed their use in experiments to test the link between MT dynamics and DNA damage. Since it has been shown that DNA damage increases the dynamicity of microtubules (Lawrimore, *et al.,* 2017), the first aim was to test whether perturbation of MTs results in the changes in DNA damage response. The answer to that question might reveal the importance of microtubule function in the DNA damage signalling.

To investigate this possibility, initially, microtubules were over-stabilised using taxol and then NCS as the most effective DNA damaging agent was added and samples were collected over a time course. The experiments were performed in three conditions with an asynchronous population of RPE-1 cells (serum treated, no starvation), with cells synchronised and left in serum-free media for the whole time of the experiments (starved, serum free), and with synchronised cells, starved for 48 hours and re-stimulated with 10 % serum (starved, serum stimulated).

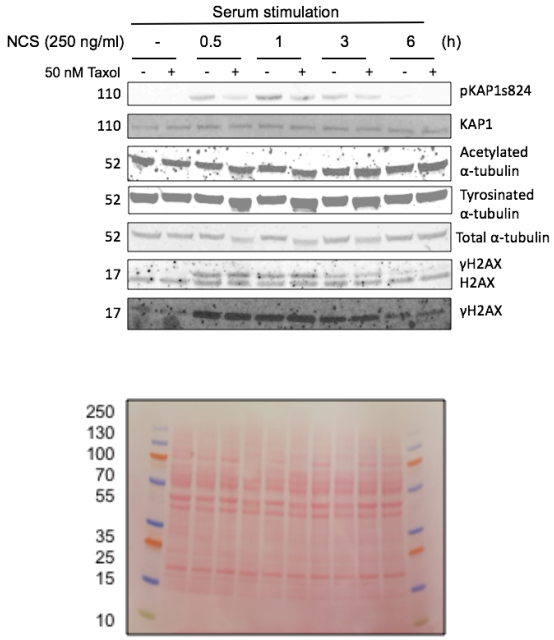
Western blot results showed that starved, serum stimulated cells, there is an increase in the DNA damage marker γH2AX compared to control in the presence of taxol, when NCS had been added to induce DNA damage (Figure 4.8.A.). The phosphorylation of KAP1 protein on serine 824 decreased following DNA damage with NCS, after over-stabilisation of microtubules with taxol in these re-stimulated cells. This modification is known to aid in chromatin state change, such as opening of DNA to allow entry of DNA repair machinery so that it can enter and repair the lesions. This finding was observed only in re-stimulated cell. Worth noting, there were no significant changes seen in KAP1 phosphorylation or γH2AX amount following taxol treatment when analysing cells grown in the other two conditions (Figure 4.8.A.).

In an attempt to confirm these western blot results, immunofluorescence was performed using serum re-stimulated RPE-1 cells treated with NCS to damage DNA, then taxol or nocodazole to over-stabilise or destabilise microtubules. As Figure 4.9. illustrates the level of 53BP1 in over-stabilised, destabilised and control cells.

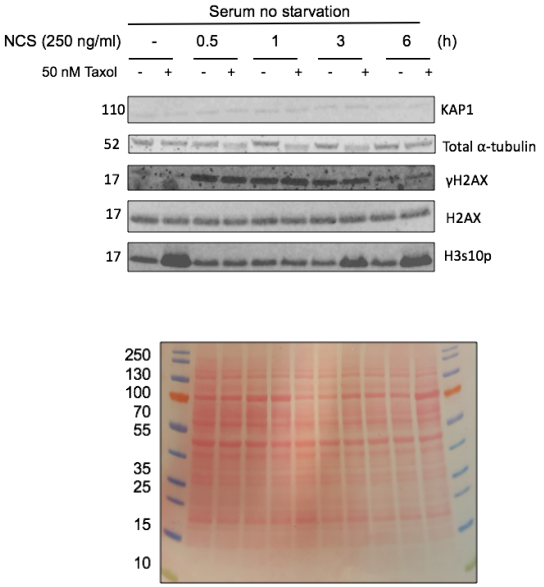
A.1.


Serum-free
NCS (250 ng/ml) - 0.5 1 3 6 (h)
50 nM Taxol - + - + - + - + - +
110 KAP1
52 Acetylated α-tubulin K40
52 Polyglutamylated α-tubulin
52 Tyrosinated α-tubulin
52 Total α-tubulin
17 γH2AX
17 H2AX
250 130 100 70 55 35 25 15 10

A.2.


Serum stimulation
NCS (250 ng/ml) - 0.5 1 3 6 (h)
50 nM Taxol - + - + - + - + - +
110 pKAP1s824
110 KAP1
52 Acetylated α-tubulin
52 Tyrosinated α-tubulin
52 Total α-tubulin
17 γH2AX H2AX
17 γH2AX
250 130 100 70 55 35 25 15 10

A.3.


Serum no starvation
NCS (250 ng/ml) - 0.5 1 3 6 (h)
50 nM Taxol - + - + - + - + - +
110 KAP1
52 Total α-tubulin
17 γH2AX
17 H2AX
17 H3s10p
250 130 100 70 55 35 25 15 10

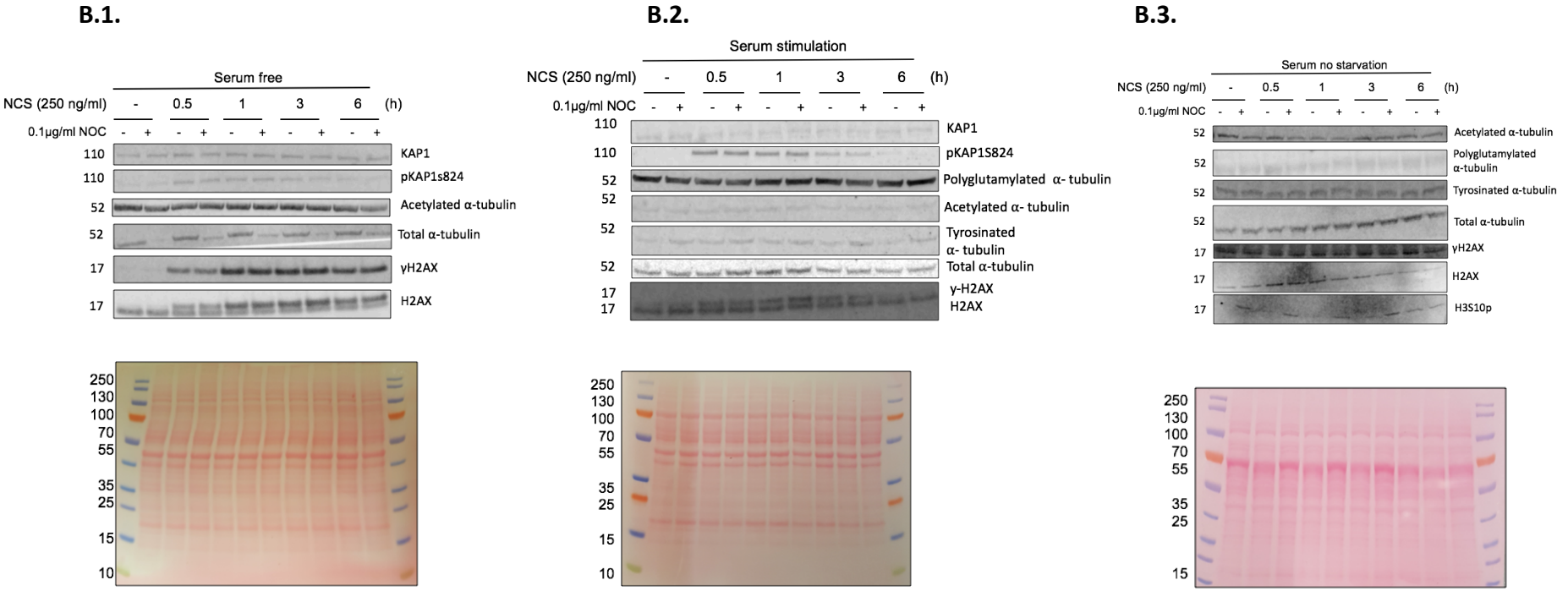
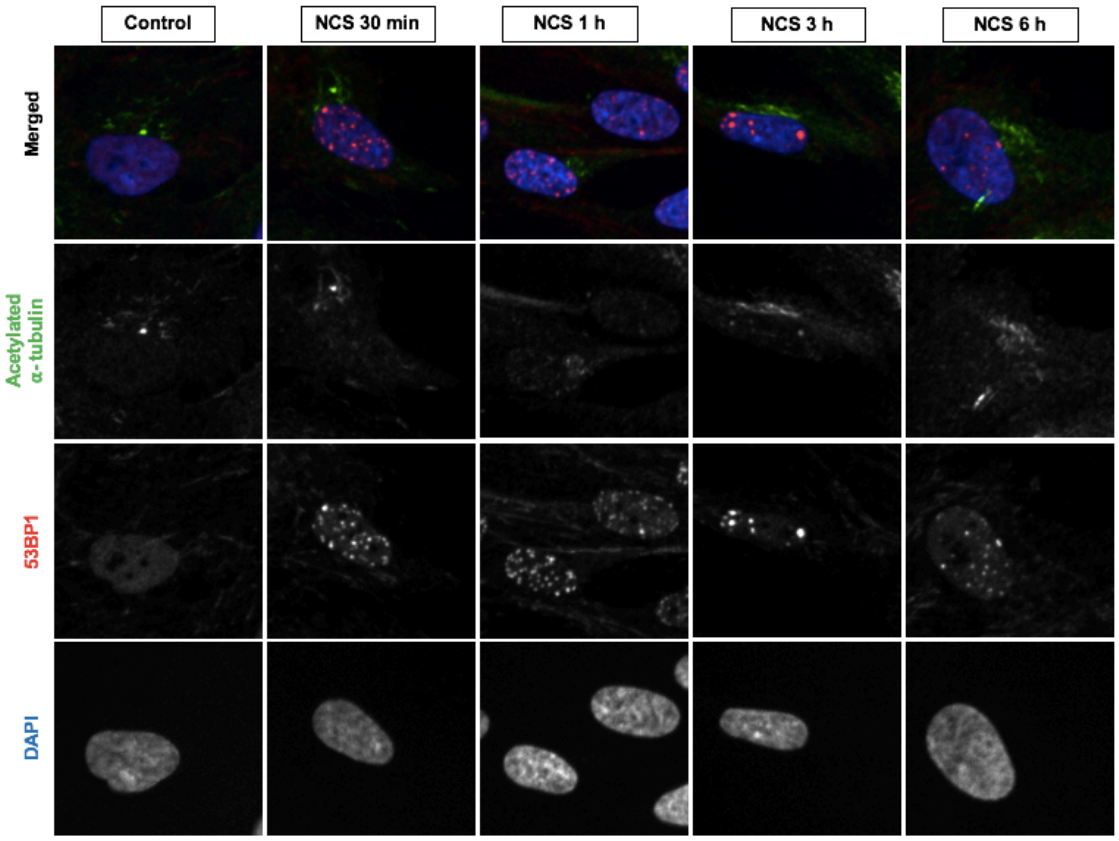


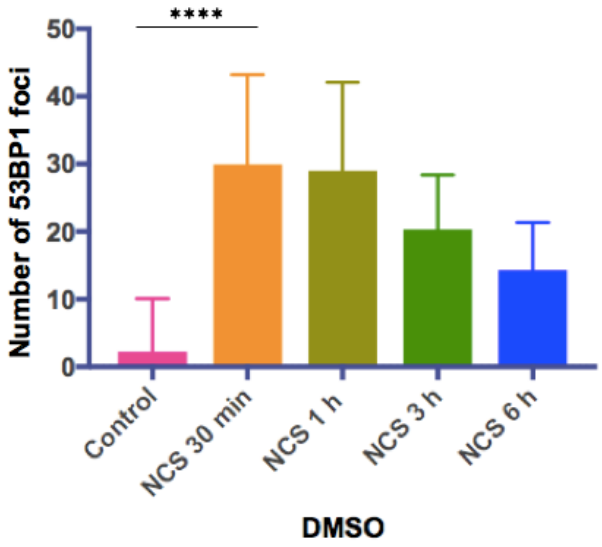
Figure 4.8. Evaluation of the effect of microtubule poisons on DNA damage in response to DNA damage on RPE-1 cells using immunoblotting.

Three conditions were assessed **A.1.** and **B.1.** cells without serum for 48 hours, **A.2.** and **B.2.** serum starved for 48 hours and then stimulated with 10 % serum cells**, A.3.** and **B.3.** controlled non-synchronised cells. The ponceau blot additionally confirm loading control. The taxol result experiments were performed three times, whereas nocodazole result experiments were performed two times.

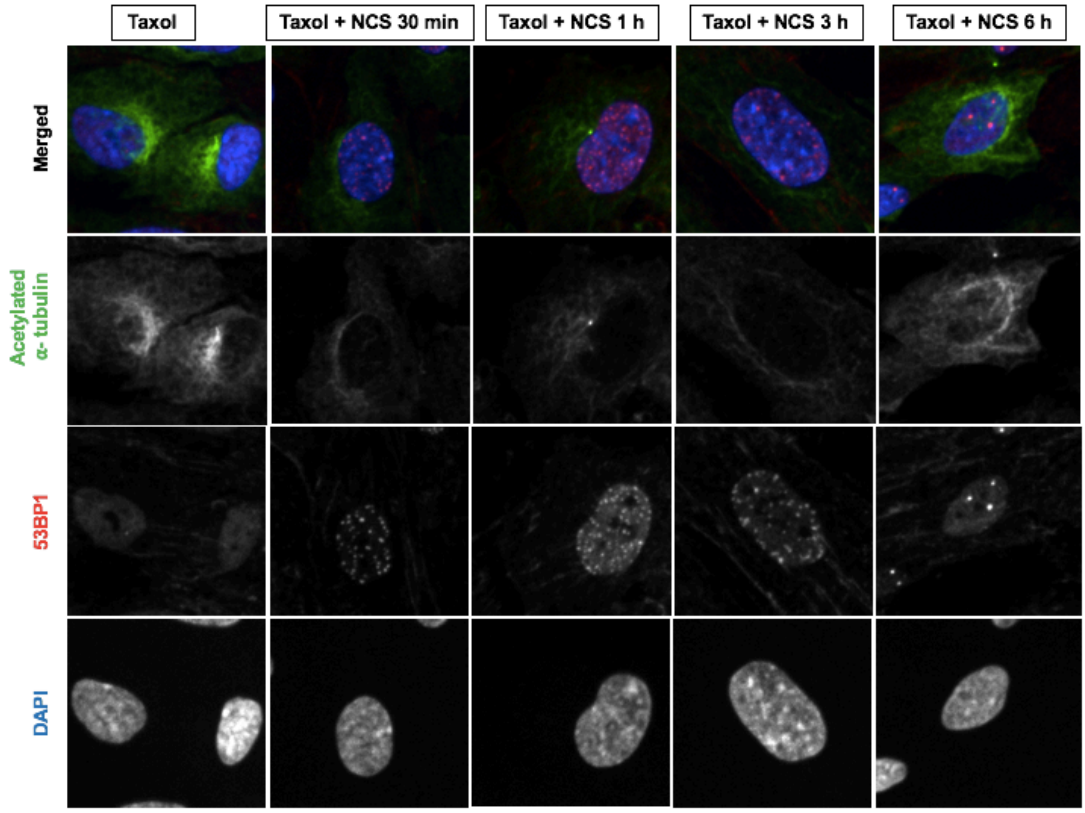
A.


Control
NCS 30 min
NCS 1 h
NCS 3 h
NCS 6 h
Merged
Acetylated α-tubulin
53BP1
DAPI

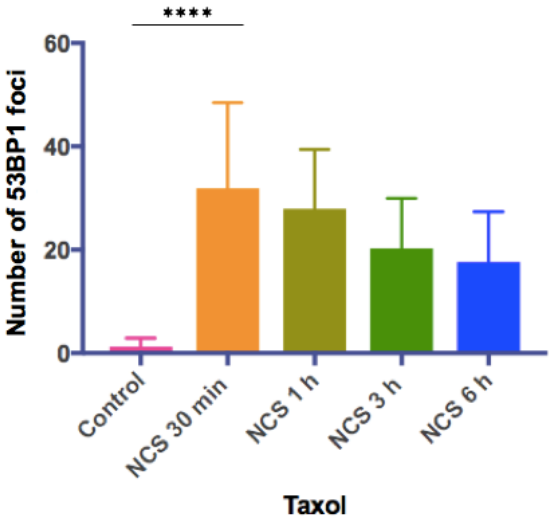
B.


****
Number of 53BP1 foci
50
40
30
20
10
0
Control
NCS 30 min
NCS 1 h
NCS 3 h
NCS 6 h
DMSO

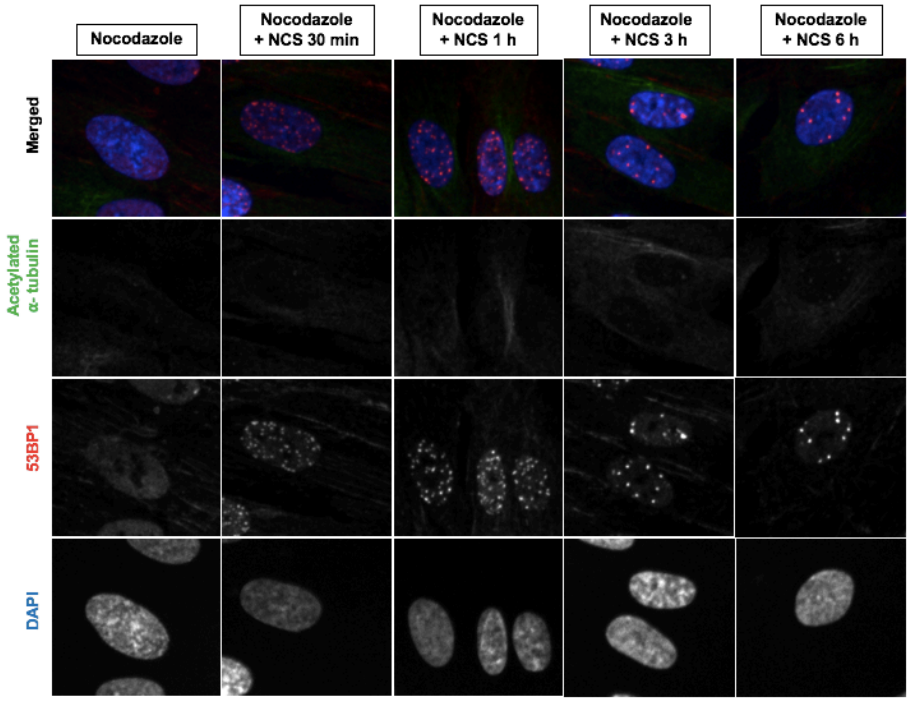
C.


Taxol
Taxol + NCS 30 min
Taxol + NCS 1 h
Taxol + NCS 3 h
Taxol + NCS 6 h
Merged
Acetylated α- tubulin
53BP1
DAPI

D.


****
60
40
20
0
Number of 53BP1 foci
Control
NCS 30 min
NCS 1 h
NCS 3 h
NCS 6 h
Taxol

E.



F.

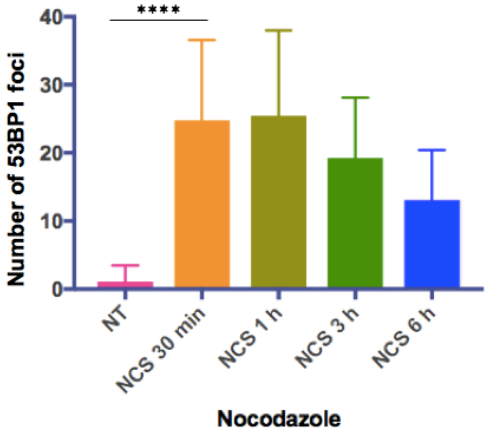


Figure 4.9. The analysis of the effect of microtubule poison drugs on DNA damage agent- 53BP1 using immunofluorescence.

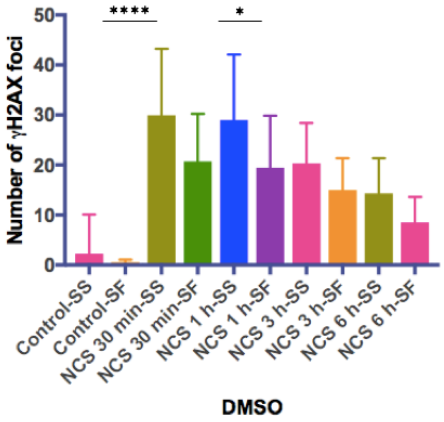
40 X images were taken with the CellVoyager microscope. images represent population of serum starved for 48 hours cells and then stimulated with 10 % serum. The analysis was performed in CellProfiler and then in GraphPad. The 0.1 ng.ml nocodazole was added to destabilise MTs or 50 nM taxol to overstabilise MTs and then 250 ng/ml NCS was added to damage DNA. ****p<0.0001, One-way ANOVA, number of technical repeats=3, total cell count= 287.

Whilst NCS increased the expression of γH2AX in all cases, no significant difference was seen in cells irrespective of MT stabilisation state. Accordingly, increase in DNA damage seen in the presence of taxol seen upon western blotting could not be reproduced using immunofluorescence.

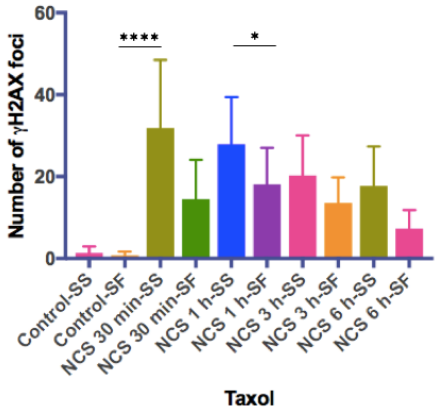
However, when γH2AX levels were compared between serum re-stimulated and serum-free conditions, NCS increased γH2AX foci number but no substantial effect of DMSO (control), taxol, or nocodazole was observed on foci number in serum-free or re-stimulated cells (Figure 4.10). Furthermore, immunofluorescence analysis showed that in serum-free condition at 3-hour time point, γH2AX levels are higher with nocodazole treatment than with DMSO (Figure 4.10.A.C.). However, this result was not observed using western blots (Figure 4.8.B.).

Importantly, phosphorylation of KAP1 on serine 824 was not changed under any of the tested conditions, suggesting that this process is affected on over-stabilised MTs and an increase in dynamicity of MT caused by nocodazole treatment facilitates DNA damage repair.

A.



B.



C.

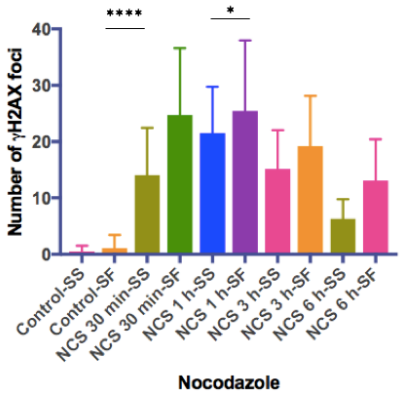


Figure 4.10. Comparison of the level of DNA damage between serum-free and serum re-stimulated cells after perturbation of microtubules over time.

RPE-1 cells were induced with **A.** DMSO, **B.** Taxol (50 nM) or **C.** Nocodazole (0.1 ng/ml) followed by DNA damage with NCS (250 ng/ml). The 40X images were taken with Cell Profiler and analysed with the GraphPad using One way ANOVA test. ****$p<0.0001$, *$p<0.05$, number of technical repeats=3.

Discussion

Since 1986, when the search and capture model was presented by Kirschner and Mitchison, dynamics of MTs has been a theme of ongoing research. After establishment that DNA damage increases MT dynamics (Chapter 3), the new question arose whether DNA damage can be influenced by microtubule dynamics.
Hence, MTs were perturbed using taxol or nocodazole, which are stabiliser and destabiliser, respectively, followed by DNA damage caused by the most effective candidate from the previous chapter- NCS.
It is now known that dynamic MTs mediate cell division via kinetochore, polar and astral MTs. Thus, any changes or lags in the MT motion could be deleterious to the cell survival in the long term, which was showed in this chapter (Figure 4.5.). After MT poison introduction, alteration of morphology was observed (Figure 4.4.). The majority of cells rounded up after the exposure to taxol for 68 hours. This might be a result of arresting cells in mitosis that is known to be the effect of taxol activity, but also this is a characteristic feature of apoptotic cells. The apoptotic assay has not been performed to distinguish the underlying cause. Nevertheless, the cells that were treated with taxol and then NCS, remained the normal cell morphology, which might indicate the possible cell cycle arrest.
In the short term effects of the MT poisons followed by DNA damage, no significant changes in γH2AX protein was observed to the cells compared to control. Thus, if dynamic microtubules are required for restoring the DNA strands, over-stabilised MTs would demonstrate an increase in that DNA damage marker protein. Therefore, this result initially implied that MTs do not affect DNA damage or this event is not γH2AX-dependent. Then it was noticed that the expression levels of phosphorylated KAP1 on serine 824 was reduced. This DDR signalling protein acts in unwinding the chromatin around the DNA lesion and hence it is imperative in DNA damage repair processes. Without the activation of KAP1, the chromatin around the damaged site remains closed and DNA repair proteins encounter difficulties to enter the site, hence the repair is prolonged. Interestingly, the hyper-stabilised and then DNA damaged RPE-1 cells demonstrated a significant decrease in the phosphorylated KAP1 on serine 824 compared to control (Figure 4.8.A.). This immunoblotting finding might suggests that highly stabilised MTs mediate partially closed chromatin that prevents from or

increases the time for DNA repair proteins from accessing and thus repairing the damaged DNA. Although this result using re-stimulated cells was readily reproducible, the finding could not be seen in the other two conditions, which could be a result of cell synchronisation in the G1 phase.

To further validate the assumption that stabilised MTs affect chromatin condensation, techniques such as FLIM- FRET could be performed to assess the chromatin organisation state in these conditions. The method presents a chromatin compaction state using the change of electrons on chromatin, therefore it is very accurate.

The immunoblotting with the nocodazole introduction demonstrated no observed changes in γH2AX, further confirming that the potential MT dynamics if alters DDR signalling, it is a non γH2AX-dependent process.

The outcome of this experiment is consistent with the previous studies, implying that microtubules are imperative in facilitating the DNA damage. Moreover, the results give more insights in the DNA damage response signalling changes and the possible chromatin state alterations. Overall, it can be concluded that MTs play role in DNA damage signalling, however, in the non γH2AX-dependent manner. Moreover, the alteration of the chromatin organisation could further indicate the missing link between changes in 53BP1 levels observed by Lottersberger and co-workers.

In order to follow-up with this question, the influence of this pathway on the LINC complex which connects MTs with the nucleus should be validated. That research could reveal the MT-activity dependent signalling pathway that leads to DNA repair.

CONCLUSIONS

Altogether, the results from chapter 3 and 4 indicate that there is a link between posttranslational modifications and dynamics of microtubules and DNA damage response signalling.

Firstly, the DNA damage alters the expression levels of PTMs of tubulin, influencing indirectly microtubules though the increase in their dynamicity.

Secondly, the work presented in this thesis supports the assumption that dynamic microtubules are required for the DNA damage repair signalling regulation and revealed the possible reason of the importance of the MT dynamicity involvement in the DNA damage repair process.

Although the preliminary data has been collected to answer the above questions, more research should be performed to fully understand the link between posttranslational modifications and dynamics of microtubules and DNA damage response signalling.

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