Investigation into the role of Aurora A kinase activity during mitosis

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Abstract

Aurora A is an important mitotic regulator that has been found to be up-regulated in a variety of tumours provoking a great deal of attention and the development of a number of small molecule Aurora kinase inhibitors. Most of these inhibitors though have predominantly targeted Aurora B, meaning that our understanding of the role of the kinase activity of Aurora A is comparatively less well developed.

MLN8054 however, is a small molecule inhibitor that has been reported *in vitro* to have a high degree of specificity towards Aurora A activity. In this thesis, I show *in vivo* that MLN8054 can be used to specifically inhibit Aurora A activity, and exploit this quality to probe the role of Aurora A activity in human cells. I was consequently able to show that Aurora A activity not only has a clear role in spindle formation, where it is required for the determination of K-fibre length and in the degree of centrosome separation, but also in the regulation of microtubule organisation. Despite the spindle deformities seen after inhibiting Aurora A activity, the majority of HeLa and DLD-1 cells were still able to form bipolar spindles capable of attaching to kinetochores. These spindle structures did not however, assert normal levels of force through the kinetochores, and cells were consequently unable to efficiently align their chromosomes, causing significant delays to mitotic progression. Cells were still able to divide in the absence of Aurora A activity, although the detection of segregation defects and aneuploid progeny indicates a role for Aurora A activity in the faithful segregation of the genetic material. Importantly however, Aurora A activity was not found to have a prominent role in the spindle assembly checkpoint.

Increasing the potency of Aurora A inhibition by using a drug-resistant cell line confirmed the observations made in HeLa and DLD-1 cells, emphasising that although Aurora A activity is required for spindle assembly, cells can still activate the spindle checkpoint and divide in its absence. I therefore propose that Aurora A activity is required for the formation of normal spindle structures capable of efficiently aligning and evenly dividing chromosomes during cell division. These roles were attributed in part to the kinase activity of Aurora A in the regulation of TACC3 and chTOG localisation on the spindle and centrosomes.

Interestingly however, Aurora A activity did not appear to be required for spindle assembly in non-transformed cells, which were able to more efficiently align their chromosomes and divide following Aurora A inhibition than the cancer cell lines. Furthermore, the non-transformed cells accumulated with 2N DNA after longer-term Aurora A inhibition, as opposed to the cancer cell lines, which exhibited profound aneuploidy following the equivalent treatment. This finding is encouraging, as consistent with recently published reports, it indicates that Aurora A inhibition may be successfully used in order to specifically target cancer cells.

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Abbreviations

A-box	D-box activating-box
ADP	Adenosine diphosphate
AIP	Aurora A kinase-interacting protein
APC	Anaphase promoting complex
Ark	Aurora related kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Bub	Budding uninhibited by Benzimidazole
Caspase	cysteine-aspartic acid protease
C.elegans	*Caenorhabditis elegans*
CENP	Centromere protein
Cdc	Cell division cycle
Cdh1	Cdc20 homolog
Cdk	Cyclin dependent kinase
CHC	Clathrin heavy chain
chTOG	Colonic and hepatic tumour over-expressed protein
CLIP	Cytoplasmic linker protein
CNN	Centrosomin
CPC	Chromosomal passenger complex
D-box	Destruction box
Drosophila	*Drosophila* melanogaster

DMEM	Dulbecco’s modified Eagle’s media
DMSO	dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Eg	Egg
FCS	Foetal calf serum
GDP	Guanosine-diphosphate
GFP	Green fluorescent protein
GSK	Gycogen synthetase kinase
GTP	Guanosine-triphosphate
Hef	Human enhancer filamentation
HRP	Horseradish peroxide
hTERT	Human telomerase reverse transcriptase
HURP	Hepatoma up-regulated protein
ILK	Integrin-like kinase
INCENP	Inner centromere protein
Ipl	Increase in ploidy
Kb	Kilobase
kDa	Kilodalton
Kif	Kinesin superfamily protein
K-fibre	Kinetochore fibre
LB	Luria Broth
Mad	Mitotic arrest-deficient

MAP	Microtubule associated protein
MCAK	Mitotic centromere-associated kinesin
MCC	Mitotic checkpoint complex
MI	Meiosis 1
Mps	Monopolar spindles
Ndc	Non-claret disjunction
PAGE	Polyacylamide gel electrophoresis
PAK1	p53 activating kinase
PBS	Phosphate buffered saline
PCM	Pericentriolar matrix
PI	Propidium iodide
Plk	Polo-like kinase
PP	Protein phosphatase
RNA	Ribonucleic acid
RNAi	RNA interference
RPE	Retinal pigment epithelial
Rpm	Revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulphate
TACC	Transforming acidic coiled coil protein
TBS	Tris buffered saline
TD-60	Disc-60kD

TMED	Tetramethylethlenediamine
TPX2	Targeting protein for Xklp2
v/v	volume/volume
w/v	weight/volume
Xenopus	*Xenopus laevis*
XMAP	*Xenopus* microtubule associated protein
ZM1	ZM447439
γ-TuRC	γ-tubulin ring complex

1 Introduction

1.1 Historical developments in the understanding of cell division

1.1.1 The birth of Cell Theory

The notion that living organisms are made up of individual units or ‘cells’ which are formed by the division of a mother cell, was both conceptually and technically difficult to develop. The ancient Greeks were the first to theorise that living things were made out of more simple components, with Aristotle hypothesising that life was the result of a ‘vital force’ which served to activate basic units. Advances to this theory were however speculative, until the development of microscopic techniques, which enabled the detailed observation of organisms. These techniques, together with a succession of meticulous studies by prolific scientific thinkers, would come to completely change our understanding of the living world.

After the development of early microscopes in the late 16th century, a number of scientists were able to examine the intricate construction of various tissues, however it was not until the first half of the 19th century that the Cell Theory was developed. This important theory was the product of two scientists called Schlieden (Schlieden, 1838) and Shwann (Shwann, 1839), who described all living things as being made out of individual units called cells. This theory was further developed by the observation that cells were not formed *de novo*, but instead were created by the formation of a partition in the parental cell, resulting in the production of two daughter cells (Amos, 2000, von Mohl, 1835). The observation in the 1840s and early 1850s that nuclear division preceded cell division brought the understanding of cell reproduction still further, however the step to understanding the relevance of chromosomes was more challenging. When analysing salamander cells, Flemming observed the strand-like entities of chromosomes and witnessed their longitudinal splitting at anaphase (Flemming, 1882) (Figure 1.1). This process was consequently named ‘mitosis’, from the Greek word for thread. Flemming’s investigations also indicated the presence of a network of fibres, which would later be described as the ‘spindle’ (Flemming, 1882). These revolutionary steps in the understanding of cell division prompted a torrent of investigations which shed further light on the intricacies of the process.

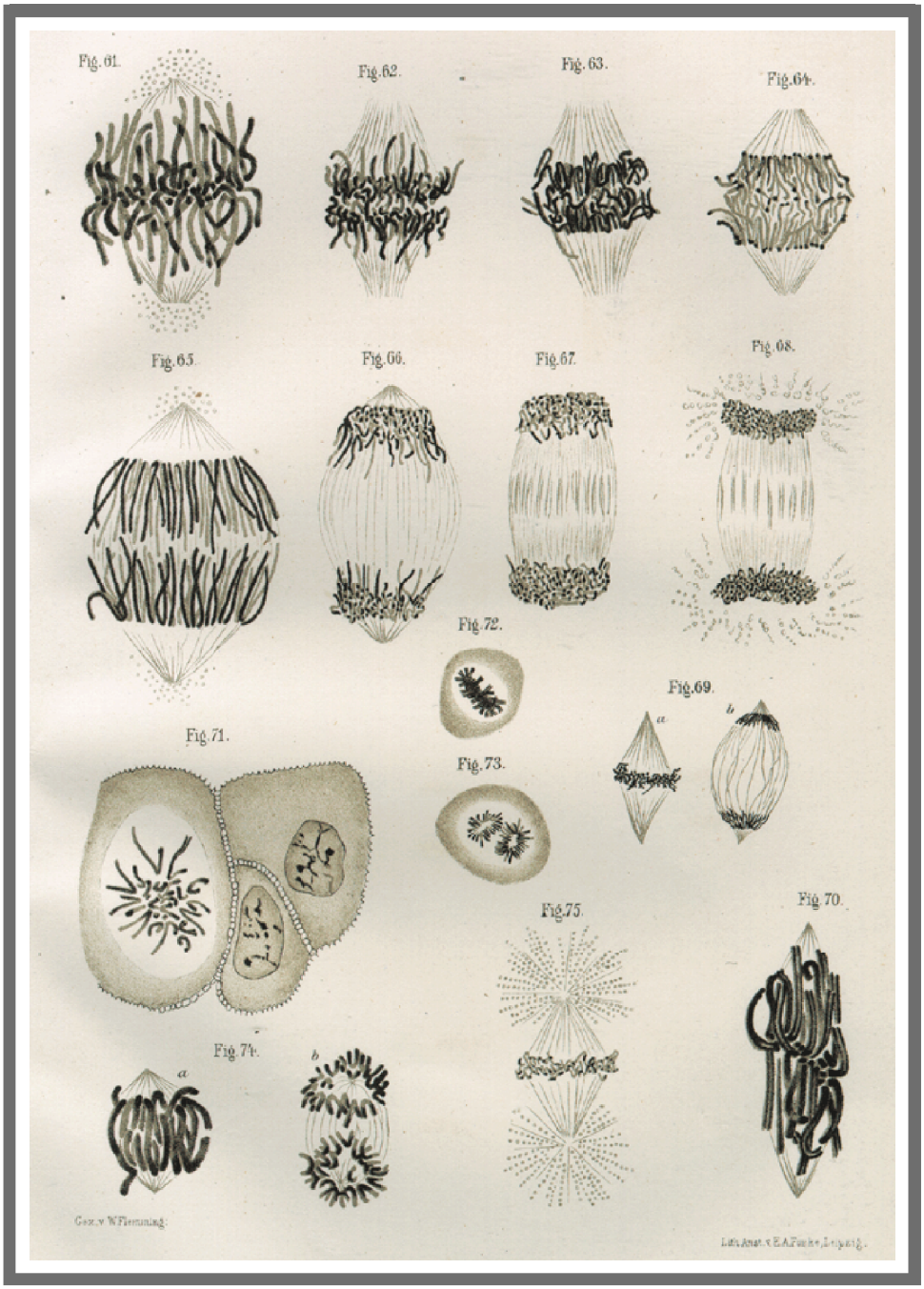

Figure 1.1 Early illustrations of the spindle and chromosomes from Flemming's book *Zellsubstanz, Kern und Zelltheilung*, published in 1882 (image taken from (Paweletz, 2001)).

1.1.2 The importance of nuclear material

Many leading thinkers in the field considered the nuclear factors of a cell to be of eminent importance in determining the character of the cell, however experimental challenges made this theory difficult to prove. In 1889 however, Boveri succeeded in fertilising an enucleate sea urchin egg with the sperm of another species to produce a distinguishably different embryo (Boveri, 1889). This appeared to be clear evidence of the nuclear rather than cytoplasmic control over cellular development. Further advances in the area also revealed that each chromosome played a distinct role in cell development, and that a complete chromosome set was required for normal development.

1.1.3 The characterisation of the spindle

The improvement of experimental techniques such as live-cell imaging and advanced microscopy between 1920 and 1950 enabled better observation and understanding of mitosis. It became possible to determine the shape of the mitotic spindle and the resulting force exerted on chromosomes (Wilson, 1928, Bajer and Mole-Bajer, 1975, Ris, 1949, Ostergren, 1951). In the late 1940s, Ostergren theorised that the movement of chromosomes toward the metaphase plate was achieved by the balancing of spindle forces which emanated from each pole. Consistent with this theory, in the 1950s a collection of data revealed that the spindle was made from protein filaments which ran parallel to the path of the chromosomes, and that it may be spindle polymerisation that drove chromosome dynamics (Inoue and Sato, 1967).

The development of electron microscopy enabled the observation of the spindle and its connection to kinetochores in even finer detail (Bajer and Mole-Bajer, 1975, Brinkley and Stubblefield, 1966, McDonald et al., 1992, Ding et al., 1993, Winey et al., 1995). Furthermore, the functioning of the spindles was revealed by a number of ingenious experiments such as spindle marking techniques that enabled the observation of microtubule dynamics and the intricate use of a micro-needle which demonstrated the pulling forces produced by the spindle (Mitchison et al., 1986, Gorbsky et al., 1988, Maddox et al., 2000, Mallavarapu et al., 1999, Nicklas, 1983). Finally, the discovery of motor proteins allowed the development of a more complete understanding of spindle construction and instigated the search for further spindle associated proteins (Sharp et al., 2000b, Walczak et al., 1998).

The culmination of all this evidence has enabled the development of the current model of cell division, and in this introduction I will discuss the current knowledge regarding the regulation of this complicated process. As my investigation specifically concerns the Aurora kinase family, I will

then explore the current understanding of these proteins and their role in the control of cell division.

1.2 The current understanding of cell division

Recent advances in experimental techniques have facilitated a greatly improved understanding of cell division, revealing the existence of various steps (Figure 1.2).

1.2.1 Phases in cell division

In a normal life cycle of a cell, division of the cellular material occurs in 'M phase' which compromises of mitosis and cytokinesis. The faithful completion of these two steps allows the even segregation of the duplicated genome, therefore preserving the hereditary information and necessary cytoplasmic content of dividing cells. For this essential process to occur, a tightly regulated sequence of mitotic stages must be completed consisting of: prophase, prometaphase, metaphase, anaphase and telophase. On successful completion of these mitotic steps, cytokinesis allows the cleavage of the mother cell to form two genetically identical daughter cells.

The progression through the cell cycle is highly regulated to ensure that mitosis is not initiated until after cellular replication has been completed and any damaged DNA is repaired. The control over the cyclical flow through the cell cycle was found to be dependent on the appropriate activation or inactivation of a series of Cdk serine/threonine kinase family members, which are controlled both by associations with partner cyclin proteins, and by a complicated system of phosphorylation and dephosphorylation reactions.

1.2.2 The control of mitotic entry: The regulation of Cyclin B1-Cdk1

Early experiments into the regulation of the cell cycle revealed the cyclic activation and inactivation of Cyclin B1 was required to drive the progression from interphase to mitosis and back again (Murray and Kirschner, 1989). Later it was shown that Cyclin B1 was actually the activating subunit for Cyclin dependent kinase 1 (Cdk1), which was required for the entry into mitosis (for review see [Nurse, 1990]).

In S phase, the transcription of Cyclin B1 is enhanced by the activation of transcription factors, which coincides with an increase in its export from the nucleus (Yang et al., 1998, Ziebold and Klempnauer, 1997, Saville and Watson, 1998, Chae et al., 2004, Major et al., 2004, Laoukili et al.,

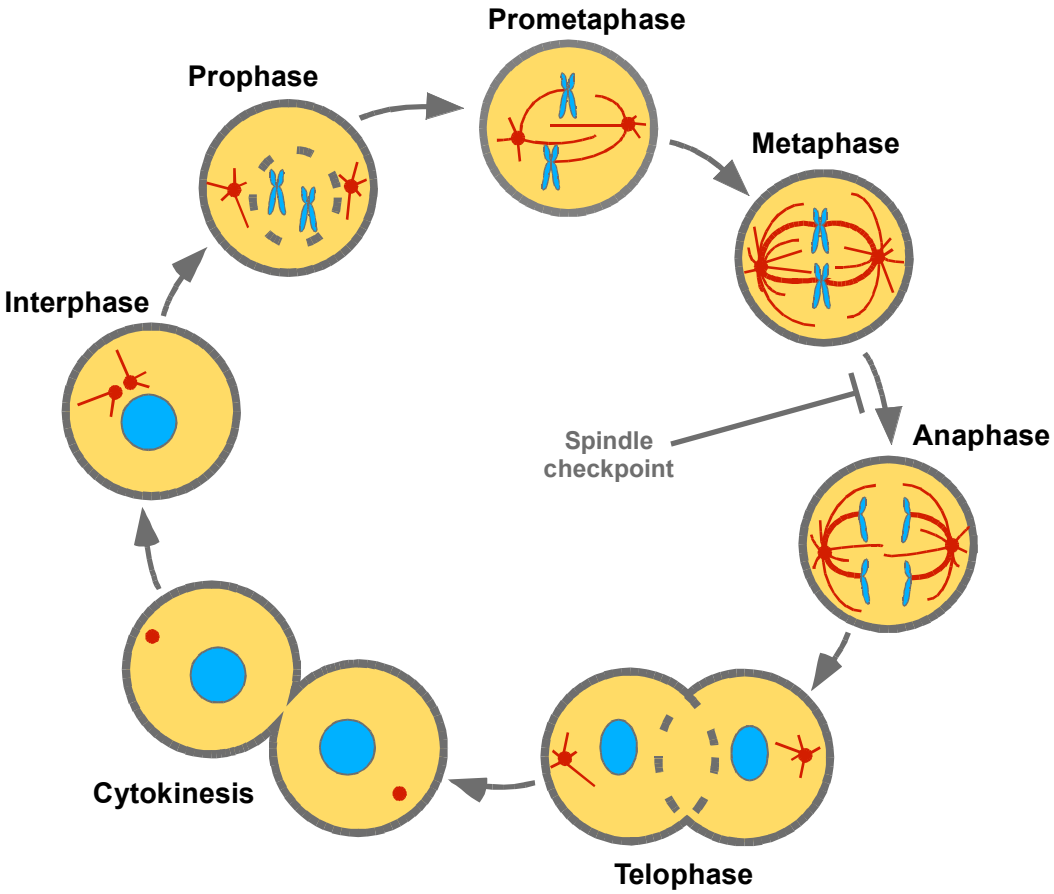

Figure 1.2 The stages of mitosis
The division of animal cells can be divided into a series of stages:
Prophase - chromatin condenses to form defined chromosomes, the nuclear envelope breaks down and centrosomes migrate to opposite ends of the cell; Prometaphase - kinetochores become attached to dynamic spindle microtubules, causing the formation of stable kinetochore-fibres, allowing chromosomes to congress towards the equatorial plane of the cell; Metaphase - Biorientation of the chromosomes is achieved, allowing the satisfaction of the spindle checkpoint; Anaphase - The satisfaction of the spindle checkpoint and the resulting loss of sister chromatid cohesion allows the sisters to be pulled towards the opposite poles of the cell; Telophase - Arrival of the sister chromatids at the cell poles promotes their decondensation and a nuclear envelope is formed around them; Cytokinesis - the formation of an actomyosin-based contractile ring brings about the division of cellular material (for review see [Nigg, 2001b]).

2008, Hagting et al., 1998). During G2, Cyclin B1 is targeted to the centrosomes by Polo-like kinase-1 (Plk1) and Aurora A, which act to amplify the effects of Cyclin B1, allowing the formation of the Cyclin B1-Cdk1 complex (Jackman et al., 2003). The activity of the Cyclin B1-Cdk1 complex is controlled by phosphorylation events which can positively and negatively regulate its activity (Tassan et al., 1994, McGowan and Russell 1995). The balancing of these activities takes the form of a delicately regulated feedback loop, which is responsible for the major regulatory step in mitotic entry (for review see [O'Farrell, 2001]; (Booher et al., 1997, Nakajima et al., 2003, Watanabe et al., 2004, Dutertre et al., 2004, Gabrielli et al., 1996, Lammer et al., 1998, Lindqvist et al., 2005, Baldin et al., 2002)). Interestingly, if the levels of Cyclin B1 are lowered, normal signalling networks do not properly function and mitoses are abnormal, possibly reflecting the improper activation of the full signalling network (Lindqvist et al., 2007).

The phosphorylation from the activated Cdk1 has also been shown to target Plk1 to Cdk1 substrates such as Cdc25C, Myt1, Wee1, Cyclin B-Cdk1, and a range of others which further promote the entry into mitosis (Elia et al., 2003, Watanabe et al., 2004, Watanabe et al., 2005, Nakajima et al., 2003, Lowery et al., 2007, Yamaguchi et al., 2005, Litvak et al., 2004, Toyoshima-Morimoto et al., 2002, Yuan et al., 2002). Cdk1 has also been shown to phosphorylate Bora, the Aurora A kinase co-factor, which may help in stimulating the Aurora A mediated activation of Plk1 (Hutterer et al., 2006, Chan et al., 2008). Indeed, the activity of both Aurora A and Plk1 has been shown to be involved in a positive feedback loop with the Cyclin B1-Cdk1 complex, in which the three proteins experience increased phosphorylation and recruitment to the centrosomes (for review see [Barr and Gergely, 2007]; (Marumoto et al., 2002, Abrieu et al., 1998, Katayama et al., 2001, Portier et al., 2007, Dutertre et al., 2004, Hachet et al., 2007)).

These findings therefore demonstrate the existence of an intertwined and complicated network of regulation, which is essential to prevent entry into mitosis in the presence of DNA damage. If DNA damage occurs in G2, this network is required to halt the progression until the damage is repaired (for review see [Smits and Medema, 2001, Bartek and Lukas, 2007]; (Lukas et al., 2001)). Interestingly, various pathways which are redundant in the mitosis of undisturbed cells, become essential for the entry into mitosis after DNA damage. In particular Aurora A and Plk1 activity were found to be required for mitotic entry after DNA damage (van Vugt et al., 2004, Macurek et al., 2008).

1.2.3 The controlled progression through mitosis: The importance of regulating Cyclin B1 levels

After the entry into mitosis, Cyclin B-Cdk1 levels are seen to increase, which allows the orchestration of normal mitotic progression (Lindqvist et al., 2007). Ubiquitination of Cyclin B1 in mitosis instigates its ubiquitin-dependent proteosome degradation, consequentially causing the reduction of the level of Cyclin B1 and the exit from mitosis (for review see [Nandi et al., 2006] Figure 1.3; (Murray et al., 1989, Glotzer et al., 1991, Hershko et al., 1991)). The E3 ubiquitin ligase found to ubiquitinate Cyclin B1 was therefore also found to be required for anaphase onset, and was subsequently coined the Anaphase-promoting complex (APC) (Zachariae and Nasmyth, 1996, Sudakin et al., 2001, Hershko et al., 1994, King et al., 1995). Further investigation into the APC has revealed that it is made up of several subunits and is only fully active when bound to one of its co-factors, such as Cdc20 and Cdh1, which have different binding affinities for the APC depending on the cell cycle phase (for review see [Peters, 2006]).

The APC co-factor Cdh1 is prevented from binding with the APC by its phosphorylation by Cdks during S phase, G2 phase and early mitosis (Zachariae and Nasmyth, 1996, Blanco et al., 2000, Kramer et al., 2000). However, during prophase, Cyclin B1-Cdk1 and Plk1 phosphorylate the APC at several sites, causing it instead to bind to Cdc20 (Kramer et al., 2000, Rudner and Murray, 2000, Golan et al., 2002, Kraft et al., 2003). The appropriately activated APC^{Cdc20} targets Cyclin B1 and Securin for proteosome-dependent degradation. Both Cyclin B1 and Securin are important regulators of the transition between metaphase and anaphase (Murray et al., 1989, Glotzer et al., 1991, Hershko et al., 1991, Clute and Pines, 1999, Hagting et al., 2002). Securin inhibits the protease called Seperase, and when Securin is degraded, Seperase is released from its inhibitory constraints, cleaving Cohesin which allows sister chromatids to separate (reviewed in [Nasmyth, 2001, Pines, 2006]; (Hagting et al., 2002, Rieder et al., 1994)).

In the presence of inappropriately attached chromosomes however, a surveillance mechanism called the spindle checkpoint ensures that the APC^{Cdc20} complex is prevented from initiating Cyclin B1 and Securin destruction, thus delaying the onset of anaphase. This delay allows a period in which cells can achieve universal chromosome biorientation thus preventing the development of aneuploidy (Kops et al., 2005, Weaver and Cleveland, 2005, Rieder et al., 1995, Rieder et al., 1994). Indeed the disruption of the spindle checkpoint in mammalian cells causes the disruption of cell proliferation and the loss of cell viability (for review see [Dobles and Sorger, 2000, Kops et al., 2005]).

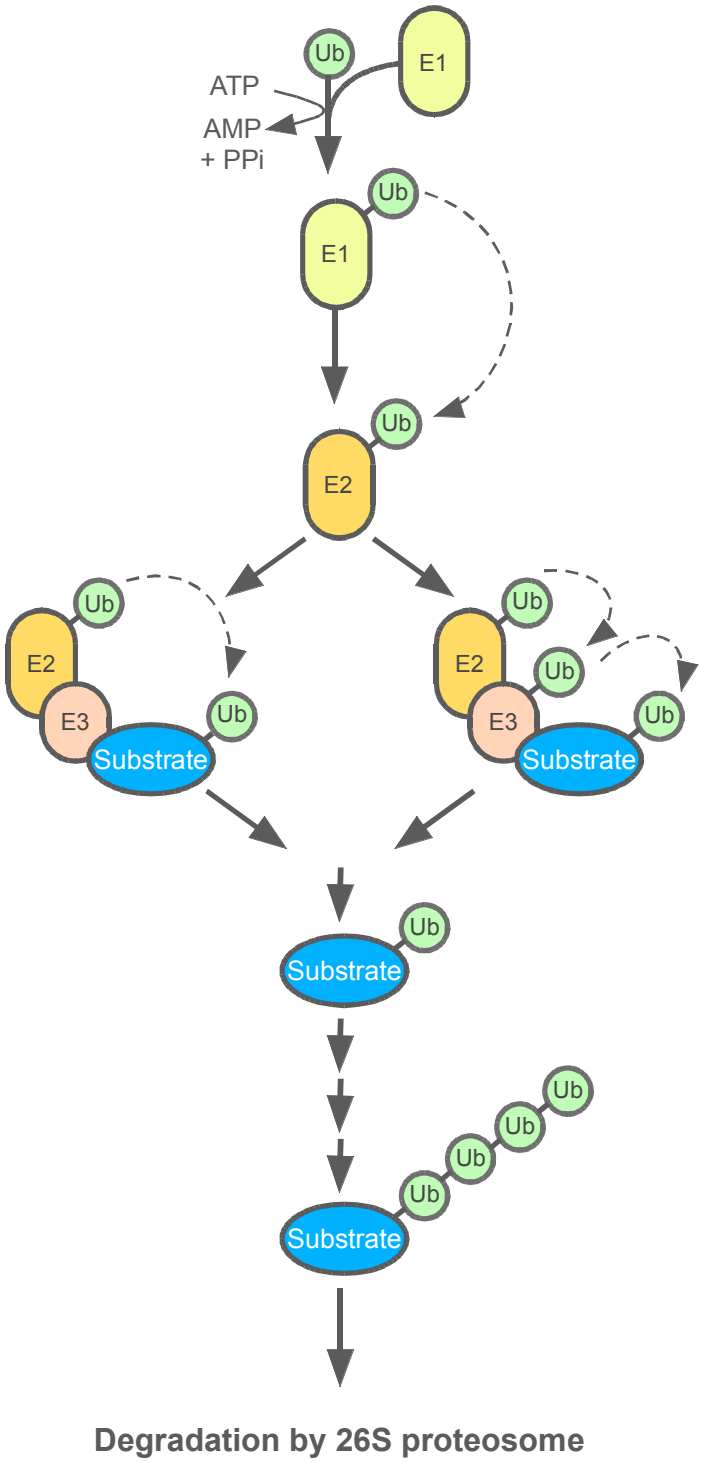

Figure 1.3 Ubiquitin-dependant protein degradation
Ubiquitin-dependant proteosome degradation commences with the action of an E1 ubiquitin-activating enzyme, which through the consummation of ATP, forms a high-energy thiolester linkage with a free ubiquitin (for review see [Ravid and Hochstrasser, 2008]). The activated ubiquitin is then transferred to an E2 ubiquitin-conjugating (UBC) enzyme, which is required for the degradation of cyclins such as Cyclin B (Hershko et al., 1994). With the help of a third enzyme, an E3 ubiquitin ligase, which determines the specificity of the substrate, the UBC enzyme transfers the ubiquitin to a lysine in the target protein. The E3 ubiquitin ligases are commonly involved in the formation of poly-ubiquitin chains which cause cells to be recognised and degraded by the 26S proteosome (Figure adapted from [Ravid and Hochstrasser, 2008]).

1.2.4 The spindle checkpoint

Spindle checkpoint activation can be maintained by the presence of just a single unaligned chromosome (Rieder et al., 1994, Rieder et al., 1995, Nicklas et al., 1995), which indicates that the incorrectly attached chromosomes instigate the production of a diffusible signal, capable of preventing APC^{Cdc20} from targeting Cyclin B1 and Securin for proteosome-dependent degradation (Figure 1.4). Interestingly however, this diffusible signal does not inhibit the activity of APC^{Cdc20}, as the complex is still capable of targeting substrates such as Cyclin A and NIMA-related kinase 2A (Nek2A) for degradation in prometaphase (Geley et al., 2001, den Elzen and Pines, 2001). Therefore the checkpoint signal may just prevent the action of APC^{Cdc20} towards specific substrates (Hayes et al., 2006).

1.2.5 The mitotic checkpoint complex

The identification of a signal produced by unattached chromosomes, prompted the search for its component parts. Following the identification of the MAD and BUB genes in budding yeast (Hoyt et al., 1991, Li and Murray, 1991), a complex was found to form between Mad2, Mad3, Bub3 and Cdc20, in budding yeast mitosis which was found to associate with the APC (Brady and Hardwick, 2000). Human homologues have since been found (Li and Benezra, 1996, Taylor et al., 1998, Meraldi and Sorger, 2005), which similarly form a complex consisting of Mad2, BubR1, Bub3 as well as Cdc20, which is known as the ‘Mitotic checkpoint complex’ (MCC) (Sudakin et al., 2001). Importantly, although recombinant Mad2 and BubR1 are individually capable of inhibiting the APC, their combination into the MCC has a synergistic inhibitory effect, preventing the APC from targeting Cyclin B1 and Securin for destruction (Sudakin et al., 2001, Fraschini et al., 2001, Morrow et al., 2005, Fang et al., 1998a, Fang et al., 1998b, Fang, 2002, Hardwick et al., 2000, Tang et al., 2001, Millband and Hardwick, 2002).

Generation of the MCC: kinetochore microtubule occupancy

In early mitosis, checkpoint proteins and Cdc20 begin to accumulate at kinetochores (for review see [Cleveland et al., 2003, Musacchio and Salmon, 2007]). These include Mad1, Bub1, Plk1, CENP-E and Aurora B which do not form part of the MCC, but function instead to increase the formation of the complex and amplify the spindle checkpoint signal or regulate the spindle checkpoint in response to chromosomal misalignments (Ditchfield et al., 2003, De Antoni et al., 2005, Kallio et al., 2002, Tang et al., 2004, Mao et al., 2005, Morrow et al., 2005).

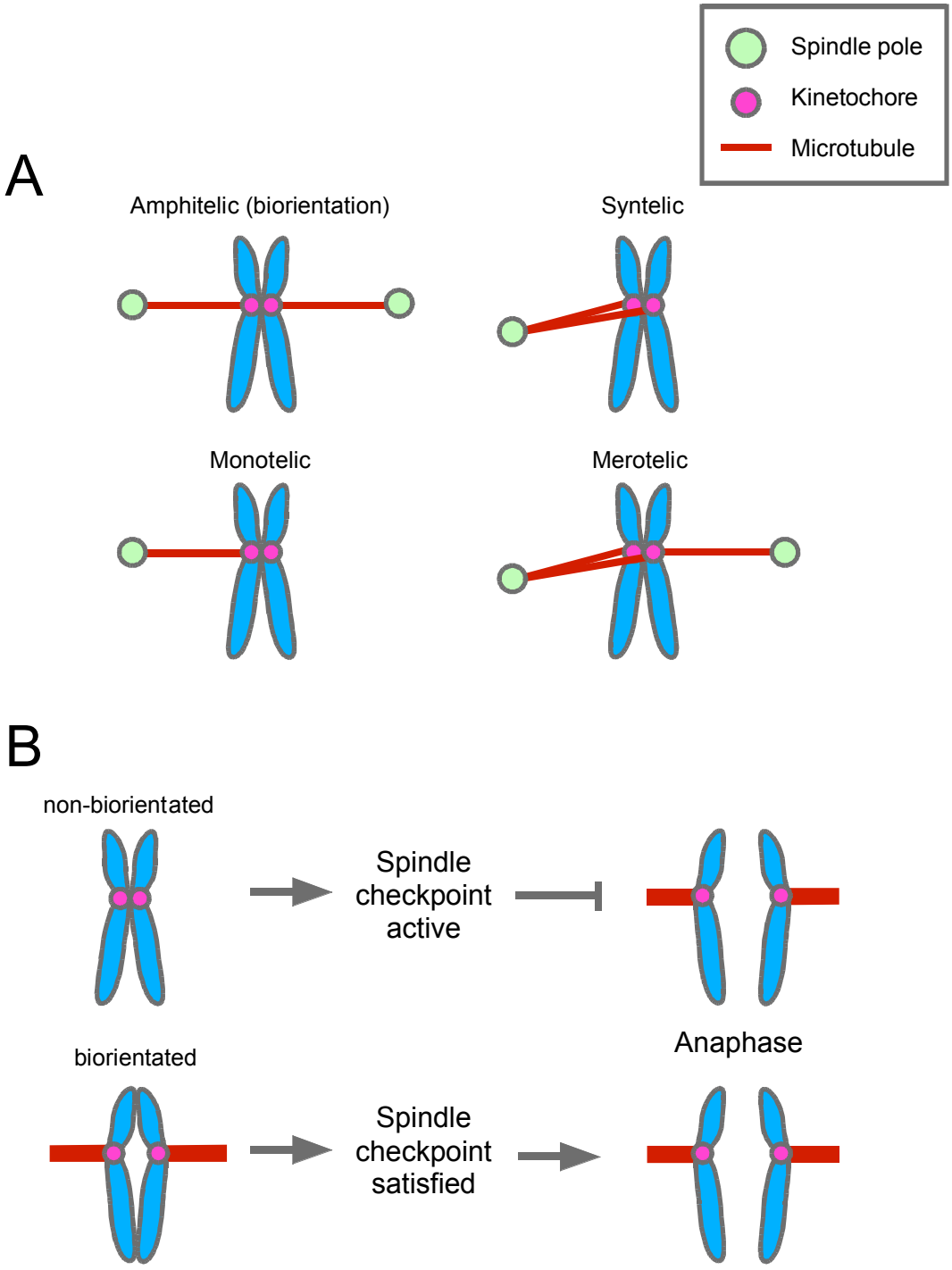

Figure 1.4 Microtubule-kinetochore attachments and the satisfaction of the spindle assembly checkpoint
(A) During the formation of the spindle, optimal chromosome orientation occurs when the two adjacent sister kinetochores are attached to microtubules which emanate from proximal spindle poles. Other forms of kinetochore-microtubule attachments do however form. These alternative attachments activate the spindle checkpoint and are eventually corrected to allow the universal biorientation of chromosomes within the cell.
(B) When chromosomes are not biorientated on the spindle they generate the checkpoint signal, which inhibits the APC and prevents the onset of anaphase. When chromosomes are biorientated however, appropriate force is applied to the kinetochore pair producing a centromere stretch, thus preventing the generation of the checkpoint signal and promoting anaphase onset (for review see [Santaguida and Musacchio, 2009, Marseca and Salmon, 2010] Figure adapted from [Maresca and Salmon, 2010]).

The assembled proteins monitor the attachment of microtubules to chromosomes. Checkpoint proteins such as Mad2 and BubR1 attach to unattached kinetochores and are removed as microtubules fill the kinetochore occupation sites (Chen et al., 1996, Chen et al., 1998, Li and Benezra, 1996, Taylor and McKeon, 1997, Taylor et al., 1998, Waters et al., 1998, Skoufias et al., 2001). This reduction in levels of checkpoint proteins at attached kinetochores is attributed to the increase in microtubules enhancing the level of motile Dynein to the area, which is capable of 'stripping' the proteins (King et al., 2000, King and Nicklas, 2000, Hoffman et al., 2001, Howell et al., 2001). BubR1 activity is also silenced via CENP-E in response to kinetochore microtubule capture, which silences BubR1 dependent checkpoint signalling (Mao et al., 2005). Mad2 at kinetochores is also proposed to be regulated by the inhibitor of the spindle assembly checkpoint $p31^{comet}$, which is thought to compete with Mad2 at kinetochores, thus inhibiting checkpoint signalling (Yang et al., 2007, Habu et al., 2002, Xia et al., 2004, Mapelli et al., 2006). It appears therefore, that many signalling networks are at work to activate the spindle assembly checkpoint in response to insufficient microtubule-kinetochore attachments. However, chromosomes can also be incorrectly aligned as a result of their inappropriate attachment to spindle microtubules.

Generation of the MCC: low inter-kinetochore tension

To prevent the onset of anaphase until chromosomes are correctly bi-orientated, it has been proposed that cells monitor the tension generated by the spindle across sister kinetochores (Nicklas et al., 1995, Nicklas, 1997). When chromosomes are incorrectly attached to K-fibres, it is not possible for the spindle to assert the same degree of tension as produced at bi-orientated chromosomes, thus presenting a method which can be used by the cell to determine incorrectly attached chromosomes (Figure 1.4). The existence of such a tension sensing mechanism is supported by the observation that kinetochores under tension experience a decrease in levels of Bub1 as well as changes in the phosphorylation of proteins recognised by 3F3/2 (Waters et al., 1998, Morrow et al., 2005, Gorbsky and Ricketts, 1993, Taylor et al., 2001, Chen et al., 1998). Furthermore, under conditions where kinetochores are monotelically or synthetically attached to the spindle, the chromosomal passenger complex (CPC) including Aurora B, is found to sense the low tension and instigate the correction of the attachment (reviewed in [Cimini and Degrassi, 2005, Vader et al., 2006b]; (Morrow et al., 2005, Dewar et al., 2004, Cimini et al., 2006, Knowlton et al., 2006)).

Despite the evidence in support of it, the existence of a tension-sensing arm of the spindle checkpoint still remains controversial. Groups arguing against the ability of cells to delay anaphase in the presence of low levels of kinetochore tension, cite experiments where cells can progress through to anaphase even when centrosome stretch is prevented by laser ablation or the exploitation

of replication defects (O'Connell et al., 2008, Dewar et al., 2004, Skibbens et al., 1995). In these experiments, the spindle checkpoint can be satisfied and cells progressed to anaphase following the attachment of microtubules. There however remains some debate on whether the distance between the inner and outer kinetochore proteins is monitored by the cell as a method of detecting erroneous low-tension attachments (Maresca and Salmon, 2009, Uchida et al., 2009, Wan et al., 2009). If this method of tension sensing exists in cells, it would still be present in the cited experiments and therefore negates the theory that tension is not a factor in the spindle checkpoint. However before this area of high debate can be resolved, further investigation must first be carried out (review in [Maresca and Salmon, 2010]).

1.2.6 Destabilisation of low tension microtubule attachments

K-fibres that do not produce appropriate levels of centromere stretch are destabilised (Hauf et al., 2003, Cimini et al., 2006, Adams et al., 2001b, Kaitna et al., 2002, Lampson et al., 2004). However, the strong relationship between attachment and tension generation at kinetochores makes it difficult to distinguish whether low levels of tension directly cause the activation of the spindle checkpoint, as changing levels of tension will probably alter the level of microtubule attachment (Zhou et al., 2002, Pinsky et al., 2003).

Aurora B kinase is involved in this destabilisation of low tension microtubule attachments by controlling the microtubule-destabilising Kin1 kinesin MCAK (mitotic centromere-associated kinesin), the Ndc80/HEC1 complex and the DASH complex, all of which are involved in causing the depolymerisation of microtubules at the kinetochore area (Andrews et al., 2004, Cheeseman et al., 2002, Ohi et al., 2004, Zhang et al., 2007, Keating et al., 2009, Tien et al., 2010). By destabilising the low tension microtubule connections, Aurora B kinase activates the spindle checkpoint by causing reduced kinetochore attachment, thus delaying anaphase onset until all chromosomes are correctly bi-oriented (Ditchfield et al., 2003, Hauf et al., 2003, Girdler et al., 2006, Biggins et al., 1999, Pinsky et al., 2006). This role for Aurora B activity was highlighted in experiments in which its inhibition permitted mitotic exit despite the absence of appropriate centromeric tension (Ditchfield et al., 2003, Hauf et al., 2003, Waters et al., 1998). Cells were however retained in mitosis following Aurora B inhibition when both kinetochore attachment and tension were lost. This demonstrates that although Aurora B activity is involved in controlling the activation of the spindle checkpoint in response to low tension, it is not involved in checkpoint activation in response to loss of kinetochore attachment.

The detection of checkpoint proteins on kinetochores indicates that the kinetochores serve as a method of contributing to the formation of the MCC (Howell et al., 2000, Howell et al., 2004, Luo

et al., 2002, Shah et al., 2004, De Antoni et al., 2005). There is however some debate over the role of kinetochores in the formation of the spindle checkpoint signal, (Sudakin et al., 2001, Fraschini et al., 2001, Poddar et al., 2005). Consequently, it has been proposed that there may be two phases of checkpoint activation, possibly involving an initial phase that occurs before kinetochores are properly matured, and a second that is more dependent on the presence of fully functional kinetochores. Mad2 and BubR1 have been put forward as controllers of mitotic timing before the maturation of kinetochores, possibly by directly binding and sequestering Cdc20, to inhibit premature mitotic exit (for review see [Musacchio and Salmon, 2007]; (Meraldi et al., 2004)).The extinguishing of the Mad2, BubR1 signal is thought to coincide with the initiation of the second phase of checkpoint signalling, which depends on the newly matured kinetochores (Meraldi et al., 2004). Therefore, the presence of mature kinetochores may not be required for inhibition of the APC and the formation of the MCC. Kinetochores may however increase the association of the MCC with the APC, an idea which is supported by the observation that while the MCC is present throughout the cell cycle, it is only found to be associated with APC during mitosis (Morrow et al., 2005).

1.2.7 Sustained spindle checkpoint activation

The sustained activation of the spindle assembly checkpoint by long-term treatment with anti-mitotic drugs, such as Nocodazole, causes cells to be maintained in mitosis for extended periods and prevents the satisfaction of the spindle assembly checkpoint (Brito and Rieder, 2006). Reports of the fate of cells after extended checkpoint activation vary greatly, with some describing mitotic exit after a prolonged mitosis without division (Jordan et al., 1996, Tao et al., 2005), while others show cell death occurring directly in mitosis (Panvichian et al., 1998), or alternatively cells are observed to experience abnormal divisions (Chen and Horwitz, 2002). Ultimately however, cells incapable of satisfying the spindle checkpoint eventually die (Rieder and Maiato, 2004).

Recent publications have revealed the existence of a variety of different responses to the sustained spindle checkpoint activation (Gascoigne and Taylor, 2008, Brito and Rieder, 2006, Brito and Rieder, 2009, Shi et al., 2008). These variations were attributed to the balancing of the death signals and Cyclin B1 levels (Gascoigne and Taylor, 2008). Cyclin B1 levels were found to be gradually degraded despite sustained spindle checkpoint activation, and when reduced below a certain threshold, cells were caused to exit mitosis without first satisfying the spindle checkpoint, through a process known as ‘slippage’ (Brito and Rieder, 2006). The variation in cell fate in response to sustained checkpoint activation was therefore thought to be derived from the different rates of Cyclin B1 degradation and slippage thresholds. Interestingly, mitotic delays inflicted by anti-mitotic drugs were shown to be further extended through their combination with caspase

inhibition (Shi et al., 2008, Gascoigne and Taylor, 2008, Brito et al., 2008). It was consequently theorised that the gradual degradation of Cyclin B1 and the activation of cell death pathways may act as two opposing networks working to define the fate of the cell (Gascoigne and Taylor, 2008). Thus while the cell death signals increased in response to sustained anti-mitotic drug treatment, the Cyclin B1 levels were also falling. The first network to reach its designated threshold would determine whether the cell died in mitosis or 'slipped' out.

1.2.8 Satisfaction of the spindle assembly checkpoint

When the chromosomes are finally aligned, the spindle checkpoint is satisfied, the APC^{Cdc20} targets Cyclin B1 and Securin for destruction and Cdk1 is inactivated, allowing the progression through to anaphase (Clute and Pines, 1999, D'Angiolella et al., 2003, Potapova et al., 2006). The inactivation of Cdk1 promotes the interaction of Cdh1 with the APC through its dephosphorylation, which in turn targets Cdc20 for degradation and maintains low levels of Cyclin B1 to enable the correct progression through G1 (Prinz et al., 1998, Pfleger and Kirschner, 2000). The appropriate phosphorylation of the APC and the dephosphorylation of Cdc20 therefore have antagonistic effects towards the binding of the co-factors to the APC. This demonstrates a network of activity, and highlights the high degree of control that is held over mitotic progression. Structural elements such as microtubule organising centres are however also required for the efficient progression through mitosis.

1.3 Microtubule organising centres

The accurate delivery of genetic material into two daughter cells requires the formation of a spindle, which is dependent on the action of microtubule organising centres. Although microtubules can form on their own, microtubule organising centres enable the efficient organisation of tubulin subunits to form functional spindles, and have been found in all eukaryotic organisms apart from higher plants and yeasts.

1.3.1 Centrosomes

When present in cells, centrosomes function as the major method of bipolar spindle formation, although bipolar spindles have been shown to still occur after removal of centrioles. Cells lacking centrosomes were however sometimes observed to have problems with cytokinesis and to eventually arrest in G1 (Hinchcliffe et al., 2001, Khodjakov and Rieder, 2001, Khodjakov et al., 2000, Basto et al., 2006).

In the absence of centrosomes a bipolar spindle can form through a 'self assembly' pathway, whereby microtubules are nucleated around chromosomes in systems which lack centrosomes (McKim and Hawley, 1995, Heald et al., 1996, Heald et al., 1997). In these cases the nucleation of microtubules is thought to occur at sites of γ-tubulin complexes which are recruited by a network of factors (Luders et al., 2006). This nucleation is mediated by the small GTPase Ran (Dasso, 2002, Hetzer et al., 2002), which has been shown to induce bipolar spindle assembly in *Xenopus* egg extracts lacking centrosomes (Carazo-Salas et al., 1999, Kalab et al., 1999, Wilde and Zheng, 1999, Ohba et al., 1999). RanGTP is thought to accumulate at chromosomes, where it creates a favourable environment for microtubule polymerisation, involving many of the proteins involved in centrosome dependent bipolar spindle formation (for review see [Walczak and Heald, 2008]; (Kalab et al., 2006)). In this way Ran instigates a cascade of activities including promoting the activation of Aurora A via TPX2 binding (Targeting protein for Xklp2), which will be discussed later in more detail (Eyers et al., 2003, Tsai et al., 2003).

1.3.2 The centrosome cycle

When present in cells, centrosomes nucleate microtubules through the incorporation of α- and β-tubulin subunits to form long polymers or microtubules (Bornens et al., 1987, Sluder and Rieder, 1985, Mitchison and Kirschner, 1984, Mitchison and Kirschner, 1986, Brinkley et al., 1981, Soltys and Borisy, 1985). The function is however dependent on the passing of the centrosomes through the highly regulated centrosome cycle (Kuriyama and Borisy, 1981) (Figure 1.5).

Centriole duplication

Centrioles are composed of α- and β-tubulin subunits arranged in a barrel shape, with a 9-fold axis of symmetry (Bornens et al., 1987, Komesli et al., 1989). A pair of centrioles begin the cell cycle within a newly formed daughter cell, just after the completion of telophase. In S phase these centrioles duplicate to produce procentrioles, which are connected to their mother by a fibrous link that prevents any further unwanted duplication (Bahe et al., 2005, Faragher and Fry, 2003, Fry et al., 1998, Mayor et al., 2000). In some cell lines such as HeLa and CHO, the presence of a mother centriole is not a necessity for the formation of a daughter centriole, although it does make the process more efficient as well as limiting the number of centrioles produced (Khodjakov et al., 2002, Kato and Sugiyama, 1971, Miki-Noumura, 1977, Marshall et al., 2001, La Terra et al., 2005, Uetake et al., 2007). In mammalian cells, the mother centriole can be distinguished from the daughter by distal and subdistal appendages which mark its state of maturity and nucleation capacity (Piel et al., 2000, Vorobjev and Chentsov, 1980, Kuriyama, 1984, Sluder and Rieder,

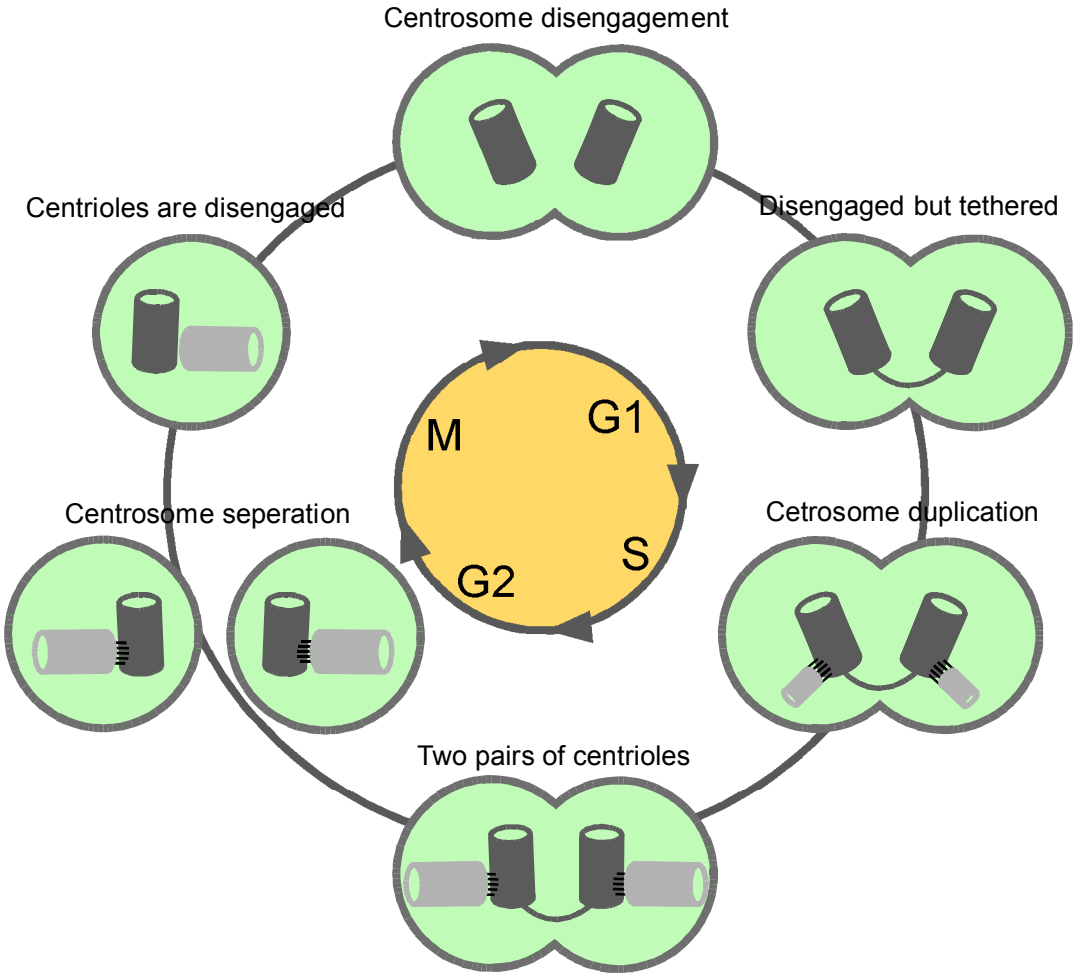

Figure 1.5 The centrosome cycle
The outer circle represents the path of the centrosome cycle, while the inner yellow circle indicates the concurrent phases of the cell cycle. The centrioles are depicted as grey cylinders, the darker version is the mature centriole while the lighter is the immature or daughter centriole. The green areas represent the pericentriolar protein matrix, the curved grey connecting line indicates the positioning of a putative tether between the mature centrioles, and the small black dashes connecting the mature and immature centrioles show that they are engaged to each other (diagram amended from [Lukasiewicz and Lingle, 2009]).

1985). The mother and daughter centrioles adopt an orthogonal orientation, with their respective ends that are situated closest to the nucleus positioned at right angles to each other (Kuriyama and Borisy, 1981).

A succession of experiments revealed that the duplication of centrioles is dependent on several proteins with homologues in human cells, and are coordinated by the activity on Cdk2/Cyclin E and/or Cyclin A and Plk4 (Meraldi et al., 1999, Hinchcliffe et al., 1999, Kleylein-Sohn et al., 2007, Pelletier et al., 2006, Delattre et al., 2006, Andersen et al., 2003, Leidel and Gonczy, 2003, Habedanck et al., 2005). Furthermore the up-regulation of Aurora A or Plk4 expression has been shown to cause centrosome amplification (Meraldi et al., 2002, Zhou et al., 1998, Goepfert et al., 2002, Shao et al., 2006, Duensing et al., 2007, Wang et al., 2006, Ohishi et al., 2010). Supernumerary centrosomes such as these have been shown to function as extra microtubule organising centres, even nucleating microtubules more efficiently than normal centrosomes (Lingle et al., 1998, Pihan et al., 1998). The misregulation of the number of centrosomes has been linked to chromosome instability and the development of cancer (Lingle et al., 1998, Lingle et al., 2002, D'Assoro et al., 2002, Nigg, 2006, Pihan et al., 1998, Dodson et al., 2004, Boveri, 1889, Lingle and Salisbury, 1999, Chng et al., 2006). Furthermore, the absence or disruption of centrosomes at S phase in many cells causes a cell cycle arrest at G1, indicating that the loss of centrosomes is also a source of major stress to the cell (Uetake et al., 2007, Srsen et al., 2006, Mikule et al., 2007). Damaged DNA however has also been shown to produce multipolar cells through causing centrosomes to lose their integrity and split, resulting in the production of multipolar spindles (Hut et al., 2003, Keryer et al., 1984, Sluder and Rieder, 1985).

Elongation and centrosome maturation

After their duplication, the newly formed procentrioles go through a process of elongation throughout S phase to G2, with some cell types recorded as even continuing the elongation process during mitosis (Kuriyama and Borisy, 1981).

The elongating centrioles sit in a fibrous bed called the pericentriolar matrix (PCM), which acts as a scaffold to localise many different proteins (Klotz et al., 1990, Sellitto et al., 1992). One such protein is the highly conserved γ-tubulin, which has been found to be localised to centrosomes, forming a γ-tubulin ring complex (γ-TuRC) mainly between prophase to metaphase (Oakley and Oakley, 1989, Oakley et al., 1990, Stearns et al., 1991, Joshi et al., 1992, Felix et al., 1994). Through electron microscopy, it was revealed that γ-tubulin forms an open ring structure and was found to be localised at the end of microtubules where it is involved in microtubule assembly and functional bipolar spindle formation (Oakley and Oakley, 1989, Oakley et al., 1990, Stearns et al.,

1991, Joshi et al., 1992, Felix et al., 1994) (Paluh et al., 2000, Sampaio et al., 2001, Zheng et al., 1995, Stearns and Kirschner, 1994, Li and Joshi, 1995, Oakley, 1992).

In late G2, the PCM undergoes a phosphorylation-dependent maturation process, in which it increases its size, the number of γ-TuRC it contains, and its nucleation ability (Khodjakov and Rieder, 1999, Casenghi et al., 2003). Plk1, Nek2 protein kinase, protein phosphatase 4 and Aurora A have been associated with the control of the centrosome maturation process, which involves Hef1, TPX2, Bora, NDEL1 and LATS2. Interestingly, all of these proteins are involved in Aurora A signalling, further highlighting the importance of the kinase in the maturation process (Mori et al., 2007, Lane and Nigg, 1996, Hutterer et al., 2006, Pugacheva and Golemis, 2005, Deluca et al., 2006, Toji et al., 2004, Prigent et al., 2005, Martin-Granados et al., 2008). In fact the inhibition of either Aurora A or Plk1 activity delays the progression into mitosis as a consequence of reduced centrosome maturation, a process which will be discussed later in more detail (Lenart et al., 2007, Hirota et al., 2003, Marumoto et al., 2002, Marumoto et al., 2003, Hannak et al., 2001, Portier et al., 2007, Hachet et al., 2007, Qian et al., 1998).

Centrosome separation and bipolar spindle assembly

In late G2 or early mitosis, the link which connects the two centrosomes together is severed by Plk1, Nek2 and possibly Aurora A (Lane and Nigg, 1996, Glover et al., 1995, Fry et al., 1998, Bahe et al., 2005, Tsou et al., 2009, Bahmanyar et al., 2008, Mayor et al., 2000, Helps et al., 2000). The disengaged centrosomes then begin to migrate away from each other, facilitating the formation of the bipolar spindle.

Centrosome migration has been found to be dependent on many factors including the plus-end directed motor protein Eg5 (Mayer et al., 1999) and the minus-end directed motor Dynein and the Dynein binding protein CLIP-170 (Fuller and Wilson, 1992, Vaisberg et al., 1993, Pfarr et al., 1990, Verde et al., 1991, Tanenbaum et al., 2008, Saunders and Hoyt, 1992). However, it is not just the balancing of motors that produces the force to allow the development of the bipolar spindle, as force generated from kinetochores has also been implicated in centrosomal movement (Toso et al., 2009, McAinsh et al., 2006). Furthermore, the movement of the centrosomes during prophase has also been attributed to the presence of astral microtubules (Verde et al., 1991, Vaisberg et al., 1993, Pfarr et al., 1990, Rosenblatt et al., 2004). The regulation of all the forces required to separate the centrosomes and create a bipolar spindle is a highly complicated process, with Aurora A and Plk1 activity playing major parts which will be explored later in this introduction (Lane and Nigg, 1996, Glover et al., 1995, Gonzalez et al., 1998).

Disengagement

The mother and daughter centrioles lose their fibrous intercentriolar link in late mitosis through the action of the APC, Seperase and Plk1 (Wang et al., 2008a, Tsou et al., 2009, Tsou and Stearns, 2006, Nasmyth et al., 2000, Wang et al., 2008b). This disengagement allows the centrioles to duplicate in the subsequent S phase, allowing the centrosome cycle to continue.

1.4 The spindle

The production of mature centrosomes aids in the formation of the highly dynamic tubulin-based structure of the spindle, which is required to arrange chromosomes so that they can be equally separated into two daughter cells. The mitotic spindle begins to form in prometaphase, and by late prometaphase it is fully formed into two radial arrays of dynamically unstable microtubules. The spindle is subject to both physical and chemical factors, enabling the proper attachment, alignment and segregation of chromosomes (Figure 1.6) (Dumont and Mitchison, 2009, Rieder, 1981, Itabashi et al., 2009, Salmon, 1975, Sharp et al., 2000a, Manning and Compton, 2008, Nicklas and Ward, 1994, Manneville and Etienne-Manneville, 2006, Grill and Hyman, 2005).

1.4.1 Microtubule composition

Each spindle fibre consists of α-/β-tubulin heterodimers, which can combine to form protofilaments that associate in sets of ten to fifteen in a parallel orientation to create a hollow tubulin cylinder known as a microtubule (Nogales et al., 1999). The asymmetry of the tubulin subunits confers different physical properties at either end of the microtubule, which allows the plus-ends to grow roughly three times faster than the minus-ends (Wiese and Zheng, 2006, Desai and Mitchison, 1997). The growth of the polymers at either end occurs through the addition of tubulin dimers, which contain GTP in the nucleotide exchangeable site (E-site) within the β-tubulin of the dimer. Depolymerisation occurs upon the hydrolysis of the β-tubulin-bound GTP, which induces a conformational change and causes the peeling away of the individual protofilaments (Mandelkow et al., 1991, Nogales and Wang, 2006, Wang and Nogales, 2005). The ‘GTP cap’ model predicts that the integrity of the microtubule can be maintained by the ‘capping’ of the microtubule polymer with GTP-tubulin subunits, and when this cap is lost the parallel tubulin filaments peel outward and the microtubule rapidly depolymerises (for review see [Gadde and Heald, 2004]). This model indicates a degree of control over the stability of microtubules, a phenomenon that is verified by the observation that the rate of mitotic microtubule turnover that is ten-fold higher than that seen in interphase (Desai and Mitchison, 1997). The dynamic instability of

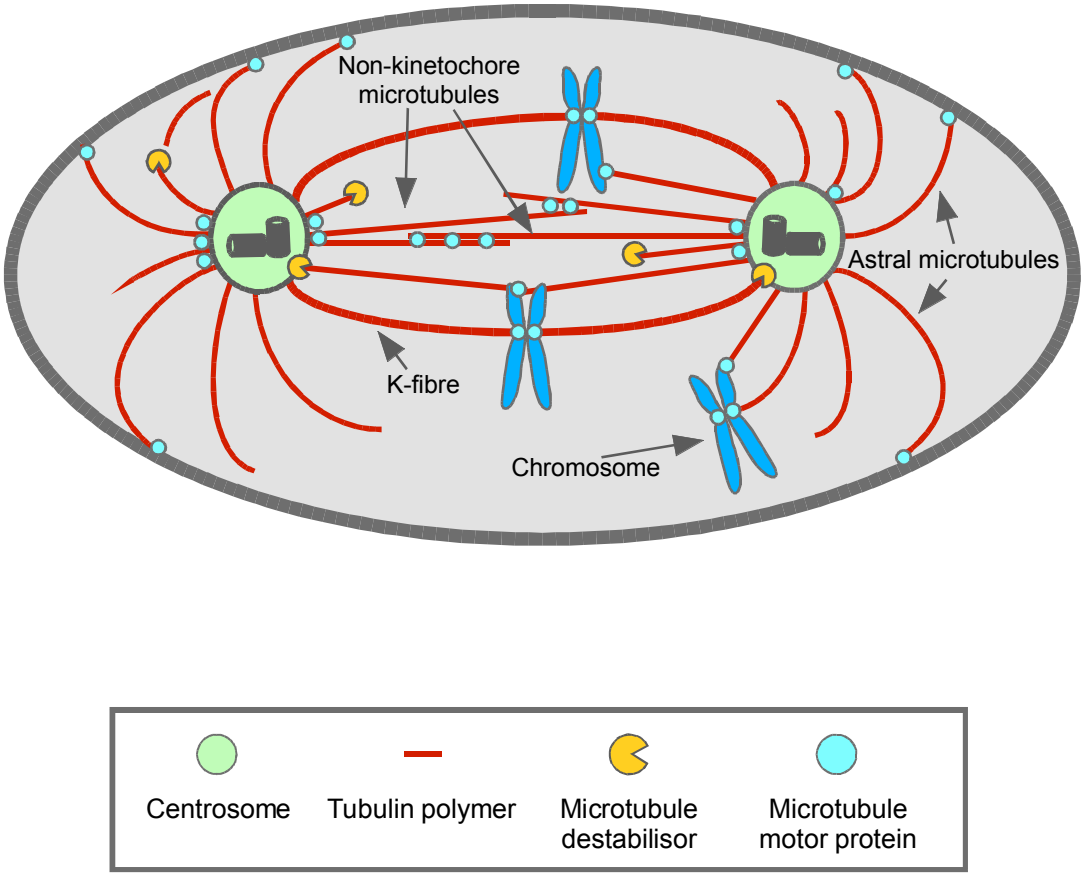

Figure 1.6 Model of the mitotic spindle
The spindle is composed of kinetochore-fibres (K-fibres), non-kinetochore fibres and astral microtubules. The structure and the function of the spindle are defined by the delicate balancing of microtubule stability and movement, which are reliant on microtubule associated proteins such as microtubule destabilisors and microtubule motor proteins (figure amended from [Mollinedo and Gajate, 2003]).

microtubules was investigated using photo-bleaching, which revealed that the turnover of tubulin subunits appeared to cause a flow or 'flux' of tubulin along the length of the microtubules towards the poles (Mitchison, 1989). These findings were advanced with the use of the 'speckling' technique, in which fluorescently labelled tubulin was incorporated into microtubules in a non-uniformed manner, causing the fluorescent striping of microtubules (Waterman-Storer and Danuser, 2002). This allowed the visualisation of both microtubule flux and the stability of the microtubule lattice, thus revealing the dynamics of the spindle during the different phases of the cell cycle (for review see [Gadde and Heald, 2004]).

1.4.2 Cellular microtubule dynamics

The changes in microtubule dynamics throughout the cell cycle and the observation that cellular microtubule dynamics are highly different from those of microtubules formed *in vitro* out of pure tubulin, indicated that cells are able to regulate microtubule stability (reviewed in [Gadde and Heald, 2004]). This regulation has been attributed to the presence of microtubule associated proteins (MAPs), which have been implicated in the control of many areas of microtubule function (Walczak and Heald, 2008, Liska et al., 2004, Mack and Compton, 2001, Gaglio et al., 1996, Goshima et al., 2005a, Sharp et al., 2000b). MAPs have been found to be required from even the very beginning of spindle formation, with γ-TuRCs initiating microtubule nucleation (Moritz et al., 1995). Additional MAPs such as the chTOG/XMAP215 and TACC family have been found to associate along the length of microtubules to increase their stability and even increase polymerisation, while other MAPs have been shown to bind exclusively to microtubule ends (for review see [Schuyler and Pellman, 2001, Howard and Hyman, 2003]; (Gard and Kirschner, 1987, Tirnauer et al., 2002, Gergely et al., 2000a, Lee et al., 2001)). EB1, EB2 and EB3 as well as Clip170 and 155 and the Clasps 1 and 2 have also been found to associate with the plus-ends of microtubules, and by doing so control microtubule stability and dynamics by reducing their depolymerisation and protecting against microtubule destabilising factors (Tirnauer and Bierer, 2000, Akhmanova et al., 2001, Mimori-Kiyosue et al., 2005). MAPs have also been found to reduce microtubule stability by inducing deploymerisation or increasing the levels of depolymerisation. MAPs such as these include the depolymerising kinesins of the Kin I family, including MCAK, which can bind to microtubule ends, subsequently distorting the microtubule lattice and causing the energy dependent peeling away of microtubule protofilaments (Desai et al., 1999). Similarly, Op18/stathmin can bind to tubulin dimmers stimulating microtubule catastrophe (Cassimeris, 2002), and Katanin can destabilise microtubules by either severing the fibres to reveal unstable ends that lack a GTP cap, or breaking microtubules directly at the centrosome (McNally et al., 2000).

The coordination of the MAPs therefore permits the regulation of microtubule dynamics, which can result in substantial pushing or pulling forces that are employed to position chromosomes and contribute to spindle positioning (Inoue and Sato, 1967, Dogterom and Yurke, 1997, Maiato et al., 2005, Faivre-Moskalenko and Dogterom, 2002, Pearson and Bloom, 2004). A mechanism by which microtubules exude a pushing force on particular cellular components has been predicted to occur through a 'Brownian ratchet' method, whereby the formation of transient gaps between the plus-end of the microtubule and the bound object, allows the binding of tubulin subunits thus creating the pushing force (Peskin et al., 1993). Whether this model is accurate remains to be determined, although the existence of proteins associated at the plus-ends of microtubules complicates the model somewhat, and debate still remains about whether kinetochores are actually pushed by spindle forces (Akhmanova and Hoogenraad, 2005, Skibbens et al., 1993, Toso et al., 2009, Waters et al., 1996, Khodjakov and Rieder, 1996). The pulling of chromosomes towards spindle poles is however an accepted method of chromosomal movement, which mainly occurs through K-fibre dynamics (Inoue and Salmon, 1995, Dumont and Mitchison, 2009).

The control over microtubule dynamics is therefore clearly an important aspect of cellular function, however when microtubules reach a certain length or are under a high level of pressure, they can bend and even break (Holy et al., 1997, Waterman-Storer and Salmon, 1997, Janson et al., 2003, Tran et al., 2001). The detection of compression forces such as these may function as a method of controlling microtubule dynamics, as when spindles are placed in a compressed situation structural changes to the spindle are observed (Dumont and Mitchison, 2009). Microtubules also have a degree of elasticity (Gittes et al., 1993, Rubinstein et al., 2009), and are supported by the surrounding elastic cytoskeleton, which together allow the spindle to sustain relatively large compressive forces (Brangwynne et al., 2006, Itabashi et al., 2009).

1.4.3 Actin cytoskeleton

An interconnected actin-myosin micro-filamentous network present within cells enables them to withstand some of the contractile stress they experience (Wang et al., 2001, Ingber, 1993). The presence of a supportive structural network is demonstrated when the disruption of cellular tubulin microtubules does not change the spreading morphology of many cells, indicating that other architectural features are also at work (Domnina et al., 1985, Middleton et al., 1988, Ingber, 1993). The tensegrity hypothesis describes how the contraction and compression forces of microtubules and the cytoskeleton are balanced for normal cellular function, demonstrating the importance of the interconnected relationship between the microtubules and the cytoskeleton (Ingber, 1993). This relationship between tubulin microtubules and the actin cytoskeleton is further demonstrated in

migrating cells, when the organisation of the actin cytoskeleton influences the organisation of microtubules (Salmon et al., 2002).

1.4.4 Spindle microtubules

The mitotic spindle is made up of three categories of microtubule: kinetochore microtubules (K-fibres), non-kinetochore microtubules, and astral microtubules (for recent review see (Dumont and Mitchison, 2009).

K-fibres

K-fibres are created from bundles of 10 to 30 microtubules which are either nucleated at centrosomes then captured by kinetochores, or are nucleated directly from kinetochores (Biggins and Walczak, 2003, Cleveland et al., 2003, Maiato and Sunkel, 2004). K-fibres are the most stable of the three types of microtubule present in the mitotic spindle (Zhai et al., 1995), and experience relatively slow rates of depolymerisation at or near the pole (Hyman and Mitchison, 1990, McDonald et al., 1992). K-fibre plus-ends that interact with chromosomes however, experience faster fluctuations (Hyman and Mitchison, 1990, McIntosh and Euteneuer, 1984). The interaction between K-fibres and chromosomes occurs via kinetochores, which are protein complexes that are intimately associated with the centromeric DNA of each chromatid (Cooke et al., 1993, McEwen et al., 1993, Rieder, 1982).

Non-kinetochore microtubules

Non-kinetochore microtubules are bundles of relatively dynamic microtubules that span the distance between two spindle poles (Mastronarde et al., 1993). Unlike K-fibres, non-kinetochore microtubules do not attach to kinetochores, but instead perform structural roles such as defining the spindle pole position, while also possibly providing a method of motor protein driven chromosomal movement (Saxton et al., 1984, Burbank et al., 2006, Burbank et al., 2007, Inoue and Salmon, 1995).

Astral microtubules

Astral microtubules are the third type of spindle microtubules, which have been found to be nucleated at the centrosomes where they are capped with γ-TuRC and have highly dynamic plus-

ends. (Grill et al., 2003, Rusan et al., 2001). The major function of astral microtubules within the cell is the positioning of the spindle within the cell (Cowan and Hyman, 2004, Manneville and Etienne-Manneville, 2006).

1.4.5 Spindle positioning

The proper positioning of the spindle within cells permits chromosomes to first be appropriately positioned during metaphase, and then evenly segregated during cell division. The prevention of proper functioning of microtubule dynamics through the mutation of β-tubulin prevents the correct positioning of the spindle within cells, demonstrating the importance of microtubules during the positioning process (Wright and Hunter, 2003, Gupta et al., 2002). Furthermore, shortened astral microtubules are unable to properly position the spindle within *C. elegans* cells (Cowan and Hyman, 2004). Astral microtubules position the spindle by making physical contacts with the plasma membrane or the actin cytoskeleton, enabling force to be applied to the spindle (Manneville and Etienne-Manneville, 2006). Dynein, Dynactin and LIS1 have been implicated in the generation of the pulling force achieved by astral microtubules. Additionally, Dynein has also been attributed to controlling microtubule polymerisation dynamics (Dujardin and Vallee, 2002, Dujardin et al., 2003, Hunter and Wordeman, 2000). Consistent with their role in astral microtubule spindle positioning, the leading-edge of migrating cells and the membranes of mitotic cells, have been shown to have enriched Dynein or Dynactin which associate with astral microtubule plus-ends (Dujardin et al., 2003, Busson et al., 1998, Skop and White, 1998). Dynein is also present at centrosomes, and has been found to have essential functions in the linking of the centrosome to the microtubule minus-ends (Askham et al., 2002). EB1, Adenomatous Polyposis Coli (APC), and Clips 170 and 155, are all also connected either directly or indirectly to Dynein to enable spindle positioning through the generation of plus-end astral microtubule forces (reviewed in [Manneville and Etienne-Manneville, 2006]).

The importance of proper spindle positioning is observed during asymmetric cell division, which is employed by both single-cell organisms (such as yeast) and during stem cell division in multicellular organisms (reviewed in [Fraschini et al., 2008]). Asymmetric cell division in multicellular organisms allows the generation of a daughter cell that is chemically different from its mother, permitting growth or the maintenance of tissue homeostasis. Through the regulation of astral microtubule pulling forces, G-proteins, PAR proteins and their regulators allow the spindle to be arranged in response to polarity factors (Gotta and Ahringer, 2001, Colombo et al., 2003, Srinivasan et al., 2003, Grill et al., 2003, Labbe et al., 2003, Ahringer, 2003). The positioning of the spindle is monitored by the spindle position checkpoint which delays cytokinesis until the DNA

is deposited into both mother and daughter cells. This mechanism inhibits the signalling of the mitotic exit network (MEN) in budding yeast, and the septation-initiation network (SIN) in fission yeast (for review see [Bardin and Amon, 2001]). Experiments in fission yeast have also revealed that the integrity of the actin cytoskeleton is also monitored by the spindle position checkpoint ((Gachet et al., 2001); reviewed in [Gachet et al., 2006]).

1.4.6 Spindle length

Similar to spindle positioning within the cell, spindle length is defined by a number of antagonistic pushing and pulling forces (Sharp et al., 1999). Molecular motor proteins cause antiparallel microtubules to slide apart, therefore when mid-zone microtubules are disrupted, the poles of spindles move in towards the centre of the cell (Leslie and Pickett-Heaps, 1983, Tolic-Norrelykke et al., 2004). In addition to experiencing connected movements, centrosomes within vertebrate prometaphase cells also experience independent movement, indicating that antiparallel sliding is not the only spindle length determinant (Waters et al., 1993). Astral microtubules have been implicated in the divergence of centrosomes in interphase and during mitosis (Waters et al., 1993, Vogel et al., 2007, Tolic-Norrelykke et al., 2004). Much of this astral microtubule-dependent movement is attributed to the connections with the cell cortex that are mediated by Dynein, which generates pulling forces, aiding both spindle positioning and elongation (Fink et al., 2006). By attaching the spindle to the cortex in this way, astral microtubules can coordinate the length of the spindle with that of the cell (Schultz and Onfelt, 2001). In addition to aiding to formation of a bipolar spindle, astral microtubules can also limit centrosome separation during anaphase, and when severed, the poles are permitted to separate at a faster pace (Aist and Berns, 1981, Aist et al., 1993).

1.4.7 Motor MAPS

As well as regulating spindle length and stability, microtubule associated proteins also promote spindle bipolarity by cross-linking microtubules, as well as by transporting cargo along microtubules (Walczak et al., 1998, Walczak and Heald, 2008, Wittmann et al., 2001). Motor MAPs walk either towards the minus- or plus-ends of microtubules, and by doing so generate force. This method of force generation allows the motors to move objects along microtubules, move and arrange microtubules or regulate microtubule stability.

The kinesin-5 motor protein family (Eg5 in human cells) associate preferentially to spindle fibres arranged in an antiparallel fashion, where they move along in a plus-end directed manner, causing

the sliding apart of the fibres, aiding the formation of bipolar spindles (Kapitein et al., 2005, van den Wildenberg et al., 2008, Sharp et al., 1999, Mayer et al., 1999, Brust-Mascher et al., 2009). The localisation of Eg5 to spindle microtubules is dependent on a phosphorylation from Cdk1, and when localised, its positioning is surprisingly unaffected by microtubule flux (Blangy et al., 1995, Sawin and Mitchison, 1995, Kapoor and Mitchison, 2001).

Similar to the kinesin-5 motor protein family, the minus-end directed kinesin-14 motor proteins (HSET in human cells) aid spindle formation by cross-linking fibres, locking together parallel microtubules and sliding apart adjacent antiparallel microtubules (Fink et al., 2009, Mountain et al., 1999, Tao et al., 2006). Similarly, Dynein and Dynactin cross-link microtubule free-ends enabling the rearrangement of microtubules and spindle pole focusing (Fink et al., 2006, Verde et al., 1991, Gaglio et al., 1997).

The balancing of forces of motor proteins such as those described, allows the maintenance of a steady state spindle length, as well as focusing the spindle poles and organising the spindle structure (Burbank et al., 2007). Adding to their roles in spindle organisation, kinesin-5 and -14 have also been associated with microtubule dynamics by promoting plus-end disassembly in yeast (Sproul et al., 2005, Gardner et al., 2008). When the finely tuned balance of kinesins is disrupted, such as when using Monastrol to allosterically inhibit Eg5 activity, the spindle structure is disrupted. Monastrol works by reducing the motorised movement of Eg5 along microtubules and even causes a decrease in Eg5 microtubule association, leading to the collapse of bipolar spindles (Kwok et al., 2006, Cochran and Gilbert, 2005, Cochran et al., 2005).

In addition to motor proteins on antiparallel microtubules, the presence of plus-end directed Kin N kinesins on chromosome arms also contributes to movement within the spindle. These motor proteins cause the chromosome arms to be pushed away from the spindle poles by their interaction with non-kinetochore microtubules, producing a phenomenon called the ‘polar ejection force’ (reviewed in [Heald, 2000]). With increased distance from the spindle pole, the polar ejection forces decrease, thus producing a steady-state chromosome position (Rieder et al., 1986, Ke et al., 2009). The kinesin, Kid, has been proposed to be responsible for producing the polar ejection force in vertebrate cells (Levesque and Compton, 2001). Indeed, Kid inhibition reduces the distance of chromosomes from the poles, highlighting its role in chromosome positioning, however because the length of bipolar spindles is only reduced by 20% in mammal cells and spindle lengths are unaffected in *Xenopus* extracts, additional methods of maintaining chromosome positioning are clearly in place (Tokai-Nishizumi et al., 2005, Funabiki and Murray, 2000, Levesque and Compton, 2001).

Lateral microtubule connections with the kinetochore also cause the movement of chromosomes towards poles, a movement that has also been attributed to the presence of motor proteins (Rieder and Alexander, 1990, Merdes and De Mey, 1990). Consistent with the existence of motor proteins at the kinetochores, the Kin N plus-end directed motor CENP-E has been detected at kinetochores and is required for chromosome positioning (Gadde and Heald, 2004). Despite the clear role for a variety of motor proteins in the movement of chromosomes and the maintenance of the spindle length, deletion of minus-end directed motor proteins in yeast did not appear to affect chromosomal movement, indicating that microtubule dynamics play a major role in the process (Grishchuk and McIntosh, 2006).

In addition to their described roles in the proper alignment of chromosomes, MAPs are also required to regulate the structure of the spindle after chromatid separation. During anaphase, many MAPs associate with the central spindle in order to regulate the process. The most well characterised of these MAPs are Centralspindlin, Protein regulating cytokinesis 1 (PRC1), and the Chromosome Passenger Complex (CPC) (reviewed in [Glotzer, 2009]). PRC1 in human cells cross-links antiparallel microtubules of the spindle, enabling the establishment of the spindle midzone to allow additional MAPs to bind and regulate cytokinesis (Zhu et al., 2006, Schuyler et al., 2003, Jiang et al., 1998, Peterman and Scholey, 2009). PRC1 also directly binds to Plk1 during anaphase, an interaction that is required from the completion of cytokinesis (Neef et al., 2007). Similarly, the Centralspindlin complex, consisting of a dimmer of a kinesin-6 motor protein (MKLP1) bound to a dimmer of the Rho family GTPase activating protein (GAP) CYK4, also promotes microtubule bundling which is required for cytokinesis (Pavicic-Kaltenbrunner et al., 2007, Mishima et al., 2002). Proteins which are localised to the spindle midzone include the CPC, who's localisation to the area is thought to be dependent on INCENP (inner-centromere binding protein) (Wheatley et al., 2001).

Some MAPs are also directly responsible for the movement and transport of cellular components throughout the spindle. NuMA is transported to the minus-ends of microtubules by Dynein/Dynactin, where it serves a role in spindle pole cohesion (Merdes et al., 2000). Dynein itself is also positioned at the microtubule ends at the cell cortex, and so can also help to orient astral microtubules (Gadde and Heald, 2004, Vaughan et al., 2002).

1.4.8 MAP regulation

MAPs clearly have a huge diversity of actions, and their delicate coordination is highly complicated. MAPs are involved in numerous signalling pathways, a feature that was uncovered through the identification of many novel phosphorylation sites on spindle proteins (Nousiainen et

al., 2006, Manning and Compton, 2008, Nigg, 2001a). The balancing of these signalling pathways enables the coordination of microtubule dynamics and the development of the spindle structure (Howard and Hyman, 2007).

It is predicted that much of the control over MAPs and their signalling pathways occurs by a gradient of morphogens, which diffuse from a source in the chromatin, producing activity gradients that define areas of microtubule nucleation and stabilisation (Caudron et al., 2005). Ran-GTP and Aurora B kinase activity have been identified as diffusible spindle morphogens (for review see [Dumont and Mitchison, 2009]; (Kalab et al., 2006, Caudron et al., 2005, Fuller et al., 2008)). In addition to chromosome derived morphogen gradients, spindle poles have also been implicated in generating gradients of regulatory factors able to contribute to the formation of the spindle (Greenan et al., 2010).

A sub-category of proteins involved in the signalling pathways are kinases, enzymes which phosphorylate specific protein substrates by transferring a phosphate group from a donor molecule, such as ATP, to specific sites on the substrate protein. This phosphorylation event can modify the activity or localisation of the involved proteins, thus allowing the transmission of chemical signals. Protein kinases mediate the majority of signal transduction in eukaryotic cells, and have been found to govern the regulation of the spindle assembly, making analysis of these enzymes an essential precursor to understanding the functioning of life processes (Manning et al., 2002). The misregulation of kinases has also been linked to the onset of disease, demonstrating the important regulatory roles they hold (Blume-Jensen and Hunter, 2001, Malumbres and Barbacid, 2007).

1.5 An overview of the Aurora kinase family

The Aurora kinases are a family of highly conserved kinases, which phosphorylate the OH group of a serine or threonine residue within specific target proteins, and are therefore known as serine/threonine kinases. The Aurora kinases contribute to the intricate control of the cell cycle from G2 through to cytokinesis in organisms ranging from yeast to humans. Yeast only have one Aurora kinase, whereas higher organisms have at least two Aurora family members called Aurora A and Aurora B in mammalian cells. A third Aurora kinase, Aurora C, is also seen in specific mammalian meiotic cells (for review see [Andrews et al., 2003, Carmena and Earnshaw, 2003]; (Chan and Botstein, 1993, Glover et al., 1995, Girdler et al., 2006, Terada et al., 1998, Bernard et al., 1998, Marumoto et al., 2003, Kimmins et al., 2007)).

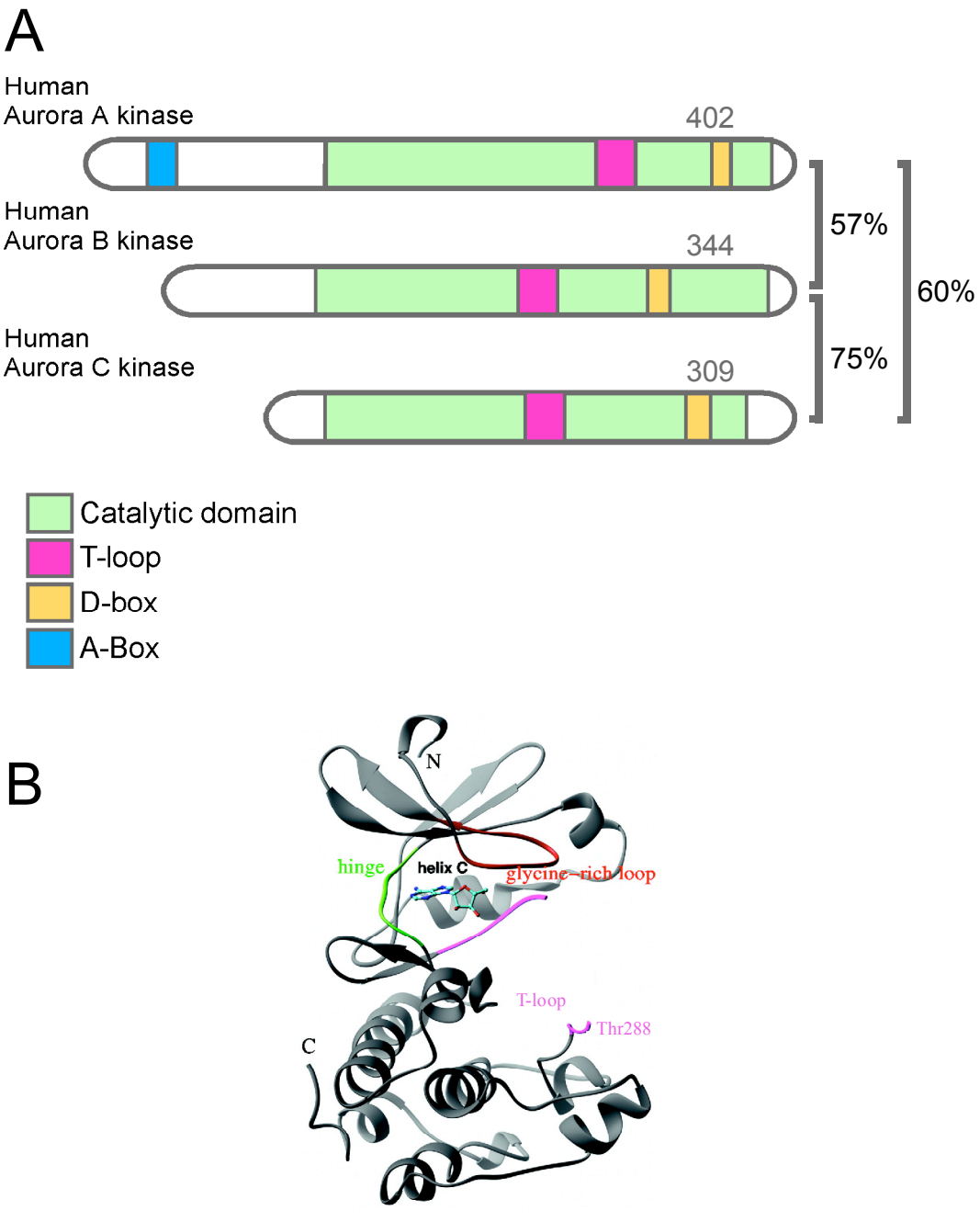

Figure 1.7 Aurora kinase structure
(A) The members of the human Aurora kinase family shown by schematic representation. The grey numbers indicate the individual sizes of the Aurora proteins in amino acids, and the percentages refer to the degree of sequence identity shared between the individual family members. The coloured portions of the diagrams represent the relative positions of the functional domains of the proteins. The catalytic domains of the kinases are shown in green, while the activation loops (T-loops) are shown in pink. The sequences that enable the targeting of Aurora A kinase for proteolysis are the destruction box (D-box) shown in yellow and the D-box activating domain (A-box) indicated in blue. Although the similar D-boxes occur in both Aurora B and C kinases, they have not been found to target the proteins for proteolysis (figure amended from [Carmena and Earnshaw, 2003] and [Keen and Taylor, 2004]). (B) The 3-dimentional structure of Aurora A kinase in complex with adenosine. The indicated hinge region (green), activation loop (pink) the glycine-rich loop (red), and the Thr288 residue are important for the activation of the kinase (image taken and amended from (Cheetham et al., 2002)).

1.5.1 The Aurora kinases in Yeast

The importance of the Aurora family was revealed after certain mutant budding yeast strains experienced a gain of chromosomes or 'increase in ploidy' after mitosis (Chan and Botstein, 1993). Molecular cloning and genetic mapping of the mutant stains identified an uncharacterised gene, which was aptly named 'Increase in Ploidy 1' (Ipl1). Ilp1 was later recognised as the only Aurora family member present in budding yeast and was found to be localised to both the mitotic spindle and kinetochores, co-localising with and phosphorylating various kinetochore proteins on an Aurora consensus site (Kang et al., 2001, Cheeseman et al., 2002, Biggins et al., 1999, Hsu et al., 2000). Ipl1 was found to govern kinetochore-microtubule interactions by altering the connections in response to the low tension they generated at kinetochores (Pinsky et al., 2006, Biggins and Murray, 2001, Biggins et al., 1999, Chan and Botstein, 1993, Tanaka et al., 2002).

Fission yeast also contains a single member of the Aurora kinase family, named Aurora related kinase 1 (Ark1). Similar to Ipl1, Ark1 localises to kinetochores and centromeres during mitosis, and transfers to the mitotic spindles during anaphase (Petersen et al., 2001). Furthermore, Ark1 has been found to phosphorylate histone H3, contribute to kinetochore activity and regulate spindle formation (Petersen et al., 2001). Unlike its budding yeast cousin however, the fission yeast Aurora kinase was found to be required for a cellular response to the lack of microtubule-kinetochore attachment, as well as controlling the alteration of low tension connections (Petersen and Hagan, 2003).

1.5.2 The Aurora kinase family in higher organisms

The three different Aurora kinase homologues in mammalian cells have varying peptide lengths but are highly similar in terms of structure and sequence, with a 70% degree of homology in their catalytic domain (Figure 1.7A)(for review see [Keen and Taylor, 2004]). Despite these similarities, the different Aurora kinase family members localise to distinct areas and exhibit divergent functions during mitosis (Giet and Prigent, 1999, Giet and Glover, 2001, Carmena and Earnshaw, 2003, Bischoff et al., 1998, Crosio et al., 2002).

1.6 Aurora A kinase

Aurora A kinase activity is the target of a number of promising cancer therapies currently in development, making it an extremely interesting kinase both clinically and academically (Karthigeyan et al., 2010, Ochi et al., 2009). Despite this, at the begining of this project, relatively

little was known regarding the kinase and its activity, making it an ideal topic for investigation and thus the focus of this research project.

1.6.1 Aurora A kinase localisation

Aurora A protein is present throughout the whole cell cycle, although from G1 to S it is only present at low levels. The protein localises to the centrosomes immediately after S phase, after which its levels increase, reaching a peak early in mitosis (Kimura et al., 1997, Bischoff et al., 1998, Roghi et al., 1998).

Human Aurora A is targeted to centrosomes by its first 310 amino acids, a localisation which is thought to be stabilised by its catalytic C-terminus interacting with centrosome components, such as the motor protein Eg5 (Giet and Prigent, 2001, Roghi et al., 1998, Stenoien et al., 2003). The protein remains in constant and rapid flux throughout mitosis, travelling between the centrosomes and the adjacent microtubules to the cytoplasm, which is indicative that Aurora A's major functions lie in regulatory signalling, rather than having structural roles (Berdnik and Knoblich, 2002, Stenoien et al., 2003).

1.6.2 Aurora A kinase Activity

Aurora A kinase is initially activated during late G2 through a phosphorylation which occurs on a T-loop residue (T288 in humans) within its catalytic domain (Figure 1.7B) (Cheetham et al., 2002, Van Horn et al., 2010, Walter et al., 2000, Ohashi et al., 2006, Dodson et al., 2010). In its active state, the flexible T-loop is positioned so that the phosphorylated T288 is exposed to the solvent, where an inactivating dephosphorylation by protein phosphatase 1 (PP1) can take place (Walter et al., 2000). Aurora A can also phosphorylate and inhibit PP1 activity during mitosis, while the regulatory subunit of PP1, Inhibitor 2, has also been shown to bind to and activate Aurora A *in vitro*, indicating the presence of a feedback loop (Katayama et al., 2001, Satinover et al., 2004). A second protein phosphatase, PP2A, has also been shown to both indirectly and directly inactivate Aurora A kinase activity either by dephosphorylating it or by stabilising Securin, which can then inhibit the kinase (Eyers et al., 2003, Horn et al., 2007, Tong et al., 2008). Similarly, p53 can also inhibit Aurora A either indirectly through regulating the transcription of a secondary protein, or directly when co-localised with the kinase at the centrosome (Shao et al., 2006).

Aurora A activity has been shown to be further regulated by the condensation of chromatin which activates Ran (a small GTPase), stimulating the release of TPX2 (targeting protein for Xklp2) from an inhibitory complex (Kufer et al., 2003, Kufer et al., 2002). TPX2 binds to the N-terminus of

Aurora A, inducing a conformational change which allows the autophosphorylation of Thr288 on the T loop of the kinase (Eyers et al., 2003). The bound TPX2 also prevents its dephosphorylation by protein phosphatase 1 (PP1) (Eyers et al., 2003, Tsai et al., 2003, Kufer et al., 2003, Kufer et al., 2002, Bayliss et al., 2003). The interaction with TPX2 localises Aurora A to the mitotic spindle, and although attachment to microtubules is not essential, it has been shown to enhance activation (Gruss et al., 2001, Eyers and Maller, 2004, Bayliss et al., 2003). TPX2 is also phosphorylated by *Xenopus* Plk1, which increases the activation of Aurora A (Eckerdt et al., 2009). Interestingly, building a spindle of the correct length requires the interaction between TPX2 and Aurora A, highlighting the importance of the TPX2 activation of Aurora A (Bird and Hyman, 2008).

Aurora A also interacts with the LIM domain-containing protein, Ajuba, which promotes the phosphorylation and full activation of Aurora A (Hirota et al., 2003, Goyal et al., 1999). The depletion of Ajuba has been shown to prevent the activation of Aurora A at centrosomes (Hirota et al., 2003). p21-activated kinase 1 (Pak1), a protein important for regulating focal adhesions, can also bind to Aurora A, promoting the phosphorylation of Aurora A at both Thr288 and Ser342 (Zhao et al., 2005). Furthermore, Arpc1b, a component of the actin related protein 2/3 (Arp2/3) complex which initiates the formation of actin filaments and has also been found to activate Aurora A and thus may aid centrosome maturation (Zigmond, 1998, Goley et al., 2006, Goley and Welch, 2006, Molli et al., 2010). The depletion or inhibition of Integrin-linked kinase (ILK), which has role in focal adhesions, also prevents Aurora A from regulating TACC3, possibly indicating a further method of Aurora A activation (Fielding et al., 2008).

When activated at centrosomes during late G2, Aurora A activates Plk1 through a phosphorylation event which is greatly enhanced by Bora, an Aurora A co-factor (Macurek et al., 2008, Hutterer et al., 2006). Plk1 has also been shown to regulate Aurora A through Bora, indicating a feed-forward mechanism in promoting the activation of both kinases (Chan et al., 2008, Seki et al., 2008).

1.6.3 The role of Aurora A kinase in bipolar spindle assembly

Aurora A kinase has been found to serve several functions within the cell, the first of which was reported through work with *Drosophila* embryos which displayed monopolar spindles after the inaction of the kinase (Glover et al., 1995). The inactivation of Aurora A kinase activity was later observed to also cause the occurrence of monopolar spindles in a range of other organisms from *Xenopus* to humans (Roghi et al., 1998, Girdler et al., 2006, Hannak et al., 2001, Mori et al., 2007, Liu and Ruderman, 2006). Indeed, Aurora A inactivation has been shown to cause the collapse of a previously assembled bipolar spindles in *Xenopus* egg extracts (Giet and Prigent, 2000). Other reports have alternatively described the formation of ‘abnormal spindles’, in the absence of Aurora

A activity, whereby centrosomes were able to separate, but spindles were disorganised (Berdnik and Knoblich, 2002, Giet et al., 2002, Schumacher et al., 1998b, Ozlu et al., 2005, Peset et al., 2005, Hoar et al., 2007, Manfredi et al., 2007). This inconsistency may be due to the different experimental strategies employed, however both reports highlight the requirement for Aurora A activity in normal bipolar spindle formation.

Another protein involved in bipolar spindle assembly is the motor protein Eg5, which has been shown to interact with and be phosphorylated by Aurora A, possibly indicating an additional method by which Aurora A activity controls bipolar spindle formation (Walczak et al., 1998, Giet and Prigent, 2000, Giet et al., 1999, Mayer et al., 1999, Kapitein et al., 2005, Koffa et al., 2006). Similarly, Aurora A kinase has also been found to interact with and phosphorylate the spindle associated protein ASAP (Aster-Associated Protein), which has been found to be required for spindle assembly and timely mitotic progression (Saffin et al., 2005). When Aurora A is prevented from doing so, ASAP is destabilised and spindle assembly, spindle pole integrity and even cell division are compromised, providing an additional method of Aurora A control over spindle formation (Venoux et al., 2008, Eot-Houllier et al., 2010). Additionally Aurora A activity is required for the efficient microtubule nucleation of astral microtubules, and therefore the role for the kinase in bipolar spindle formation may also be derived from its regulation of these pulling forces (Kinoshita et al., 2005, Motegi et al., 2006, Giet et al., 2002, Rosenblatt et al., 2004).

Aurora A also regulates the activity and localisation the microtubule depolymerisors Kif2a and MCAK (or Kif2c), which have been shown to have distinct roles, although both contribute to the formation of bipolar spindles (Ganem and Compton, 2004). In *Xenopus* egg extracts, MCAK activity and localisation to Ran asters and spindle poles was found to be regulated by Aurora A activity (Zhang et al., 2008). These phosphorylation events were required to aid both centrosome-independent pole focusing and bipolar spindle formation (Zhang et al., 2008). Aurora A also interacts with and phosphorylates Kif2a, a microtubule depolymerise required for spindle assembly and chromosome congression, consequently suppressing Kif2a depolymerise activity (Jang et al., 2008, Jang et al., 2009). Aurora A inhibition was shown to increase Kif2a on spindle microtubules, causing a reduction in microtubule polymers (Jang et al., 2009). Kif2a is also positively regulated by Plk1, and due to the interconnected nature of Aurora A and Plk1 throughout mitosis discussed previously in this introduction, this antagonistic regulation demonstrates an intriguing and complex method of controlling Kif2a activity (Jang et al., 2009). The balancing of depolymerises such as MCAK and Kif2a enables the highly dynamic structure of the spindle to remain remarkably constant.

1.6.4 The role of Aurora A kinase in centrosome-independent bipolar spindle assembly

Aurora A kinase has also been found to be required for the nucleation, stabilisation and focusing of microtubules as well as the formation of bipolar spindles in environments which lack centrosomes. In *Xenopus* egg extracts which lack chromosomes and centrosomes Aurora A is responsible for enhancing Ran-GTP-induced bipolar spindle assembly through promoting the formation of a particular complex. This complex comprises of the microtubule cross-linking and bipolar spindle promoting proteins Eg5 and TPX2 (Kapitein et al., 2005, Manning and Compton, 2007), the microtubule stabiliser chTOG/XMAP215, the microtubule bundler HURP (Koffa et al., 2006, Sillje et al., 2006) and Aurora A itself (Berdnik and Knoblich, 2002, Liu and Ruderman, 2006). When correctly assembled, the different components of the complex work together to provide an method of microtubule nucleation and spindle assembly, which is completely independent of centrosomes. The requirement for Aurora A in centrosome-independent bipolar spindle assembly highlights the kinase's strong connection with the organisation of microtubules and regulation of microtubule stability and prominent role in bipolar spindle formation.

1.6.5 The role of Aurora A kinase in centrosome maturation

In addition to regulating the formation of a normal bipolar spindle, Aurora A promotes the proper maturation of centrosomes (Hannak et al., 2001, Mori et al., 2007, Berdnik and Knoblich, 2002, Giet et al., 2002, Barros et al., 2005, Terada et al., 2003, Hirota et al., 2003, Greenan et al., 2010). The maturation of centrosomes increases both their size and nucleation capacity, enabling the organised growth of the mitotic spindle (Greenan et al., 2010, Hannak et al., 2001, Khodjakov and Rieder, 1999, Piehl et al., 2004). By causing the maturation of the centrosomes, a positive feedback loop is created, which promotes further Aurora A recruitment to the centrosome, thus amplifying the maturation (Portier et al., 2007).

As discussed earlier, centrosomal maturation is an essential step in the organisation and nucleation of the mitotic spindle, involving the recruitment of many different proteins, a process found to involve Aurora A activity (Hannak et al., 2001, Berdnik and Knoblich, 2002). Aurora A stimulates the recruitment of the essential centrosomal component γ-tubulin to the spindle poles, through controlling Centrosomin (CNN) in *Drosophila* and Lats2 in human cells (Berdnik and Knoblich, 2002, Toji et al., 2004, Terada et al., 2003, Abe et al., 2006). The integrity of the centrosome and the organisation of the spindle poles are also proposed to be regulated by Aurora A which, through an interaction with Astrin, controls Seperase activity (Gruber et al., 2002, Thein et al., 2007, Yuan et al., 2009). Astrin has also been shown to be involved in the localisation of Aurora A to mitotic

spindles, and depleting Astrin levels causes a mitotic arrest similar to that seen after the depletion of Aurora A protein (Du et al., 2008).

The recruitment of TACC3 (transforming acidic coiled coil protein 3) to the centrosome and spindle microtubules is also controlled by its direct phosphorylation by Aurora A, or by an indirect phosphorylation by Plk1 (Giet et al., 2002, Mori et al., 2007, Barros et al., 2005, Kinoshita et al., 2005, LeRoy et al., 2007). Interestingly, cells with inactivated Plk1 have reduced centrosome maturation and form monopolar spindles, thus highlighting the importance of the kinase in centrosome maturation (Lane and Nigg, 1996, Sumara et al., 2004, Lenart et al., 2007) Furthermore, the immunodepl**e**tion of Aurora A or the prevention of Aurora A phosphorylation of TACC, reduces the level of microtubules nucleated from centrosomes, particularly in the case of astral microtubules (Barros et al., 2005, Wang et al., 2008a).

The recruitment of phosphorylated TACC3 stabilises centrosomal microtubules through loading chTOG/XMAP215 to the minus-ends of centrosome microtubules (Barros et al., 2005, Kinoshita et al., 2001, Kinoshita et al., 2005, Peset et al., 2005, Lee et al., 2001, Gergely et al., 2003). The protein known as XMAP215 was first identified in *Xenopus* egg extracts, were it was found to increase the elongation rate of microtubules *in vitro* (Gard and Kirschner, 1987). The function of the protein in increasing microtubule length and mass was determined to be derived through the promotion of the polymerisation rate, the slowing in the switching between microtubule polymerisation and depolymerisation states, and encouraging the formation of highly dynamic microtubules, all of which have particular importance in mitosis (for review see [(Peset and Vernos, 2008]). This was later found to be due to the proteins ability to counter the microtubule destabilising activity of MCAK (Tournebize et al., 2000). XMAP215 was subsequently discovered to be related to a human protein which was over-expressed in tumour cells called chTOG (colonic and hepatic tumour over-expressed protein). Depletion of chTOG/XMAP215 produces disorganised spindles consisting of relatively robust microtubules (Gergely et al., 2003, Cassimeris and Morabito, 2004). The interaction between Aurora A and TACC3/chTOGXMAP215 is maintained by the serine/threonine kinase and scaffold protein Integrin-like kinase (ILK), and its depletion or inhibition disrupts spindle organisation (Fielding et al., 2008).

1.6.6 The role of Aurora A in mitotic entry

The part played by Aurora A in centrosome maturation extends to the regulation of the entry into mitosis. Cells depleted of Aurora A experience delays in their nuclear envelope breakdown, although the timing of chromosome decondensation is not altered. This phenotype is also seen after the prevention of centrosome maturation, further supporting the notion that Aurora A activity is

involved in the maturation of the centrosome (Liu and Ruderman, 2006, Hachet et al., 2007, Portier et al., 2007). Consistent with its role in promoting mitotic entry, Aurora A has been shown to phosphorylate Cdc25B (Dutertre et al., 2004, Cazales et al., 2005), which promotes the activation of Cyclin B1-Cdk1and the entry into mitosis (Hirota et al., 2003). Aurora A has also been linked to the regulation of the translation Cyclin B1 in late G2, which can then be recruited to the centrosome to further promote mitotic entry (Hirota et al., 2003, Warner et al., 2003). Aurora A kinase may also control mitotic entry through regulating the threshold of Cyclin B1 that permits the entry into mitosis, with delays in the entry to mitosis reported to occur due to an increase in Cyclin B1 threshold in interphase cells following the depletion of Aurora A (Satinover et al., 2006, Satinover et al., 2004). Cyclin B1-Cdk1 activation is also accentuated by the activation of Plk1 by Aurora A and Bora, which initiates mitotic entry following a checkpoint dependent arrest (Lenart et al., 2007, Qian et al., 1998, van Vugt et al., 2004, Macurek et al., 2008, Seki et al., 2008, Van Horn et al., 2010).

1.6.7 Aurora A kinase degradation

At anaphase and early G1, the presence of Aurora A is reduced through ubiquitin-dependent proteolysis by the APC^{Cdh1} complex (Marumoto et al., 2002, Gurden et al., 2010, Marumoto et al., 2003). Degradation of the protein is dependent on the recognition of two motifs: a destruction box (D box) at the C-terminus, and an A-box in the N-terminus (Honda et al., 2000, Castro et al., 2002). This process is regulated by a phosphorylation at Ser51, which renders the kinase resistant to APC mediated degradation and by AIP (Aurora A kinase-interacting protein), which counters this event by initiating its proteosome dependent degradation (Kiat et al., 2002).

1.7 Aurora B kinase

1.7.1 Aurora B activation and localisation

Aurora B kinase forms part of a multimeric chromosome passenger complex (CPC) made up of INCENP, Survivin and Borealin, which brings about the activation of Aurora B kinase by causing a conformational change in its structure and localising it to the chromosomes (Vader et al., 2006a, Uren et al., 2000, Wheatley et al., 2001, Bishop and Schumacher, 2002, Bolton et al., 2002, Vagnarelli and Earnshaw, 2004, Sessa et al., 2005, Jeyaprakash et al., 2007, Kelly et al., 2007, Jelluma et al., 2008).

On entry to mitosis the CPC is localised to the arms of chromosomes and inner centromere. The proportion of the CPC localised to the chromosome arms decreases as cells progress through mitosis, with the majority targeted instead to the inner centromere (Beardmore et al., 2004). Aurora A may also be indirectly responsible for the concentration of Aurora B to the inner centromere and even kinetochore function through the phosphorylation of CENP-A on Ser7 during prophase (Kunitoku et al., 2003). Similarly, Disc-60kD (TD-60), a protein required for the progression from prometaphase to metaphase, is also involved in localising Aurora B to centromeres and is required for Aurora B to reach its full activation (Mollinari et al., 2003, Rosasco-Nitcher et al., 2008). At anaphase the localisation of the complex changes again, relocalising to the spindle midzone. The activation of Aurora B at its various localisations is balanced by inhibitory agents such as PP1, PP2A and BubR1, which negatively regulate its action (Sugiyama et al., 2002, Lampson and Kapoor, 2005, Sun et al., 2008).

1.7.2 The role of Aurora B kinase and chromosome alignment

Like the yeast Aurora kinase Ipl1, Aurora B is proposed to sense microtubule-kinetochore interactions, and consequently promote chromosome biorientation through destabilising kinetochore attachments that induce low tension (Ditchfield et al., 2003, Hauf et al., 2003, Tanaka et al., 2002, Sampath et al., 2004, Liu and Lampson, 2009). Microtubule connections are altered by Aurora B through targeted phosphorylation of MCAK and Kif2b, which controls their localisation and therefore their action at centromeres (Andrews et al., 2004, Gorbsky, 2004, Tanaka et al., 2002, Ducat and Zheng, 2004, Kline-Smith et al., 2004, Lan et al., 2004, Bakhoum et al., 2009). Aurora B within the CPC also controls the recruitment of a number of additional proteins to centromeres such as CENP-E, Dynein, Ndc80 and Plk1, which have been linked to kinetochore function and microtubule-kinetochore stability, indicating a possible method of control over microtubule attachment (Ditchfield et al., 2003, Murata-Hori and Wang, 2002, Cheeseman et al., 2006, Deluca et al., 2006, Goto et al., 2006).

1.7.3 The role of Aurora B kinase in cytokinesis

The CPC has also been implicated as a key instigator of the segregation of cellular material and cytokinesis, a process which begins with furrow ingression during anaphase and ends with the cleavage into two separate daughter cells (Giet and Glover, 2001, Guse et al., 2005, Fuller et al., 2008). Aurora B activity also regulates the abscission of cells by stabilising the bridge when chromatin from unsegregated chromosomes is trapped in the cleavage furrow (Steigemann et al., 2009). The various roles of Aurora B and the CPC in cytokinesis demonstrates the kinase's

importance for protecting against tetraploidy. Indeed, the use of small molecule inhibitors to probe the role of Aurora B activity have revealed that the inhibition of the kinase produced chromosome alignment defects, problems with cytokinesis and aneuploidy (Ditchfield et al., 2003, Hauf et al., 2003).

1.8 Aurora C kinase

A high degree of homology occurs between the kinase domains of Aurora B and C (83%), however out of the Aurora kinase family members, the function of Aurora C remains the most elusive (Katayama et al., 2003, Keen and Taylor, 2004).

Aurora C kinase has been found to be expressed in meiotically dividing spermatocytes and oocytes (Yanai et al., 1997, Bernard et al., 1998, Tseng et al., 1998, Kimura et al., 1999, Li et al., 2004, Tang et al., 2006, Yang et al., 2010b, Chen et al., 1998). Similar to the described localisation of Aurora B kinase during mitosis, in mouse meiosis Aurora C has been detected on chromosome arms and centromeres, before accumulating on the spindle midzone and midbody during anaphase and telophase (Tang et al., 2006, Yang et al., 2010b). This sequence of localisation has given rise to the theory that Aurora C may function as a meiotic chromosomal passenger protein like Aurora B during female mouse meiosis (Yang et al., 2010b). Indeed, Aurora C has been shown to be recruited by INCENP (Chen et al., 2005a), and prologues of additional components of the CPC such as Survivin, Borealin and INCENP have been identified in *Drosophila* meiosis, possibly representing a method of meiosis specific Aurora C regulation (Gao et al., 2008). Furthermore, an Aurora C kinase-dead dominant negative mutant was found to prevent the localisation of Bub1 and BubR1 to the kinetochore in female mouse meiosis (Yang et al., 2010b).

Merotelic and syntelic attached chromosomes were also seen in meiosis after depletion of active Aurora C, indicating that, like Aurora B kinase, Aurora C may also have a role in correcting microtubule kinetochore attachments (Yang et al., 2010b). Cells exposed to dominant negative kinase-dead Aurora C exhibit cytokinesis failure after MI, consequently producing a polyploidy after meiosis in mice (Dieterich et al., 2009, Dieterich et al., 2007).

Importantly however, Aurora C knockout mice were viable, despite being sterile (Kimmins et al., 2007), and the loss of a functional Aurora C gene in female oocytes inferred only minor effects to oogenesis (Kimmins et al., 2007, Hu et al., 2000, Dieterich et al., 2009). These relatively mild effects of the Aurora C kinase's depletion with no discernable effects to mitotic cells, are in stark contrast to the more severe effects seen after the depletion other two Aurora kinase family members (Girdler et al., 2006).

1.9 Cancer and the Aurora Kinase family

In recent years the Aurora kinases have raised significant interest due to reports of their over-expression in various cancers (Mountzios et al., 2008).

1.9.1 Cancer and Aurora A kinase

Aurora A is mapped to the chromosome region 20q13.2, which has also been found to be frequently amplified in a range of cancers, indeed the over-expression of Aurora A had been detected in tumours including breast, bladder, ovarian, colon and pancreatic human cancers (Katayama et al., 2003, Karthigeyan et al., 2010, Bischoff et al., 1998, Mountzios et al., 2008). This strong correlation between Aurora A over-expression and transformation consequently makes it a potential prognostic or malignancy marker as well as a promising anti-cancer target (Dutertre et al., 2002).

Further supporting the link between Aurora A activity and cancer susceptibility, the over-expression of the kinase has been correlated with genetic instability, the override of spindle toxin induced spindle checkpoint, augmentation of the G2 arrest in response to DNA damage, and resistancy to chemotherapeutic apoptosis (Meraldi et al., 2002, Goepfert et al., 2002, Marumoto et al., 2002, Anand et al., 2003, Jiang et al., 2003a). Aurora A over-expression has also been linked to the stabilisation of Cyclin B1 and centrosome amplification, both of which have been attributed to tumorigenesis (Meraldi et al., 2002, Goepfert et al., 2002, Yang et al., 2006, Zhou et al., 1998, Qin et al., 2009). Additionally, Aurora A has been shown to regulate the tumour suppressors p53 and BRCA1 (Pascreau et al., 2009, Katayama et al., 2004, Ouchi et al., 2004, Liu et al., 2004). Aurora A has been shown to directly interact with p53 through its N-terminal A-box motif, and phosphorylate p53 at two locations which either abrogates its transactivation activity or causing its ubiquitination and eventual proteolysis (Chen et al., 2002, Katayama et al., 2004, Liu et al., 2004). Aurora A over-expression has also been proposed to extend the life-span of the cells by both increasing the expression of the human telomerase reverse transcriptase (hTERT), and activating telomerase activity possibly through its regulation of c-Myc levels (Otto et al., 2009, Yang et al., 2004). Aurora A may also promote metastasis of tumour cells through activating factors such as RalA which is involved in the transformation pathway, causing enhanced collagen I-induced cell migration and anchorage-independent growth (Wu et al., 2005). Considering this large amount of data, it is unsurprising to learn that the over-expression of Aurora A has been shown to cause the oncogenic transformation of cells (Zhou et al., 1998, Bischoff et al., 1998).

In addition to Aurora A being over-expressed or hyper-activated in cancer cell lines, reports have also described its deletion and down regulation of the kinase to be apparent in cancer cells (Bibby et al., 2009). Intriguingly, these findings were associated to the dual reduction of wild-type p53 in cells, with Aurora A inhibition also found to increase the growth of tumours with reduced p53 levels (Mao et al., 2007, Bibby et al., 2009). Although additional reports have described how Aurora A inhibition can cause cell death in p53-deficient cancer cell lines (Dar et al., 2008). This ability of Aurora A inhibition to provide a growth advantage to selected tumours therefore highlights the requirement for tumour-specific therapies in the fight against cancer progression. Whether these changes to Aurora A are however directly involved in cell transformation or are simply a product of the numerous mutations that take place during the development of the tumour is still to be determined.

1.9.2 Cancer and Aurora B kinase

Unlike Aurora A, Aurora B is located at a chromosome position that has not been associated with amplification. Aurora B over-expression has however, been reported in some tumour samples such as colorectal tumours, with an increase in Aurora B kinase levels has been attributed to poor prognosis, although this may simply represent the heightened mitotic index of the samples (Tatsuka et al., 1998, Katayama et al., 2003, Ota et al., 2002). Instead of being a sole inducer of tumorigenesis, Aurora B over-expression may cause cell transformation, possibly through the hyperphosphorylation of its substrates, when in combination with other misregulated factors (Meraldi et al., 2002, Ota et al., 2002, Kanda et al., 2005).

1.9.3 Cancer and Aurora C kinase

Although Aurora C expression is limited to germ cells involved with reproduction, the expression of the protein has also been described in human leukaemia cells, with its depletion shown to cause G0/G1 arrest and apoptosis, indicating that Aurora C kinase may have a role in transformation (Kobayashi, 2006).

1.9.4 Aurora kinase-directed therapies

The association of the increased expression of the Aurora kinases with transformation, cancer progression and metastasis make the Aurora family attractive targets for anti-cancer drugs. Furthermore, the up-regulation of Aurora kinases in cancerous cells and in cells undergoing mitosis

also allow Aurora directed therapies to weald a degree of targeted action towards tumours cells, which may help avoid damage to the healthy tissues.

1.10 The development of small molecule Aurora A kinase inhibitors

A range of Aurora targeted therapies have been developed as anti-cancer treatments, a number of which are presently in clinical trials (Karthigeyan et al., 2010). Small molecule inhibition allows a high degree of temporal control over kinase inactivation, as they are easily diffusible so have a rapid speed of action, but their inhibitory effects can also be washed out and therefore reversed with relative ease. These properties have meant that small molecule inhibitions have been both a useful method of therapy and exciting investigative tools which can be used to dissect the complex and intricate roles of the Aurora kinases (Garcia-Echeverria et al., 2000, Ditchfield et al., 2003, Hauf et al., 2003, Girdler et al., 2006, Girdler et al., 2008, Dar et al., 2008).

A drawback of small molecule Aurora inhibition in both clinical and experimental usage however, is the difficulty of specifically targeting one kinase in isolation. The three members of the Aurora kinase family share a high degree of sequence homology between their catalytic domains (reviewed in [Karthigeyan et al., 2010]), and also bear close structural similarities to CDK-2, GSK-3β and SRC kinase (Cheetham et al., 2002, Dancey and Sausville, 2003, Garber, 2005). These similarities make it extremely difficult to design and develop inhibitors that are specific to just one of the Aurora kinase family members.

This problem with specificity became evident following the attempted development of ZM447439 (herein referred to as ZM1) and VX680 as inhibitors of Aurora A activity (Ditchfield et al., 2003, Harrington et al., 2004). ZM1 was generated from a screen of 250,000 compounds using Aurora A kinase activity as an inhibition target, while VX680 was synthesised using knowledge regarding the structure of Aurora A (Andrews, 2005). Application of either of the drugs caused a reduction of histone H3 phosphorylation on Ser10 as well as the inhibition of cell division, producing tetraploid cells (Girdler et al., 2006, Ditchfield et al., 2003, Harrington et al., 2004). These phenotypes were suggestive of Aurora B inhibition, rather than Aurora A, and it was revealed that VX680 was equipotent for the two kinases as well as inhibiting a number of additional kinases, while ZM1 inhibited Aurora B more potently than Aurora A (Girdler et al., 2006, Cheetham et al., 2007, Tyler et al., 2007).

Despite the lack of specificity towards Aurora A activity, both ZM1 and VX680 have been shown to selectively kill tumour cells *in vitro*, although ZM1 never progressed to clinical trials and VX680 has now been removed from trials due to complications in the development process (Ditchfield et

al., 2003, Girdler et al., 2006, Harrington et al., 2004, Keen and Taylor, 2009, Georgieva et al., 2010).

The majority of small molecule inhibitors characterised to date predominantly inhibit Aurora B kinase activity. However, in 2006 the ZM-related inhibitor ZM3 was reported to cause the formation of monopolar spindles, which is consistent with Aurora A inactivation (Girdler et al., 2006, Barr and Gergely, 2007). Following ZM3 development, another small molecule named MLN8054, was also found to cause the development of monopolar spindles. It has been claimed that *in vitro,* MLN8054 can inhibit Aurora A activity over 40 times more potently than Aurora B, and more than 100 times more potently than a panel of selected kinases (Hoar et al., 2007). MLN8054 is an ATP competitive and reversible inhibitor of Aurora A kinase activity, and has been shown to cause the induction of apoptosis and senescence in laboratory tests (Huck et al., 2010, Manfredi et al., 2007). Clinical treatment of the drug did however cause somnolence in patients, prompting the development of a secondary compound called MLN8237, which at the time of writing, is in phase II clinical trials and has been found to produce encouraging levels of cytotoxicity when used as a combination therapy (Moore et al., 2010, Karthigeyan et al., 2010, Nawrocki, 2008).

1.11 Methods of investigating Aurora A function

Many techniques have been successfully employed in the past to characterise the role of the Auroras within cells. *In vitro* and *in vivo* methods have been detailed in the literature, ranging from RNAi and conditional knockouts of Aurora A, to micro-injection of anti-Aurora A antibodies and the use of Aurora A mutant proteins (Giet and Prigent, 2001, Marumoto et al., 2002, Glover et al., 1995, Girdler et al., 2006, Girdler et al., 2008, Hannak et al., 2001, Cowley et al., 2009, Mori et al., 2007, Berdnik and Knoblich, 2002). It is however important to distinguish between reports specifically concerned with the role of the kinase activity, and those that investigate the function of the entire protein. Removing a protein from the system (using techniques such as null models and RNAi) induces effects that reflect the prevention of the kinase from performing its structural function as well as any chemical roles. To specifically investigate the role of kinase activity, the physical protein must still be present in the cell to allow the activity of the kinase to be examined in isolation.

Clearly, developing an understanding of the role of a particular kinase's activity is a delicate and intricate process. However, investigation into Aurora A kinase activity has been elegantly conducted in the past by creating phospho-mimics of Aurora A substrates, and by using catalytic inactive Aurora A mutants (Mori et al., 2007, Glover et al., 1995, Berdnik and Knoblich, 2002,

Girdler et al., 2006). The creation of mutant proteins has been used to collect valuable information, however it is not possible with the current techniques to have temporal control, a feature that has extensive advantages when investigating temporally controlled kinases. The mutant proteins must either be continually expressed, or their expression must be induced over a relatively long period (Berdnik and Knoblich, 2002, Hannak et al., 2001). In comparison, the ability to rapidly manipulate small molecule inhibitors means that they can be used in experiments to examine intricate and specific areas of the cell cycle without the need for long treatment periods (Sawyers, 2004, Keen and Taylor, 2009).

1.12 Outline of this investigation

The Aurora kinase family are integral to the regulation of mitotic progression, and numerous reports detail their importance in the process. Aurora A kinase has attracted particular interest due to the strong links between its over-expression and cancer. Despite a good number of discoveries made regarding the role of Aurora A however, the limitations of experimental techniques have meant that relatively little is known about the role of its kinase activity. The aim of the study presented in this thesis was therefore to exploit the experimental benefits of small molecule inhibition in order to probe the role of Aurora A activity in mitosis. During the course of this thesis I will describe the experimental findings that resulted from my exploration of the role of Aurora A kinase activity.

In the first results section described in chapter 3, I describe how the small molecule inhibitor MLN8054 was selected as the best tool with which to investigate Aurora A kinase activity. Then in chapter 4, I describe how MLN8054 was employed to examine the role of Aurora A activity within cells, showing that its inhibition resulted in the formation of bipolar spindles of a reduced length, with fewer long K-fibres and a lower level of microtubule organisation. Despite demonstrating that Aurora A activity has a role in spindle formation, the results were in contrast to previous reports which associated the removal of Aurora A activity with the production of a monopolar spindle phenotype (Roghi et al., 1998, Glover et al., 1995, Liu and Ruderman, 2006, Girdler et al., 2006).

The functional consequences of the spindle abnormalities seen after Aurora A inhibition are demonstrated in chapters 5 and 6, with data showing that Aurora A inhibition prevents cells from being able to efficiently align their chromosomes and maintain genetic integrity. These findings are consistent with reports of Aurora A depletion and Aurora A antibody micro-injection, which described the treatments as causing chromosomal misalignments despite the presence of a bipolar spindle (Marumoto et al., 2003, Kunitoku et al., 2003). When examining the role of Aurora A activity in chromosomal alignment, it became apparent that Aurora A activity was not required for

the attachment of kinetochores to the spindle. Additionally, when causing the dramatic reduction in centromeric stretch by inducing the formation of monopolar spindles in cells treated with MLN8054, monotelically orientated chromosomes were observed. This ability of MLN8054-treated cells to produce monotelically orientated chromosomes when kinetochores are under reduced tension thus confirmed that Aurora B activity was not inhibited by the MLN8054 treatment (Hauf et al., 2003). Furthermore, contrary to predictions, the finding also appeared to demonstrate that Aurora A does not cooperate with Aurora B in regulating chromosomal attachment (Marumoto et al., 2003). Therefore, the role of Aurora A activity in the alignment of chromosomes was instead predicted to be derived from its function in regulating spindle formation.

When exploring the role of Aurora A activity in the regulation of the formation of a functional mitotic spindle, TACC3 was found to be completely mislocalised following Aurora A inhibition, which is consistent with reports in the literature, and chTOG localisation to the centrosomal area was also diminished (Giet et al., 2002, Kinoshita et al., 2005, LeRoy et al., 2007, Lee et al., 2001). Aurora A localisation to spindle microtubules was also found to be partially destabilised by its inhibition and centrosome size appeared reduced.

Despite the clear role for Aurora A activity in spindle assembly and chromosomal alignment, the majority of cells performed cytokinesis after extended mitotic delays. This observation conflicts with data from Aurora A RNAi or antibody micro-injection experiments, which may therefore highlight divergent structural and catalytic roles for Aurora A in the process (Marumoto et al., 2003). Despite the ability of cells to divide in the absence of Aurora A activity, segregation defects were observed and the cells became aneuploid. This requirement for Aurora A activity to evenly segregate segregation the genetic material is consistent with *Drosophila* data and a report published during the investigation (Hoar et al., 2007, Glover et al., 1995).

Despite the clear role for Aurora A activity in spindle assembly and mitotic progression, the effects of its inhibition were not as profound as indicated in many reports in the literature (Roghi et al., 1998, Glover et al., 1995, Liu and Ruderman, 2006, Girdler et al., 2006). Therefore, to address whether Aurora A activity could be inhibited more potently, in chapter 7 I used a cell line which made it possible to increase the concentration of inhibitor applied to cells, while maintaining its specificity towards Aurora A over Aurora B activity. These cells exhibited a monopolar spindle phenotype in response to Aurora A inhibition, although they were still able to divide, thus supporting the notion that although Aurora A activity is required for spindle formation it is not involved in cytokinesis.

In comparison to the cancer cell lines tested, investigation into non-transformed cells during chapter 8, revealed that they could form bipolar spindles of a normal length, which were able to

more efficiently align their chromosomes in the absence of Aurora A activity compared to the cancer cell lines tested. The non-transformed cells tested also only suffered relatively short mitotic delays following Aurora A inhibition and did not accumulate a substantial sub-G1 population, unlike the cancer cells, which became profoundly unviable after the equivalent treatment.

In summary therefore, I will describe my findings which demonstrate that Aurora A activity is involved in the formation of a functional spindle and efficient alignment of chromosomes. Through the development of a functional spindle, I will demonstrate that the kinase activity of Aurora A enables the appropriate detection of alignment defects, allowing the even segregation of genetic material, although it does not play a prominent part in the spindle assembly checkpoint or cytokinesis. Finally, I will describe a number of intriguing differences between the cell lines in response to Aurora A inhibition, which have important connotations for the targeting of Aurora A as an anti-cancer therapy.

2 Materials and Methods

2.1 Cell culture

The cell lines used during this investigation (described in Table 2.1) were grown in Dulbecco's modified Eagle's media (DMEM) (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine (all from Lonza) and 10 % (v/v) foetal calf serum (FCS) (Invitrogen). GFP-H2B HeLa and RPE cell lines were also treated with 0.5 and 3 µg/ml puromycin respectively, to select for their pLPCX-based plasmids.

Cell Line	*Origin*	*Source*	*p53*
DLD-1	Human colorectal adenocarinoma	American Type Culture Collection	WT
TA-HeLa	Human cervical carcinoma	(Taylor and McKeon, 1997)	WT (inactive)
HCT116	Human colon carcinoma	American Type Culture Collection	WT
HCT116 R12	Human colon carcinoma	(Girdler et al., 2008)	WT
hTERT RPE	Telomerase immortalised human retinal pigment epithelial cell	A gift from Dr. S, Doxsey, University of Massachusetts	WT
GFP H2B TA-HeLa	Human cervical carcinoma virally infected with pLPCX	(Gascoigne and Taylor, 2008)	WT (inactive)
GFP H2B hTERT RPE	Telomerase immortalised human retinal pigment epithelial cell virally infected with pLPCX	(Gascoigne and Taylor, 2008)	WT

Table 2.1: ***Cell lines.*** *The tissue and cell type of the cell line origin are described as in the ATCC database, and the p53 status are designated as either wild type (WT) of inactivated form of the p53 gene (WT (inactive)), as detailed in the Mutant P53 loss of activity database.*

The cells were cultured as monolayers in a humidified atmosphere at 37ºC and 5% CO_2 and passaged when necessary by first washing with phosphate buffered saline (PBS) then incubating at 37°C for 5 minutes with 1% (v/v) trypsin in PBS (Invitrogen). After detachment of the cells by trypsinisation, fresh media was used to both quench the trypsin and appropriately dilute the cells.

For storage, the cells were harvested as above then centrifuged at 1000 rpm for 5 minutes. The resulting pellet was resuspended in freezing media (FCS with 10% (v/v) dimethyl sulfoxide (DMSO) (Sigma)) and aliquoted into cryotubes (Nunc). The tubes were incubated at -80°C in polystyrene boxes for a minimum of 24 hours before being transferred to liquid nitrogen for long-term storage.

2.2 Drug treatment

The drugs used were stored at -20ºC at stock concentrations in DMSO or, in the case of MLN8054, sterile water (Table 2.2). To achieve the final concentrations, drugs were diluted with media, although DMSO was never more that 0.1% (v/v) of the final concentration. Samples with equivalent concentrations of DMSO were used as controls for experiments using drugs stored in DMSO.

Drug	*Stock concentration*	*Final concentration*
Nocodazole (Sigma)	5 mg/ml	30 ng/ml
AZ138 (A gift from AstraZeneca)	10 mM	1 μM
Monastrol	100 mM	100 μM
ZM447439 (Tocris)	10 mM	2 μM
MLN8054 (A gift from Millennium Pharmaceuticals)	10 mM	1 μM

Table 2.2: ***Drug concentrations.*** *The stock concentrations of the drugs were diluted to make the indicated final concentrations with media.*

2.3 Cell Biology

2.3.1 Flow Cytometry

Cells were seeded into 10 cm Petri dishes and cultured for 24 hours before drug treatment. Following the drug treatment, media was collected from the plates and the cells were carefully washed with 2 ml PBS, which was also subsequently collected. The cells were harvested by trypsinisation, pooled with the media and PBS collected earlier, then centrifuged at 1000 rpm for 5

minutes to form a pellet. The pellet was resuspended in 150 µl PBS and fixed using 350 µl 100% ice-cold ethanol, which was added in a drop-wise fashion while gently vortexing. The fixed cells were then incubated at -20ºC for at least 16 hours.

To assess the DNA content and mitotic index of the cells were stained with propidium iodide (PI) and an antibody against MPM-2 respectively. Before the staining however, the cells were washed to remove ethanol by adding 10 ml PBS and centrifuging at 1000 rpm for 5 minutes. The supernatant was removed and the cells were incubated at 4ºC in the dark for 2 hours with 500 µl mouse monoclonal MPM-2 (Upstate) diluted to 1:2000 in PBS. The samples were then washed again by adding 10 ml PBS, centrifuging at 1000 rpm for 5 minutes and discarding the supernatant. Anti-mouse-FITC secondary antibody (Jackson Labs), diluted to 1:1000 in PBS, was added for 1 hour after which samples were washed with PBS once more and then resuspended in 500 µl propidium iodide solution (40 µg/ml PI, 50 µg/ml ribonuclease A in PBS: Sigma) and incubated in the dark at 4°C for 30 minutes. If it was not necessary to observe the mitotic index of the cell population, the addition of primary and secondary antibodies against MPM-2 was omitted from the protocol. The stained cells were analysed using CyAn™ (DakoCytomation) and Summit analysis software (Dako), with 10,000 cells analysed from each sample.

2.3.2 Phase-contrast time-lapse microscopy

To observe individual cell progression through mitosis, cells were plated into a clear-bottomed 24-well dish (Corning) and cultured for 24 hours before drugs were applied. To reduce the evaporation of media from the cells and therefore maintain the allocated drug concentrations, the unused wells of the 24-well dish were filled with media in order to create a humidified environment. Filming of the cells was started 1 hour after drug application, with images being taken every 2 minutes with a CoolSNAP HQ camera (Photometrics) operated by MetaMorph software (Universal Imaging). Phase contrast was used to visualise cells with a 32x Apo Plan objective and 200 ms exposure using a Zeiss Axiovert 200 fitted with a PZ-2000 motorised stage (Applied Scientific Instrumentation) to enable point revisiting. Culture conditions of 37ºC and 5% CO_2 were maintained during filming through the use of an environmental control chamber and heater (Solent Scientific). A syringe needle was inserted into the plastic dish lid to deliver humidified CO_2 to the cells.

The images produced from the procedure were analysed using Metamorph software (Universal Imaging), and the time taken from cells to progress from the point of nuclear envelope breakdown to chromosome decondensation was used to determine the mitotic period of the cells.

2.3.3 Fluorescent spinning-disc time-lapse microscopy

Spinning-disc time-lapse microscopy enabled the collection of high-resolution imaging, allowing the observation of individual fluorescently marked chromosomes within live cells. The cells in question expressed GFP-histone H2B to enable the visualisation of the DNA, and were plated in a 35 mm Petri-dish with a 14 mm^2, 0.16 – 0.19mm thick glass insert (Mat Tek). The cells were left to settle for around 24 hours, after which the media was removed and replaced with the appropriate drug concentration diluted using fresh media. The culture conditions of 37ºC and 5% CO_2 were maintained by enclosing the cell plate in a heated environmental control chamber (Solent Scientific). Following a 1 hour period which was used to allow the cell environment to stabilise, a 100x objective was used to take a series of 7 z-sections every 2 minutes with a CoolSNAP HQ camera (Photometrics) and spinning disc (Yokogawa) operated by MetaMorph software (Universal Imaging).

2.3.4 Immunofluorescent staining of cell samples

Immunofluorescence microscopy was used to observe mitotic spindles within cells as well as the positioning of various proteins in response to various treatments. To do this, the cells were first seeded onto 19 mm glass cover slips using 450 µl of 1.2 or 2.4 x 10^5/ml cell suspension. 24 hours after plating the cells, the media was removed and replaced with the appropriate drug treatment. Following the specifically allocated treatment period, the cell samples were fixed, washed and blocked according to Table 2.3, and 100 µl of primary antibody in blocking agent was applied to each slip (Table 2.4). After washing the cells three times with PBS plus 0.1% (v/v) Triton X-100 (PBST), 100 µl of secondary antibody conjugated to Cy2, Cy3 or Cy5 (all used at 1:500 from Jackson Immunoresearch Laboratories) diluted in blocking agent was applied to each slip and incubated in the dark for 30 minutes. To stain the DNA, after washing with PBS three further times with PBST to remove unbound secondary antibodies, Hoechst No. 33358 (Sigma) diluted to 1 µg/ml in PBS was applied to the samples for 2 minutes. After washing with PBST three final times to remove the Hoechst, the cover slips were inverted and positioned onto mounting media (90% glycerol (v/v) with 20 mM Tris HCL pH 8.0) which had been placed on glass slides. Nail varnish was applied to the edges of the cover slips to seal them to the glass slide.

Protocol step	*Fixation techniques*			
	Methanol	*Formaldehyde*	*PEM extraction*	*PEM/methanol*
Microtubule extraction	None.	None.	Aspirate off media, wash with PBS, and then apply microtubule extraction buffer PEM (100 mM PIPES pH 6.9, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 % triton) for 90 seconds.	Aspirate off media, wash with PBS, then apply PEM for 90 seconds.
Fixation	Aspirate off media and carefully wash once with PBS. Remove PBS by and submerge into ice-cold 100% ethanol for 10 seconds. Fix cells by placing in fresh ice-cold 100% ethanol and incubate at –20°C for 10 minutes.	Aspirate off media and wash once with PBS, then apply 1% (v/v) formaldehyde in PBS for 5 minutes.	Aspirate off PEM and apply 4% (v/v) formaldehyde in PEM for 10 minutes.	Remove PEM and submerge into ice-cold 100% ethanol for 10 seconds. Fix cells by placing in fresh ice-cold 100% ethanol and incubate at –20°C for 10 minutes.
Wash	3-times with PBST	3-times with PBST	3-times with PEM	3-times with PEM
Block	5% (w/v) fat-free milk powder (Marvel) in PBST for 10 minutes.	Glycine in PBS for 5 minutes.	Glycine in PEM for 5 minutes.	% (w/v) fat-free milk powder (Marvel) in PEM for 10 minutes.
Experiment	Figures 5.3 and 5.5.	Figures 3.4B, 3.4C, 4.2, 4.4, 4.5, 4.6, 5.1, 7.1A, 7.4 and 8.3.	Figures 3.4A, 4.1, 5.4A, 4.7A, 4.7B, 8.6A and 8.6B.	Figures 4.7C. 4.7D and 8.6C.

Table 2.3: ***Fixation techniques.*** *Four different protocols were selected for the fixation of samples from immunofluorescence, which involved specifically tailored methods of microtubule extraction, sample fixation, washing and blocking. The figures in which each of the methods were used are indicated at the bottom of the table.*

2.3.5 Immunofluorescence microscopy

Immunofluorescently stained samples were analysed in a range of different methodologies. When simply counting cells, a Zeiss Axioskop 2 microscope with a 100x objective and epifluorescence was used. Single plane images were taken of the cell samples through the use of a CoolSNAP HQ CCD camera (Photometrics) and Metamorph software (Universal Imaging).

For the acquisition of z-sectioned fluorescence images of the cells or the quantitation of areas of fluorescence through measuring pixel intensity, a wide-field optical sectioning microscope (Delta Vision; Applied Precision) driven by SoftWorx (Applied Precision) was used. Image stacks were

created by taking z-sections at 0.2 μm intervals to create image stacks, which were then deconvolved using 10 cycles of enhanced ratio deconvolution (SoftWorx). These stacks could either then be projected to make 2D images or used to observe the cell image in 3D.

Antibody	*Antigen*	*Dilution for IF*	*Dilution for WB*	*Source*
SAA-1	Sheep anti-Aurora A	1:5000	1:5000	(Girdler et al., 2006)
TAT1	Mouse monoclonal anti-α-tubulin	1:200	-	Gull Lab (Woods et al., 1989)
Pericentrin	Rabbit polyclonal anti-Pericentrin	1:1000	-	Abcam
4B12/6	Mouse anti-Bub1	1:10	-	(Taylor and McKeon, 1997)
pH3	Rabbit anti-phospho-S10-Histone H3	1:500	1:500	Chemicon
SAB.1	Sheep polyclonal anti-Aurora B	1:1000	1:1000	(Ditchfield et al., 2003)
SM2.1	Sheep polyclonal anti-Mad2	1:500	-	(Johnson et al., 2004)
ACA	Human anti-centromere	1:800	-	Bill Earnshaw
TACC3	Rabbit anti-TACC3	1:500	1:1000	(Gergely et al., 2000a)
Phospho-TACC3	Rabbit anti-phospho(Ser558)TACC3	-	1:1000	A gift from Fanni Gergely (University of Cambridge)
chTOG	Rabbit polyclonal anti-chTOG	1:500	-	Abcam
2914	Rabbit anti-Phospho-Aurora A (Thr288), Aurora B (Thr232), and Aurora C (Th198)*	-	1:1000	Cell Signalling
SB1.2	Sheep anti-Bub1	1:1000	-	(Taylor et al., 2001)
MIL	Rabbit anti-pT288	-	1:2000	Millennium Pharmaceuticals

Table 2.4: ***Antibodies used for immunofluorescence and Western blotting.*** *The concentrations of primary antibodies used for immunofluorescence microscopy (IF) or immunoblotting of Western blots (WB). NB: Antibody 2914 was to detect phospho-Aurora in Figures 3.1 and 3.3, while MIL was used in Figure 3.2.*

**It is claimed by the manufacturer that the antibody 2914 recognises a phophorylation event on Aurora C, I however have no evidence to support this claim.*

2.3.6 Immunoblotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were used to observe the presence of different forms of proteins within cell samples that had been exposed to a range of drug treatments. Before the treatment of the cells, they were plated in 6-well dishes and allowed to settle 24 hours, then mitotically enriched with Nocodazole for 16 hours. After this period the cells were treated for further 2 hours with the indicated Aurora A inhibitor concentrations in combination with MG132 and Nocodazole. A 500 µl portion of the media was then collected from the wells and the rest was discarded. The cells were gently washed with PBS, which was pooled with the collected aliquots of media. The cells were harvested by trypsinisation and combined with the collected media and PBS, which served to quench the trypsin. The samples were centrifuged at 1000 rpm for 5 minutes, the supernatant was discarded, the pellet was resuspended in 2 x Laemmli SD sample buffer (10% (v/v) β-mercaptoethanol, 125 mM Tris, 4% SDS, 20% (v/v) glycerol, 0.02% (w/v) bromophenol blue) and then incubated at 100ºC for 5 minutes to lyse the cells and denature proteins. After allowing the samples to cool to room temperature, they were centrifuged for 15 seconds at 14,000 rpm, after which the samples could then either be stored at –20°C or loaded into a 12% polyacrylamide gel (see Table 2.5).

Reagent	*Resolving gel (12%)*	*Stacking gel*
Water	5.1	5.7
Tris 1.5M pH 8.8	3.75	-
Tris 0.5M pH 6.8	-	2.5
SDS 10%	0.15	0.1
Acrylamide 30%	6	1.7
Ammonium persulphate (APS) 10%	0.15	0.1

Table 2.5: ***Resolving and stacking gels for SDS PAGE.*** *Volumes of the indicated reagents are in ml. '12%' refers to the final percentage of acrylamide within the resolving gel. All the reagents are from Sigma, apart from the acrylamide, which is from National Diagnostics.*

The SDS PAGE gels were made with the ingredients indicated in Table 2.5, and polymerisation of the resulting solutions was aided and/or induced by the addition of TEMED (Sigma). To make a gel, a mould was made from a 10 cm x 10.5 cm glass plate clamped to a notched ceramic plate separated by 1.5 mm spacers (all from Hoefer). The gel solution was prevented from flowing out of the vertical plates by standing on a section of Para-film (Scientific Laboratory Supplies). To make a gel plug, 1 ml aliquot of resolving gel solution was combined with 10 µl of TEMED and poured

into the mould, and after it had polymerised, the remaining resolving solution was combined with 15 µl of TEMED and poured between the plates. To help remove bubbles from the gel and flatten the top, a 0.1% (w/v) SDS solution was poured onto the hardening resolving gel. When the resolving solution had solidified, the SDS solution was poured away and the sacking solution was poured into the mould after adding 12 µl of TEMED. Wells for the samples were made by inserting a fifteen-tooth comb (Hoefer) into the solidifying resolving gel. When the gel was fully solidified, the comb was removed and the wells were washed thoroughly. The gel, which as still held between the two plates, was then clamped into a running tank (Hoefer) filled with running buffer (25 mM Tris, 200 mM glycine, 0.1% (w/v) SDS) and a 30 mA charge was applied to each gel for 90 to 120 minutes to separate the proteins.

To observe the separated proteins, the SDS PAGE gel was carefully removed from the plates and immersed in transfer buffer (25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS, 20% methanol). The gel was sandwiched between six Whatman paper squares and Immobilon-P membrane (Millipore) all of which were also soaked in transfer buffer. The gel, paper squares and membrane were then placed in an EB10 or EB20 Electro-Blot Unit (Wolf Laboratories), and a preferential difference of 15 V was applied in the Electro-Blot unit, causing the proteins to be transferred from the gel onto the membrane.

After the transfer, the membrane was removed from the unit and incubated for 1 hour in 5 % (w/v) fat-free dried milk powder (Marvel) which had been diluted in TBST (100 mM Tris, 150 mM NaCl, 0.1 % (v/v) Tween-20, pH 7.5). To detect specific proteins, the membrane was incubated with primary antibodies which had been diluted to specific concentrations in blocking agent overnight at 4ºC (Table 2.4). Unbound antibody was removed by agitating the membranes for 30 minutes in TBST, after which secondary antibodies conjugated to horseradish-peroxidease-conjugated (HRP) (Zymed) diluted to 1:2000 in blocking agent, were applied to the membrane for 1 hour at room temperature. After a another 30 minute agitated wash in TBST to remove any unbound secondary antibody, the HRP signal was detected using Super Signal west pico luminal based chemiluminecent substrate (Pierce), which is oxidised by HRP to produce luminescence. The luminescence was imaged on Biomax MR film (Kodak), enabling the visualisation of the HRP signal.

3 The characterisation of the Aurora A inhibitors

3.1 Introduction

Aurora A kinase plays a key role in several stages of mitosis, in addition Aurora A expression has been implicated in oncogenesis (Bischoff et al., 1998, Zhou et al., 1998, Carmena and Earnshaw, 2003, Keen and Taylor, 2004). These observations have made Aurora A an exciting area of research for those studying both mitosis and potential anti-cancer therapies. Numerous groups have consequently endeavoured to characterise the kinase, a line of investigation that has been greatly advanced by the development of RNA interference (RNAi) (Elbashir et al., 2001, Marumoto et al., 2003, Hannak et al., 2001, Motegi et al., 2006). Despite the many benefits of using RNAi as an investigative technique, the depletion of endogenous protein induced by the treatment does not allow for the distinction between any possible catalytic roles and the identification structural function. To address this issue, groups have induced the expression of mutated proteins in combination with using RNAi to deplete endogenous protein, to mimic the effects of Aurora A kinase inhibition. This treatment has been described to cause the development of monopolar spindles, a phenotype that was consequently attributed to the inactivation of Aurora A kinase activity (Girdler et al., 2006, Liu and Ruderman, 2006, Mori et al., 2007). Despite these promising results, Girdler and colleagues (2006) warned against assuming that such experiments demonstrate a solid role for Aurora A activity in bipolar spindle formation, as it was argued that there was little evidence to indicate Aurora A was truly inactivated. Instead, the writers called for the use of antibodies, which recognise both the phosphorylated T-loop of Aurora A and phosphorylated forms of Aurora A downstream targets, to enable the accurate depiction of the role of Aurora A kinase activity in cells.

Subsequent to the introduction of RNAi techniques, the development of small molecule inhibitors opened new doors for the investigation of kinase activity (for review see [Taylor and Peters, 2008]). The highly penetrant and often reversible effects of small molecule inhibitors, in conjunction with their rapid speed of action, aptitude for dose-response investigations and precise temporal control, make them a highly attractive method of experimentation (Sawyers, 2004, Keen and Taylor, 2009). Most importantly however, the use of small molecule inhibitors enables the specific inhibition of enzymic activity, without also affecting the protein’s levels or structural interactions, thus enabling the role of the catalytic action to be more efficiently characterised. The many advantages of using small molecule inhibitors to probe catalytic activity therefore make them ideal tools for investigating the role of Aurora A kinase activity.

To date, a number of small molecule inhibitors have been developed to target the Aurora kinases, however the high degree of homology shared between Aurora A and B, has made the identification of a specific Aurora A inhibitor rather challenging (for review see [Keen and Taylor, 2004, Giet et al., 2005, Carmena and Earnshaw, 2003]). Although Aurora C also shows a high degree of structural similarity to the other Aurora family members, it has been observed to be expressed in specific mammalian meiotic cells, with no endogenous protein detected in HeLa or DLD-1 cells (Yanai et al., 1997, Tang et al., 2006, Bernard et al., 1998, Tseng et al., 1998, Li et al., 2004, Kimura et al., 1999, Yang et al., 2010b, Girdler et al., 2006).

Frustratingly, the majority of Aurora inhibitors characterised to date principally inhibit Aurora B activity (Hauf et al., 2003, Ditchfield et al., 2003, Girdler et al., 2006, Girdler et al., 2008, Bebbington et al., 2009, Harrington et al., 2004). Indeed the first small molecule developed to inhibit Aurora A activity, ZM1, was later revealed to actually inhibits Aurora B activity more potently than Aurora A (Ditchfield et al., 2003, Girdler et al., 2006). Encouragingly however, three further small molecule Aurora inhibitors, VX680, ZM3 and MLN8054, have recently been reported to target the activity of Aurora A kinase. At certain concentrations though, these three inhibitors have also been shown to also affect Aurora B activity (Harrington et al., 2004, Bebbington et al., 2009, Girdler et al., 2006, Hoar et al., 2007). Therefore, before any one of these three inhibitors could be used to probe the role of Aurora A kinase activity, I first needed to determine whether they could be used to specifically inhibit Aurora A without also effecting Aurora B activity in my chosen experimental system. Human tumour DLD-1 and HeLa cell lines were selected for use in the investigation, as both have previously been well characterised in the fields of cell division and anti-mitotic drug response (Jordan et al., 1996, Gascoigne and Taylor, 2008).

To determine the selectivity of the small molecule inhibitors for Aurora A activity in the chosen cell lines, I used a combination of immunofluorescence and immunoblotting techniques to analyse changes in specific phosphorylation events in response to individual inhibitor treatments (Jackman et al., 2003, Girdler et al., 2008). The level of Aurora A inhibition achieved by the inhibitors was gauged using immunoblotting to observe changes in the auto-phosphorylation on the T-loop (residue Thr288) in the catalytic region of Aurora A (see Figure 1.7), as well as the phosphorylation of TACC3 on an Aurora A phosphorylation site (residue Ser558) (Bischoff et al., 1998, Littlepage et al., 2002, Walter et al., 2000, Liu and Ruderman, 2006, Ohashi et al., 2006, Barros et al., 2005, Giet et al., 2002, LeRoy et al., 2007, Peset et al., 2005, Tyler et al., 2007). In addition, I also assessed whether any unwanted Aurora B inhibitory effects were produced by the inhibitors, which may occur as a result of the close structural similarities between the active sites Aurora A and B. Changes in Aurora B activity were determined by observing its phosphorylation on Thr232, together with detecting the kinase's effect on one of its down-stream targets by

analysing the phosphorylation of histone H3 (Ser10) (Nigg, 2001b, Carmena and Earnshaw, 2003, Vagnarelli and Earnshaw, 2004, Ditchfield et al., 2003). In addition to this, immunofluorescence microscopy was used to compare the effects of the three inhibitors on cell morphology, thereby highlighting any other off-target effects caused by the inhibitors. By addressing whether VX680, ZM3 and MLN8054 produced off-target cellular effects as well as assessing their potency towards Aurora A activity, I aimed to determine their suitability as experimental tools to investigate the role of Aurora A kinase activity in mitosis.

3.2 VX680 inhibits both Aurora A and Aurora B activity

Following the development of ZM1, VX680 was the subsequent Aurora kinase inhibitor to be characterised (Harrington et al., 2004). VX680 was originally designed to target all three members of the Aurora family, however encouragingly it was found *in vitro* to be most potent against Aurora A activity, with tests showing IC50 values of 0.6, 18 and 4.6 nM for Aurora A, B and C respectively (Harrington et al., 2004, Bebbington et al., 2009). I therefore set out to determine if this specificity of VX680 indicated in the *in vitro* tests could also be seen *in vivo*, and thus whether it could be used as a tool to investigate Aurora A kinase activity.

To characterise the inhibitory action of VX680, I treated both HeLa and DLD-1 cells with a range of concentrations of VX680, before lysing and then separating the samples by SDS PAGE. Immunoblotting of the separated cell lysates revealed that increasing the concentration of VX680 caused the reduction of both phospho-Aurora A and phospho-TACC3 signals in both HeLa and DLD-1 cell samples (Figure 3.1). Therefore, consistent with the published *in vitro* data, VX680 could be used to inhibit Aurora A activity in an *in vivo* environment (Harrington et al., 2004, Bebbington et al., 2009). This inhibition was determined to be maximally achieved by 350 nM and 900 nM of VX680 in HeLa and DLD-1 cells respectively. Crucially however, at these concentrations, a reduction could also be seen in the presence of phosphorylated Aurora B and histone H3 in the immunoblotted samples. This indicated that Aurora B activity was inhibited at equivalent concentrations to that of Aurora A. This finding conflicts with the results of the kinase assays of Harrington and colleagues (2004), but is consistent with two reports published during the course of my investigation (Scutt et al., 2009, Tyler et al., 2007). This lack of selectivity demonstrated by VX680 thus reduced its value as a tool to investigate the role of Aurora A kinase activity. Consequently, I turned my attention to the other small molecule Aurora inhibitors available for investigation, to assess whether they would make more suitable investigative tools.

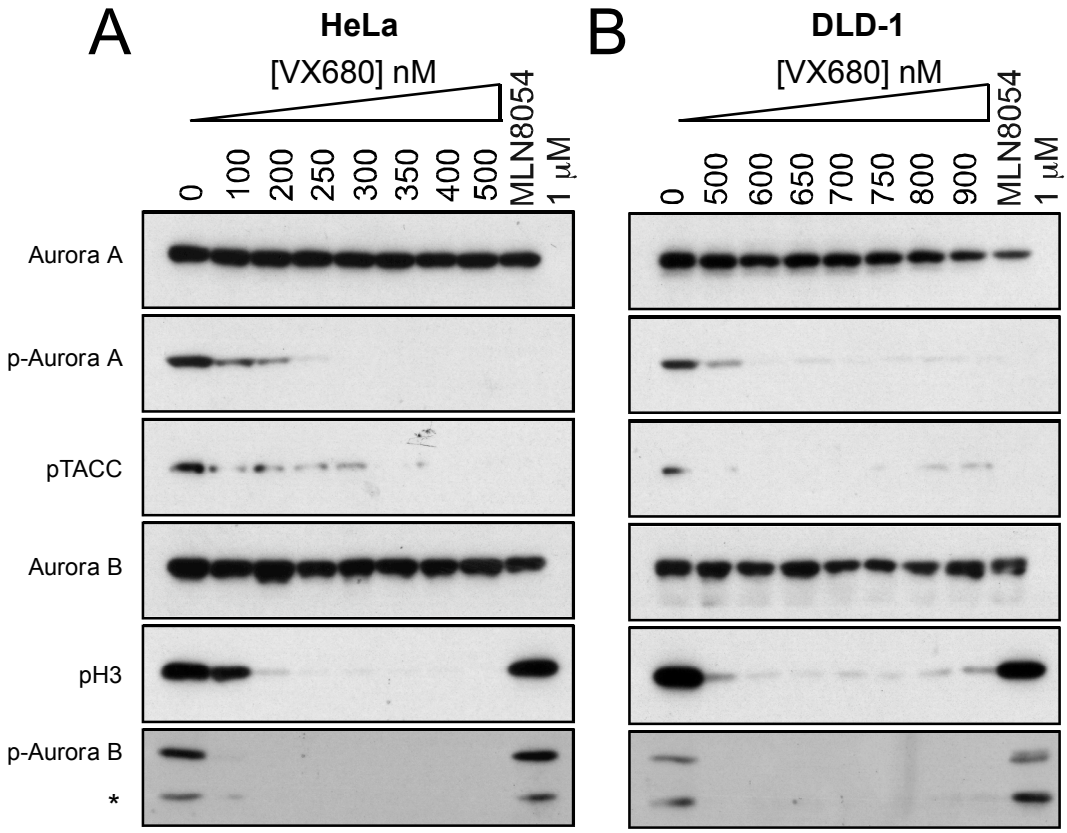

Figure 3.1 VX680 inhibits both Aurora A and B kinase activity
HeLa (A) and DLD-1 cells (B) were treated with Nocodazole for 16 hours to mitotically enrich the cell population, after which the cells were treated with MG132 together with a range of concentrations of VX680 for a further 2 hours. To compare the inhibition produced by VX680 with that of MLN8054, the last lane of the gel was used for cells which had been treated with 1 μM MLN8054 and MG132 in parallel to the VX680 treatments. After the various drug treatments, the cells were harvested and lysed, and whole cell lysates were separated using SDS PAGE. Immunoblotting was used to detect the presence of: Aurora A, Phopho-Aurora A (Thr288), phopho-Aurora B (Thr232), phopho-TACC (Ser558), Aurora B and phospho-histone H3 (Ser10). The images displayed represent results that were consistent over three independent experiments.
* It is claimed by the antibody manufacturer that the indicated band is generated by antibodies against the phosphorylated form of Aurora C (Thr198), however I have no evidence to support this.

3.3 ZM3 inhibits both Aurora A and Aurora B activity

The next small molecule inhibitor selected for further characterisation was ZM3. This ZM-related compound had previously been reported to inhibit Aurora A activity 20-times more potently than ZM1 in kinase assays, making it an exciting new candidate for use in the investigation (Girdler et al., 2006). However, to corroborate the *in vitro* data and determine whether ZM3 could be used to specifically inhibit Aurora A activity *in vivo*, I treated both HeLa and DLD-1 cells with a range of ZM3 concentrations, then lysed and separated the samples by SDS PAGE. Immunoblotting the separated cell lysates revealed that, like VX680, ZM3 caused a reduction in phospho-Aurora A signal, confirming that ZM3 could also be used to inhibit Aurora A activity in both HeLa and DLD-1 cells (Figure 3.2). The immunoblots showed that Aurora A activity was inhibited in HeLa cell samples treated with 1 μM of ZM3, whereas in DLD-1 it took 4 μM of ZM3 before Aurora A activity was fully inhibited. Despite these encouraging results, Aurora B activity was also inhibited at similar concentrations as Aurora A, an observation that was deduced from the reduction in phospho-histone H3 band intensity. In fact, Aurora B activity even appeared to be affected at only 0.25 μM of ZM3 in both cell lines, a finding which is consistent with published *in vitro* data, which described ZM3 as being more potent towards Aurora B activity than Aurora A (Girdler et al., 2006). Therefore, although both ZM3 and VX680 can be used to inhibit Aurora A activity, their dual inhibition of Aurora B activity reduced the potential of using them as tools to specifically investigate Aurora A activity.

3.4 MLN8054 can be used to inhibit Aurora A activity without also inhibiting Aurora B activity

Subsequent to the finding that both VX680 and ZM3 lacked selectivity towards Aurora A, my search for a suitable investigative tool was extended to a third small molecule inhibitor: MLN8054. This is a compound that has been shown *in vitro* by it's manufacturing company to be the first truly specific Aurora A inhibitor, thus making it an attractive potential tool investigate Aurora A activity (Hoar et al., 2007, Manfredi et al., 2007).

To verify these claims and confirm MLN8054 specificity for Aurora A activity, I treated the cells with a range of MLN8054 concentrations before lysing and separating them by SDS PAGE. The immunoblotting of the separated lysates showed that both auto-phosphorylated Aurora A and phospho-TACC3 antibody signal were reduced following their treatment with increasing concentrations of MLN8054 (Figures 3.3A and 3.3B), showing that, consistent with the published literature, MLN8054 could be used to inhibit Aurora A activity (Hoar et al., 2007, Manfredi et al.,

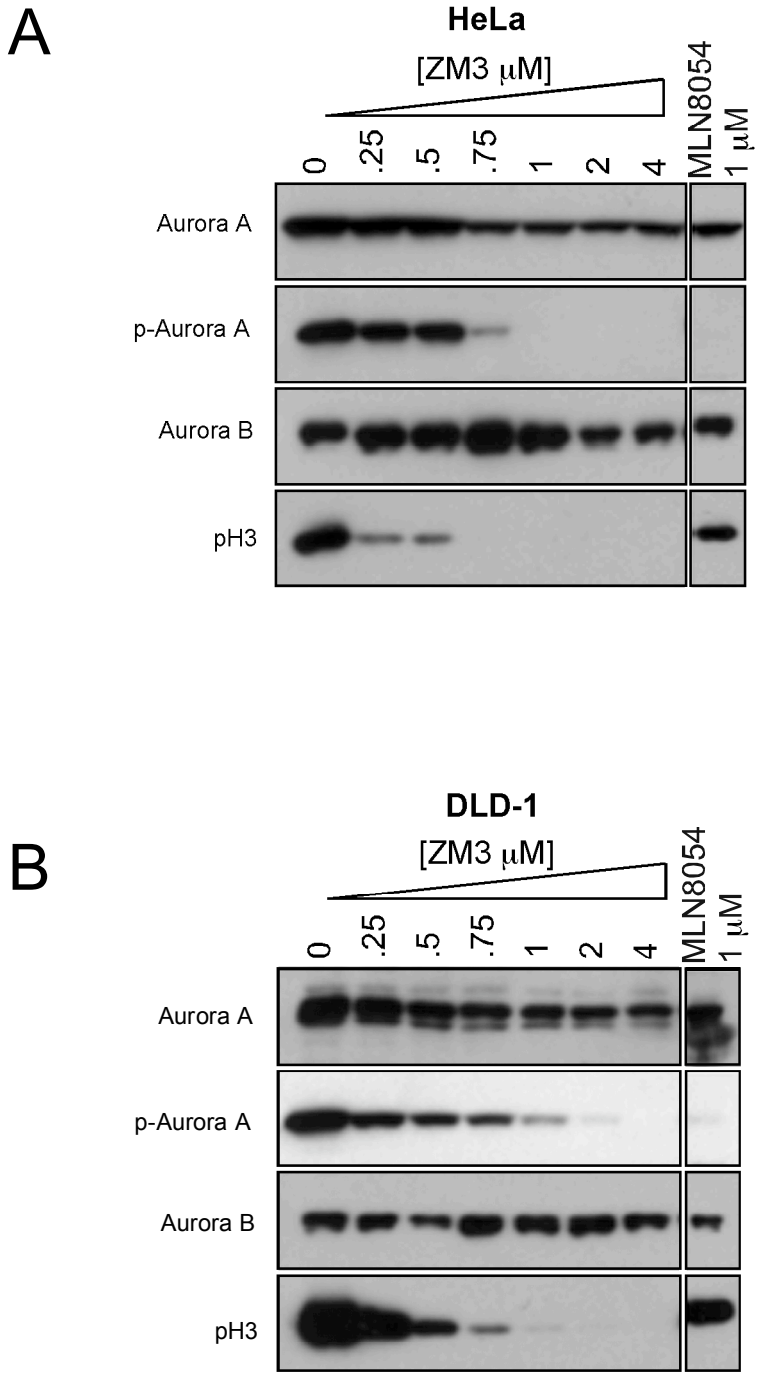

Figure 3.2 ZM3 inhibits both Aurora A and B kinase activity
HeLa (A) and DLD-1 cells (B) were treated with Nocodazole for 16 hours to mitotically enrich the cell population. The cells were then treated with MG132 together with a range of concentrations of ZM3 for a further 2 hours. In parallel, both cell lines were treated with 1 μM MLN8054 to allow the comparison of the inhibitors (right-hand lane). After the drug treatments, the cells were harvested and lysed, and whole cell lysates were separated using SDS PAGE. Immunoblotting was used to detect: Aurora A, Phopho-Aurora A (Thr288), Aurora B and phospho-histone H3 (Ser10).

2007). Aurora A activity was inhibited in both HeLa and DLD-1 cell lines by 1 μM MLN8054, although importantly Aurora B activity suffered no observable decrease at this concentration, as judged by levels of antibodies against the phosphorylated Aurora B and Histone H3 (Figures 3.3A and 3.3B). Importantly, Aurora B activity appeared to only be affected at 2 μM MLN8054 and above in DLD-1 cells, but not until 4 μM in HeLa cells. MLN8054 can therefore inhibit Aurora A activity without also inhibiting Aurora B *in vivo*, making it an ideal tool to probe the role of Aurora A activity.

To quantitate the degree of Aurora A inhibition achieved by 1 μM MLN8054, I compared the level of phospho-Aurora A antibody staining seen on treated immunoblotted samples, with that of a series of increasingly diluted control samples presented on the same gel. Importantly however, phospho-Aurora antibody signal was not detectable on samples which were diluted below 5% of the total cell lysate (Figures 3.3C and 3.3D). Taking this antibody limitation into consideration, immunoblotted HeLa lysates from cells treated with 1 μM MLN8054 showed only an extremely weak phospho-Aurora A band, even when overexposing the blot (Figure 3.3C). In comparison, DLD-1 cell lysates treated with 1 μM MLN8054 showed a band similar to that observed by diluting down to 5% of the total control cell lysates. Therefore, by comparing diluted control samples with samples of cells treated with MLN8054, it was deduced that 1 μM MLN8054 inhibited Aurora A activity to more than 95% in HeLa cells and to around 95% in DLD-1 cells.

3.5 ZM3 and MLN8054 produce similar cellular phenotypes, while the comparative severity of the cellular effects of VX680 treatment may be indicative of its off-target effects

All three small molecule inhibitors tested were shown to be capable of inhibiting Aurora A activity, however MLN8054 was the only compound that could potently inhibit Aurora A without also affecting the activity of Aurora B (Figure 3.3). These results were encouraging, although it was not yet clear whether MLN8054 exhibited inhibitory action towards kinases other than those of the Aurora family. Previously conducted tests show MLN8054 to be more potent for Aurora A activity *in vitro* than a panel of selected kinases, however the hundreds of remaining kinases that make up the human kinome have, as yet, not been tested (Hoar et al., 2007, Manning et al., 2002). It was therefore essential to further understand the specificity of MLN8054 before it could be used to investigate the role of Aurora A kinase activity.

Therefore to explore whether MLN8054 exhibited inhibitory action in addition to Aurora A activity, I compared the cellular effects of MLN8054 with those seen after VX680 and ZM3

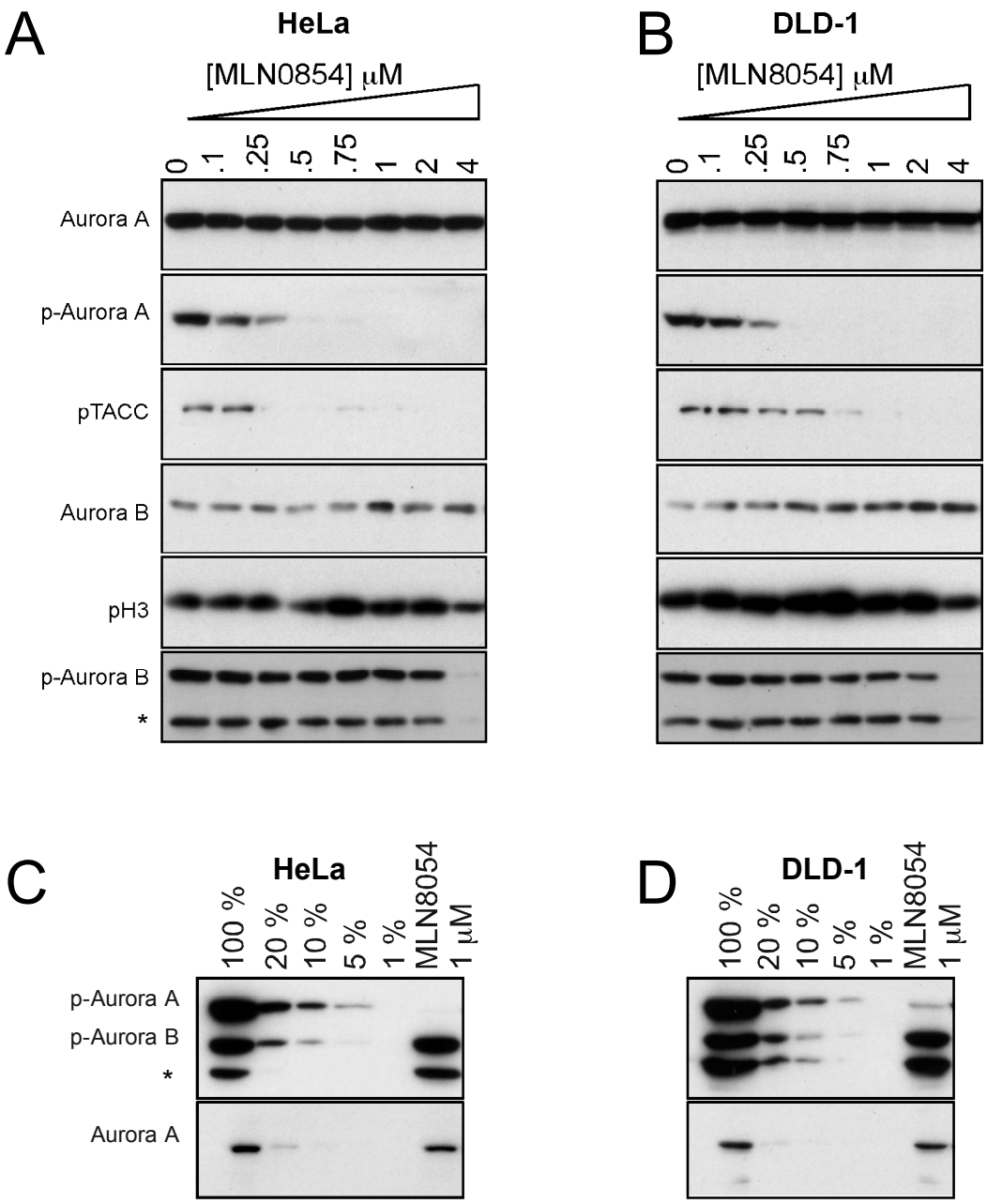

Figure 3.3 MLN8054 can be used to specifically inhibit Aurora A activity
HeLa (A and C) and DLD-1 cells (B and D) were treated with Nocodazole for 16 hours to mitotically enrich the cell population. The cells were then treated with MG132 in combination with a range of concentrations of MLN8054 for a further 2 hours, after which the cells were harvested and lysed, and whole cell lysates were separated using SDS PAGE. Immunoblotting was used to detect the presence of: Aurora A, Phospho-Aurora A (Thr288), phospho-Aurora B (Thr232), phospho-TACC (Ser558), Aurora B and phospho-histone H3 (Ser10). A range of increasingly diluted control cell lysates was used as a comparative tool to enable the determination of the level of active Aurora A remaining in HeLa (C) and DLD-1 cells (D) after treatment with 1 μM of MLN8054. The images displayed represent results that were consistent over three independent experiments.
*It is claimed by the antibody manufacturer that the indicated band is generated by antibodies against the phosphorylated form of Aurora C (Thr198), however I have no evidence to support this.

treatments. The thinking behind this methodology was that if used at concentrations that equivalently inhibited Aurora A activity, one would expect that that all three inhibitors would simply cause the equivalent range of spindle defects to the treated cells. This theory is based on a considerable body of evidence implicating Aurora A activity in spindle assembly (Peset et al., 2005, Cowley et al., 2009, Liu and Ruderman, 2006, Roghi et al., 1998, Marumoto et al., 2003, Glover et al., 1995, Hannak et al., 2001, Girdler et al., 2006, Schumacher et al., 1998a, Berdnik and Knoblich, 2002, Hoar et al., 2007). The fact that Aurora B activity is also dually inhibited by ZM3 and VX680 makes the comparison of their inhibition with that of MLN8054 a little more complex. However, because Aurora B activity is involved in resolving improper kinetochore-microtubule interactions and its inhibition has been shown to cause chromosome misalignment, any difference in chromosome alignment between the three inhibitors was disregarded so as to allow for a fair comparison of the inhibitors (Ditchfield et al., 2003, Hauf et al., 2003, Girdler et al., 2006). Therefore by comparing the cellular effects of the three small molecule inhibitors, and ignoring any differences in chromosome alignment caused by Aurora B inhibition, I hoped to highlight any additional off-target effects of the inhibitors, with particular emphasis on those of MLN8054.

To enable the effective comparison of the effects of the three inhibitors, equivalent levels of Aurora A inhibition were determined by the examination of Western blots which exhibited the various inhibition profiles (Figures 3.1, 3.2 and 3.3). Analysis of the inhibitors inhibitory profiles in this way revealed that equivalent Aurora A inhibition was achieved by VX680 at 350 nM in HeLa and 800 nM in DLD-1 cells, by ZM3 at 1 μM in HeLa and 4 μM in DLD-1 cells, and by MLN8054 at 1 μM in both cell lines.

When the selected inhibitor concentrations were applied to cells, which were consequently fixed and immunostained, all three inhibitors were found to produce a range of cellular effects in both HeLa and DLD-1 cells (Figure 3.4). Out of the three inhibitors however, VX680 appeared to cause the most severe cellular effects, with around 44% of DLD-1 cells observed to be monopolar, and the rest showing severely reduced spindle lengths (Figure 3.4B), which bears close resemblance to the findings of two recently published reports (Scutt et al., 2009, Tyler et al., 2007). In HeLa cells, VX680 caused the majority (76%) of HeLa cells to appear to have numerous centrosomal foci (Figure 3.4C), which is similar to a report of high numbers of multipolar HeLa cells following the microinjection of an inhibitory Aurora A antibody (Marumoto et al., 2003).

In contrast to the effects of VX680 in HeLa cells, the treatment with MLN8054 or ZM3 allowed a large proportion the cells to form bipolar spindles (68% and 55% of MLN8054 and ZM3 treated cells respectively) (Figure 3.4C). Similarly, DLD-1 cells were also able to form bipolar spindles following MLN8054 or ZM3 treatment, although at slightly reduced levels compared to HeLa cells

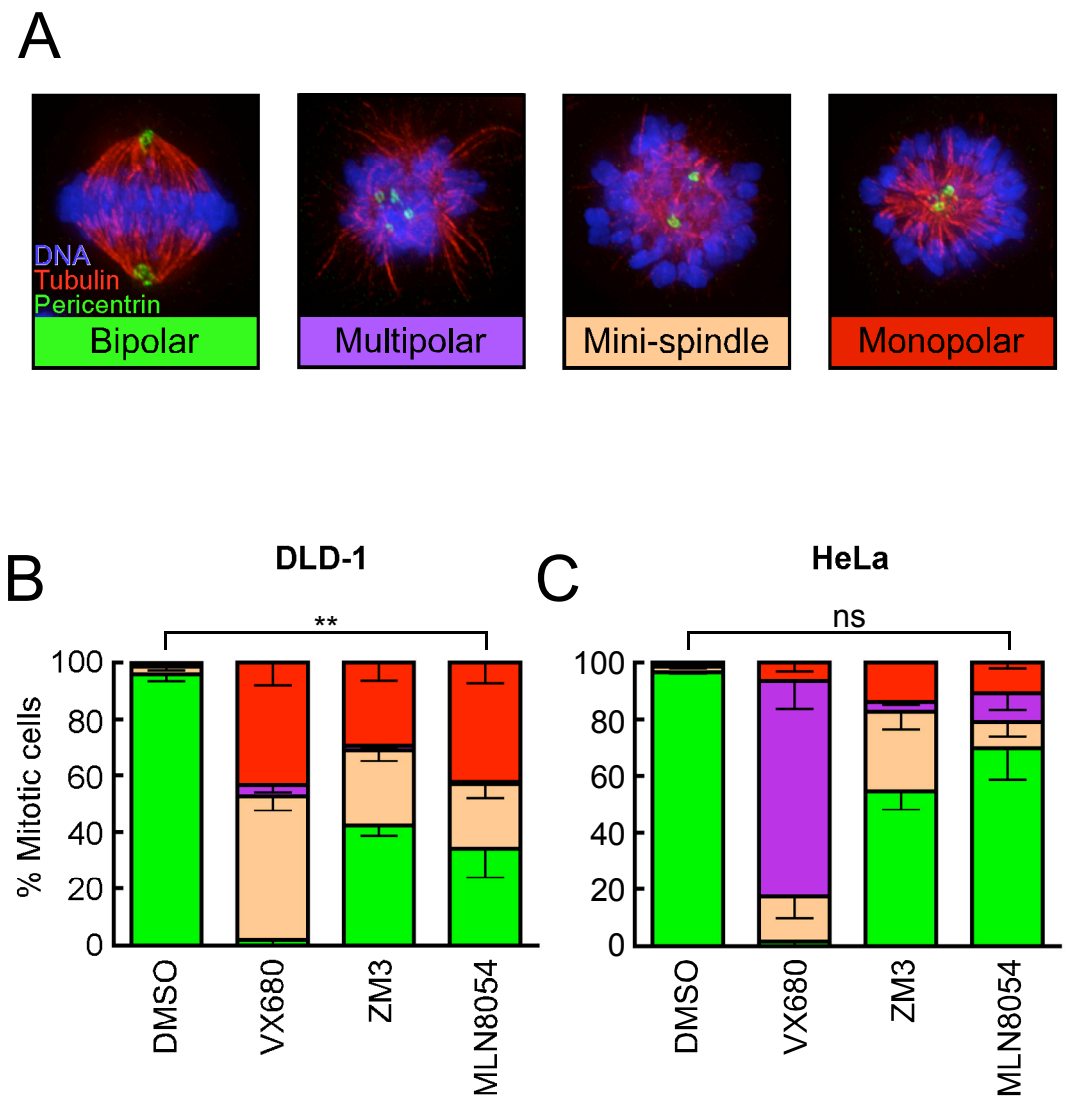

Figure 3.4 The Aurora inhibitors produce a range of spindle abnormalities
(A) Example images of spindle phenotype categories. HeLa cells were immunostained with antibodies against Tubulin (Red) and Pericentrin (green), and Hoescht was used to observe the DNA (Blue). DLD-1(B) and HeLa cells (C) were treated with either DMSO or an Aurora inhibitor together with MG132 for 2 hours. Appropriate Aurora inhibitor concentrations were chosen on the basis of their equivalent levels of Aurora A inhibition, determined from western blots such as those displayed in Figures 3.1, 3.2 and 3.3. MLN8054 was used at 1 μM in HeLa and DLD-1 cells, VX680 at 350 nM in HeLa and 800 nM in DLD-1 cells, and ZM3 at 1 μM in HeLa and 4 μM in DLD-1 cells. After treatment with the Aurora inhibitors at these concentrations, the samples were fixed, immunostained with antibodies against Aurora A to observe centrosomes, and quantitated using the 4 categories shown in (A). The graphs represent the average counts from three independent experiments with a minimum of 300 cells counted for each treatment. A two-tailed t-test was used to determine the significance of the differences in the proportion of bipolar spindles observed following each treatment. ** = $P<0.01$; ns = $P>0.01$. Error bars represent s.e.m.

(54% of MLN8054-treated cells and 42% of ZM3 treated cells) (Figure 3.4B). The observation of bipolar spindle formation following Aurora A inhibition in both DLD-1 and HeLa cells is surprising when considering the large amount of evidence implicating Aurora A in bipolar spindle assembly (Figures 3.4B and 3.4C) (Peset et al., 2005, Cowley et al., 2009, Liu and Ruderman, 2006, Roghi et al., 1998, Marumoto et al., 2003, Glover et al., 1995, Hannak et al., 2001, Girdler et al., 2006, Schumacher et al., 1998a, Berdnik and Knoblich, 2002, Hoar et al., 2007).

In addition to permitting bipolar spindle formation, treatment of cells with MLN8054 and ZM3 produced very similar cellular phenotype ranges. DLD-1 cells exposed to the designated concentrations of MLN8054 or ZM3 exhibited a near-even mixture of monopolar, bipolar and mini-spindles (Figure 3.4B). This finding is similar to a published report of the effects of ZM3 inhibition, which described around 29% of cells becoming monopolar following the treatment, while others were detected as having a range of normal and shortened spindle lengths (Girdler et al., 2006). The equivalent effects of MLN8054 on spindle length had not been described in the literature at the time of writing, however during the investigation a report was published describing the creation of around 75% abnormal spindles in HCT116 cells in response to treatment with 0.25 μM MLN8054 for 5 hours (Hoar et al., 2007). This bears a close resemblance to the 66% of 'abnormal spindles' (referred in my analysis as monopolar and mini-spindles) produced by 1 hour of 1 μM MLN8054 treatment in DLD-1 cells.

Consistent with DLD-1 cells, HeLa also were observed to exhibit similar morphology profiles when exposed to the selected concentrations of MLN8054 or ZM3 (Figure 3.4C). HeLa cells did however not seem as affected by either drug treatment as DLD-1 cells, with only 11% and 14% of the cells having monopolar spindles after MLN8054 or ZM3 treatment respectively. Interestingly though, proportionally more HeLa cells were observed as having multipolar spindles after MLN8054 or ZM3 treatments than in DLD-1 cells. This increase in multipolar spindles following Aurora A inactivation is similar, although not as pronounced, as seen that seen after VX680 treatment or the microinjection of Aurora A antibodies into HeLa cells (Marumoto et al., 2003). Interestingly however, increasing the concentration of microinjected antibody was also reported to allow 85% of cells to form bipolar spindles, as opposed to the high proportion of multipolar spindles seen at the lower concentration (Figure 3.4C) (Marumoto et al., 2003). This surprising effect of apparently further reducing Aurora A activity, produces cellular effects that are similar to those seen after MLN8054 or ZM3 inhibition, instead of the more profound multipolar spindle phenotype seen after VX680 treatment of HeLa cells.

Therefore, the initial hypothesis that all three Aurora inhibitors would produce similar cellular effects was incorrect. Instead, VX680 treatment caused distinct array of cellular phenotypes,

appearing to be the ‘odd one out’ of the three small molecule inhibitors, possibly indicating ‘off-target’ inhibition (Figures 3.4A and 3.4B). Comparatively, the cellular effects of ZM3 and MLN8054 treatments are highly similar, suggesting that they may not affect the activity of ‘off-target’ kinases. ZM3 however, was shown to inhibit Aurora B activity at equivalent concentrations to Aurora A. In comparison, MLN8054 acts specificity towards Aurora A activity over other the other family members, thus making it an ideal tool to probe the role of the kinase in mitosis.

3.6 Summary

In this chapter I have described my work into determining a small molecule inhibitor that could be used to specifically inhibit Aurora A activity in HeLa and DLD-1 cultured cells. Western blotting was used to select concentrations of three small molecule inhibitors, MLN8054, ZM3 and VX680, at which Aurora A activity could be potently inhibited. Consistent with the current literature however, Aurora B activity was inhibited in combination with Aurora A activity by both VX680 and ZM3 (Bebbington et al., 2009, Harrington et al., 2004, Girdler et al., 2006). MLN8054 was found to be the only inhibitor out of the three tested that could be used to specifically inhibit Aurora A activity over Aurora B. This finding is consistent with published *in vitro* data, in which MLN8054 was found to be more than 40-times more selective for Aurora A activity than for that of Aurora B (Manfredi et al., 2007). In the same publication, *in vitro* data was used to show MLN8054 was more potent towards Aurora A activity than a panel of 226 other kinases. In agreement with these findings, comparative immunofluorescence microscopy revealed no obvious off-target effects produced by the MLN8054 or ZM3 treatment. Surprisingly however, treatment of cells with either MLN8054 or ZM3 appeared to only affect a small proportion of mitotic spindles despite potent Aurora A inhibition. This result appeared to conflict with findings described in the published literature, which links Aurora A inactivation with the observation of a high proportion of monopolar spindles (Roghi et al., 1998, Glover et al., 1995, Liu and Ruderman, 2006, Girdler et al., 2006). In comparison, VX680 treatment caused more acute cellular phenotypes, which instead of being linked with more potent Aurora A inhibition, was predicted to reflect inhibition of off-target kinases due to the inconsistency with the other two inhibitor effects. Consistent with this idea, VX680 has previously been reported to target kinases other than the Aurora family (Harrington et al., 2004, Carter et al., 2005, Giles et al., 2007).

Therefore in summary, all three of the small molecule inhibitors tested inhibit Aurora A activity with varying degrees of potency, which is consistent with reports in the published literature (Hoar et al., 2007, Manfredi et al., 2007, Girdler et al., 2006, Harrington et al., 2004, Bebbington et al., 2009). MLN8054 however, was identified as the only small molecule out of the three, which could

be used to inhibit Aurora A activity without also inhibiting Aurora B *in vivo*. Furthermore, the comparison of the cellular effects of the three inhibitors indicated that, unlike ZM3 and MLN8054, VX680 may have had additional cellular effects other than to the Aurora family. The lack of specificity demonstrated by both VX680 and ZM3 therefore strengthened the decision to select MLN8054 as the best tool available for probing the role of Aurora A kinase activity.

In the next chapter, I describe how MLN8054 was used as a tool to understand why a large proportion of spindles appeared to be unaffected by Aurora A inhibition, despite a large amount of literature to the contrary (Hoar et al., 2007, Manfredi et al., 2007, Girdler et al., 2006, Harrington et al., 2004, Bebbington et al., 2009).

4 Examining the role of Aurora A activity in spindle assembly

4.1 Introduction

In the last chapter I analysed the effects of three small molecule inhibitors and evaluated their specificity towards Aurora A activity. Surprisingly, it was revealed that most cells were capable of forming bipolar spindles in the absence of Aurora A activity despite reports describing a monopolar spindle phenotype after the inactivation of Aurora A kinase (Roghi et al., 1998, Glover et al., 1995, Liu and Ruderman, 2006, Girdler et al., 2006). The unexpected occurrence of bipolar spindles after Aurora A inhibition in my experiments therefore warranted further investigation.

Despite the large body of evidence implicating a prominent role for Aurora A activity in spindle assembly, descriptions of this role do however vary throughout the published literature. Some groups have shown that bipolar spindles are present after the inactivation of Aurora A, although their spindles were often reported as being disorganised (Berdnik and Knoblich, 2002, Peset et al., 2005, Giet et al., 2002, Hoar et al., 2007). Therefore it may be possible that effects of Aurora A inhibition may have gone undetected, as spindle organisation was not intricately scrutinised in the previous chapter. Thus to determine whether this was indeed the case, I performed a more detailed examination of spindles formed during MLN8054 treatment on to assess whether Aurora A activity had a more prominent role in spindle assembly than indicated in the previous chapter.

4.2 Aurora A activity has a key role in spindle assembly

Immunofluorescence microscopy was used to closely examine the spindles of immunostained control and MLN8054-treated HeLa cells. Clear images of the spindles were produced by extracting the cells before fixation, using conditions that maintained stable K-fibres but caused the loss of spindle and astral microtubules (Mitchison et al., 1986). The imaging of the cells revealed some distinct differences between the spindles exposed to the different treatments (Figure 4.1). Control cells displayed robust and clearly visible K-fibres, which formed bipolar spindles with well separated poles and chromosomes that were neatly aligned on the metaphase plate (Figure 4.1A). In contrast, bipolar spindles formed during MLN8054 treatment exhibited poorly defined K-fibres, with spindles that appeared to be shorter than those seen in control cells (Figure 4.1B). The vast majority of K-fibres seen in MLN8054-treated cells seemed to be attached to both kinetochores and the centrosomal area, however some long microtubules could also bee seen to extend away from the centrosomes towards the cytosol. The chromosomes were also often less organised, forming

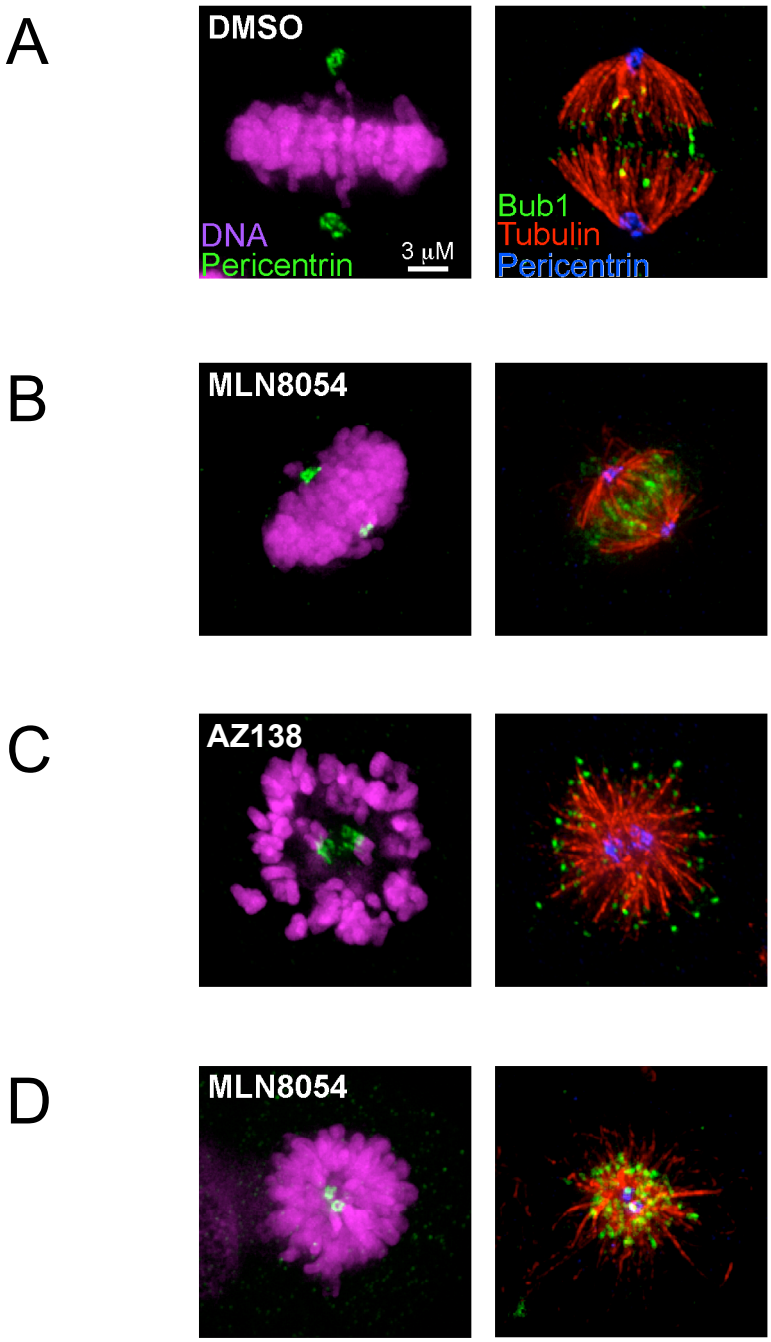

Figure 4.1 Aurora A activity is required for the formation of normal spindle structures
HeLa cells were treated with DMSO (A), 1 μM MLN8054 (B and D) or the Eg5 inhibitor AZ138 (C) for 2 hours in combination with MG132. After treatment the cells were fixed and immunostained with antibodies against Bub1 (green), Tubulin (red) and Pericentrin (blue and green). Hoescht was used to identify the DNA (pink). Z-sectioned images were taken of each of the cells and projected to produce the images shown.

broader metaphase plates, with some chromosomes pulled closely into the poles. This showed that, although Aurora A inhibition did not prevent bipolar spindles from occurring, significant differences in spindle morphology were clearly visible when compared to control cells. Thus, Aurora A activity did appear to play a more prominent role in spindle assembly than indicated in the previous chapter.

Having inspected the spindles of bipolar cells, I extended the investigation to monopolar cells, comparing those created by Aurora A inhibition with those seen after inhibiting Eg5 activity. Eg5 is a plus-end directed motor protein, whose inhibition produces monopolar spindles by causing the collapse of the centrosomes through the inability to maintain their separation, without affecting the length of spindle fibres (Mayer et al., 1999, Walczak et al., 1998). By comparing the morphologies of the two monopolar spindle types, I hoped to highlight any further spindle abnormalities produced by Aurora A inhibition. The same extraction and immunostaining process was used to examine spindles, with the imaging of the cells showing that consistent with the published literature, treatment with the Eg5 inhibitor caused spindles to collapse (Mayer et al., 1999, Walczak et al., 1998). The resulting monopolar spindles exhibited straight, robust K-fibres, which radiated out from the centrosome area towards the attached chromosomes (Figure 4.1C). The chromosomes were mono-orientated with just one kinetochore of the pair attached to a K-fibre. These monotelic attachments were highlighted by weak Bub1 staining on the attached kinetochore sisters, while the unattached sisters showed strong Bub1 staining, reflecting the different level of microtubule connections with the kinetochores (Taylor et al., 2001). In comparison, the monopolar spindles formed during MLN8054 treatment and therefore in the absence of Aurora A activity, displayed noticeably shortened K-fibres, which caused the chromosomes to be pulled closely into the centrosomal area (Figure 4.1D). Furthermore, long non-kinetochore microtubules could sometimes also be seen to protrude from the centrosomal area, similar to those observed in MLN8054-treated bipolar cells (Figure 4.1B). These spindle abnormalities therefore demonstrate a prominent and complex role for Aurora A kinase activity in spindle assembly.

4.3 Aurora A activity is involved in the separation of centrosomes

Out of the many structural abnormalities seen after MLN8054 treatment, two of the most notable were the reduction of K-fibre length and degree of centrosome separation (Figures 3.4 and 4.1B). To test whether these observations represented a quantifiable reduction in the average spindle length, I measured the distance between the centrosomes of fixed and immunostained cells (Figure 3.2A). The collection of the data revealed that populations of both control and MLN8054-treated cells exhibited a range of spindle lengths, presumably reflecting the different stages of centrosome

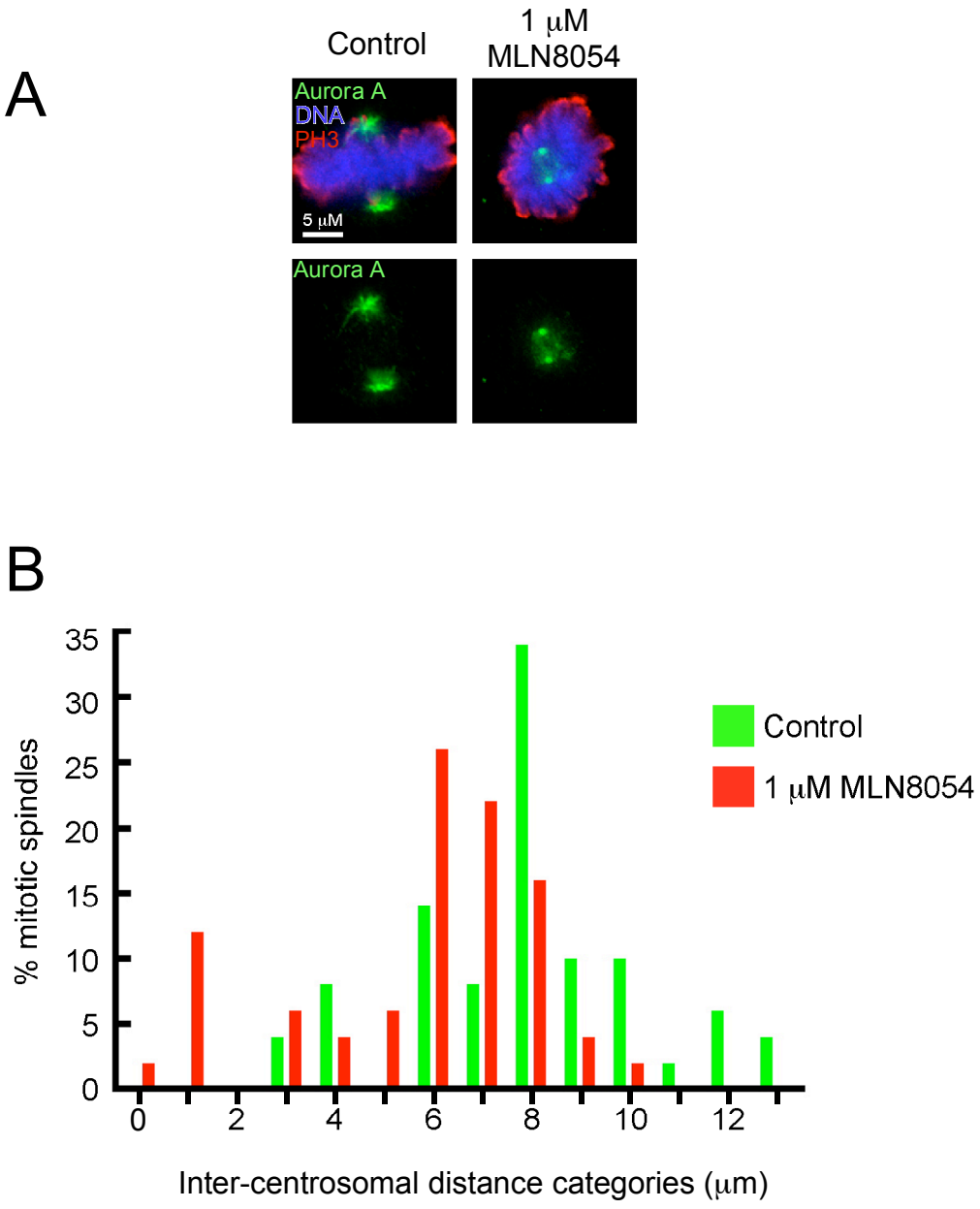

Figure 4.2 Aurora A activity is required for normal centrosome separation
HeLa cells were treated with 0 μM MLN8054 (control) or 1 μM MLN8054 for 2 hours, fixed then immunostained with antibodies against Aurora A and phospho-histone H3 (Ser10) as well as Hoescht to observe the DNA. Z-sectioned images were taken of cells that were between the stages of nuclear envelope breakdown and anaphase. (A) Projections of z-sectioned images of control and 1 μM MLN8054 treated HeLa cells, with Aurora A (green), DNA (blue) and phospho-histone H3 (Ser10) (red). (B) Graph representing the qunatitation of the inter-centrosomal distances of HeLa cells treated with no inhibitor (control in green) and 1 μM MLN8054 (red). 50 cells were measured in total for each condition as part of three independent experiments. The stated values for inter-centrosomal distances on the x-axis represent categories of grouped inter-centrosomal measurements, which include measurements up to 0.99 μm more than the indicated value. Treating the cells with 1 μM MLN8054 was found to significantly reduce inter-centrosomal distances ($P<0.0001$) using a two-tailed t-test to analyse the individual distances.

separation (Figure 4.2). When the inter-pole distances were plotted on a graph, the control cell data formed a bell-shaped distribution, with the most common category of inter-pole distance being 8 μm to 8.99 μm (Figure 4.2B). In contrast, the largest category of MLN8054-treated cells (26%) had inter-pole distances of only 6 μm to 6.99 μm, which was notably shorter than the most common spindle length in control cells. A second peak could also be seen in MLN8054-treated samples, representing an increase in the proportion of cells with greatly reduced inter-pole measurements. Interestingly, the proportion of cells with greatly reduced inter-pole distances (between 0 and 1.99 μm long) is consistent with the proportion with monopolar spindles produced after MLN8054 treatment described in chapter 3 (14% and 11% respectively) (Figure 3.4C and 4.1D). Therefore, although only a small proportion of cells become monopolar after Aurora A inhibition, Aurora A activity has a definite role in the separation of centrosomes in mitotic cells.

4.4 Inhibiting Aurora A activity reduces the number of long K-fibres

The examination of MLN8054-treated spindles appeared to indicate that they consisted of shortened K-fibres (Figures 4.1B and 4.1D). It was therefore hypothesised that the shortened K-fibres may cause reduced spindle length, thus providing a possible explanation as to how Aurora A activity is involved in the determination of centrosome separation (Figure 4.2).

To determine whether the reduction in K-fibre length observed in images of cells was quantifiable, I treated DLD-1 and HeLa cells with MLN8054, an Eg5 inhibitor or a combination of the two treatments. The samples were then extracted to remove all the tubulin polymers apart from K-fibres, before fixing and immunostaining the cells. Antibodies against tubulin were used to observe K-fibres, while antibodies recognising Bub1 and Aurora A were used to detect kinetochores and centrosomes respectively. Using DNA and centrosomal staining as a guide for spindle polarity, images were taken only of cells with monopolar spindles. Circles of a fixed size were then placed over the cell images, and the number of long K-fibres per cell was determined by counting Bub1 foci that fell outside of the circle circumference (Figure 4.3A).

As a negative control for the experiment, cells were treated with an Eg5 inhibitor, which caused the formation of monopolar spindles consisting of K-fibres radiating out from the centrosomal area towards the connected kinetochores (Figure 4.3A). The chromosomes of within cells treated with the Eg5 inhibitor were situated at a distance from the centre of the cell, producing a relatively disperse effect. In contrast, the chromosomes of cells treated with MLN8054 were seen to be tightly clustered around the centrosomal area. Similarly, the dual inhibition of Eg5 and MLN8054 also caused chromosomes to appear gathered in towards the collapsed centrosomes. To determine whether this positioning of chromosomes close to the centrosomes reflected a shortening of K-

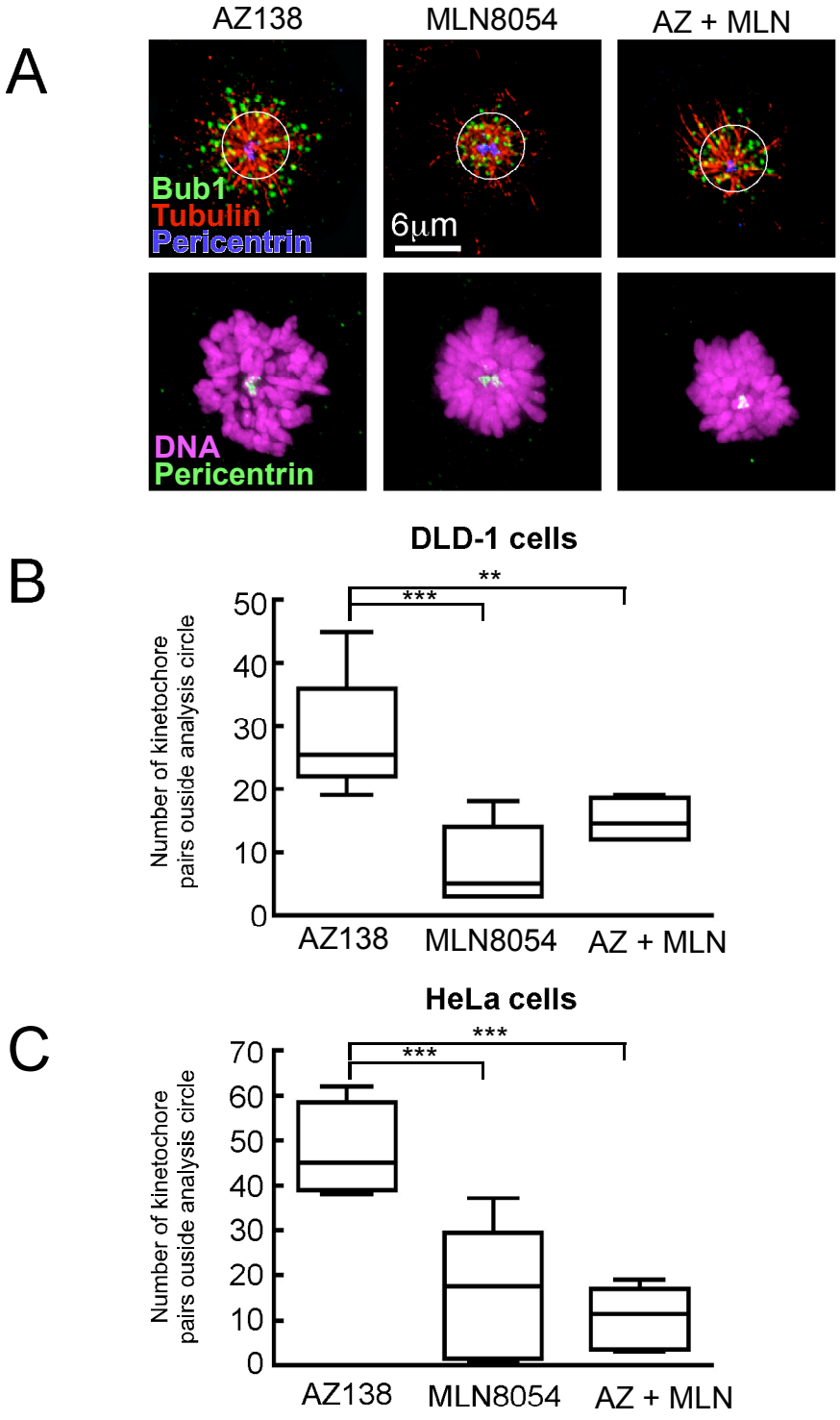

Figure 4.3 Aurora A inhibition causes a reduction in the presence of long K-fibres
(A) DLD-1 and HeLa cells were treated with AZ138, 1 μM MLN8054 or the combination of the two treatments for 2 hours. The cells were then fixed and immunostained with antibodies against Tubulin (red), Bub1 (green) and Pericentrin (blue and green), and Hoescht to visualise DNA (pink). Z-sectioned images were taken of monopolar mitotic cells and circles of a fixed size (6 μm for DLD-1 cells and 7 μm for HeLa cells) were placed in the centre of the cell images. The number of kinetochore pairs (indicated by Bub1 antibody staining) which fell outside the circles were counted and plotted on box and whisker graphs to represent DLD-1 (B) and HeLa cells (C). 12 cells were counted for each condition as part of 2 independent experiments. Two-tailed t tests were used to assess the significance of the differences ** = P<0.01; *** = P<0.001.

fibres, the number of Bub1 foci pairs present outside of the analysis circles was analysed. DLD-1 cells treated with an Eg5 inhibitor had an average of 28 Bub1 foci pairs outside of the analysis circles, which dropped to only 7 when cells were treated with MLN8054 or 15 when combining the Eg5 and MLN8054. Similarly, Eg5 inhibition in HeLa cells allowed an average of 48 Bub1 foci pairs to be counted outside analysis circles, compared with 16 and 15 after MLN8054 and the combined treatments respectively. Therefore, the relatively low number of Bub1 foci pairs recorded after MLN8054 treatment, indicated that Aurora A inhibition did indeed cause a reduction in the presence of long K-fibres. Therefore, Aurora A activity must therefore have a role in determining K-fibre length, which may explain the diminished inter-pole distances also seen after MLN8054 treatment (Figure 4.1 and 4.2).

4.5 Aurora A activity is required to maintain the separation of centrosomes

The data so far in this chapter has shown that Aurora A activity is required the formation of normal bipolar spindles, although it is not clear whether Aurora A activity is also required to maintain a bipolar spindle (Figures 3.4, 4.1, 4.2 and 4.3). Previous investigations, carried out in *Xenopus* egg extracts, describe the collapse of previously formed bipolar spindles after the addition of a recombinant kinase-dead Aurora A protein, a monoclonal Aurora A antibody or the non-catalytic region of Aurora A (Giet and Prigent, 2000, Giet and Prigent, 2001). These observations therefore demonstrate that, in *Xenopus* egg extracts at least, Aurora A activity is required to maintain a stable bipolar spindle. Using a similar methodology, I examined whether this was also the case in human cells, by inhibiting Aurora A activity in cells with previously assembled bipolar spindles. However, before I could investigate the role of Aurora A activity in bipolar spindle maintenance, I first explored potential positive controls for the experiment, a line of investigation which was to give an intriguing and unexpected outcome.

An ideal positive control would be a condition which would both prevent the development of a normal bipolar spindle (as seen after Aurora A inhibition), as well as hindering the maintenance of spindle bipolarity. Eg5 inhibition was selected as a potential positive control, as it has previously been found to be required for the formation of bipolar spindles, and was therefore predicted to also be required for bipolar maintenance (Figure 3.1C; (Walczak et al., 1998, Mayer et al., 1999). Indeed, consistent with the published literature, inhibiting Eg5 activity in DLD-1 cells revealed that the cells were unable to separate their centrosomes (Figure 4.4A). In comparison, although the majority of HeLa cells also were unable to separate their centrosomes after Eg5 inhibition, 33% were still able to form bipolar spindles (Figure 4.4B). Despite this proportion of bipolar HeLa cells,

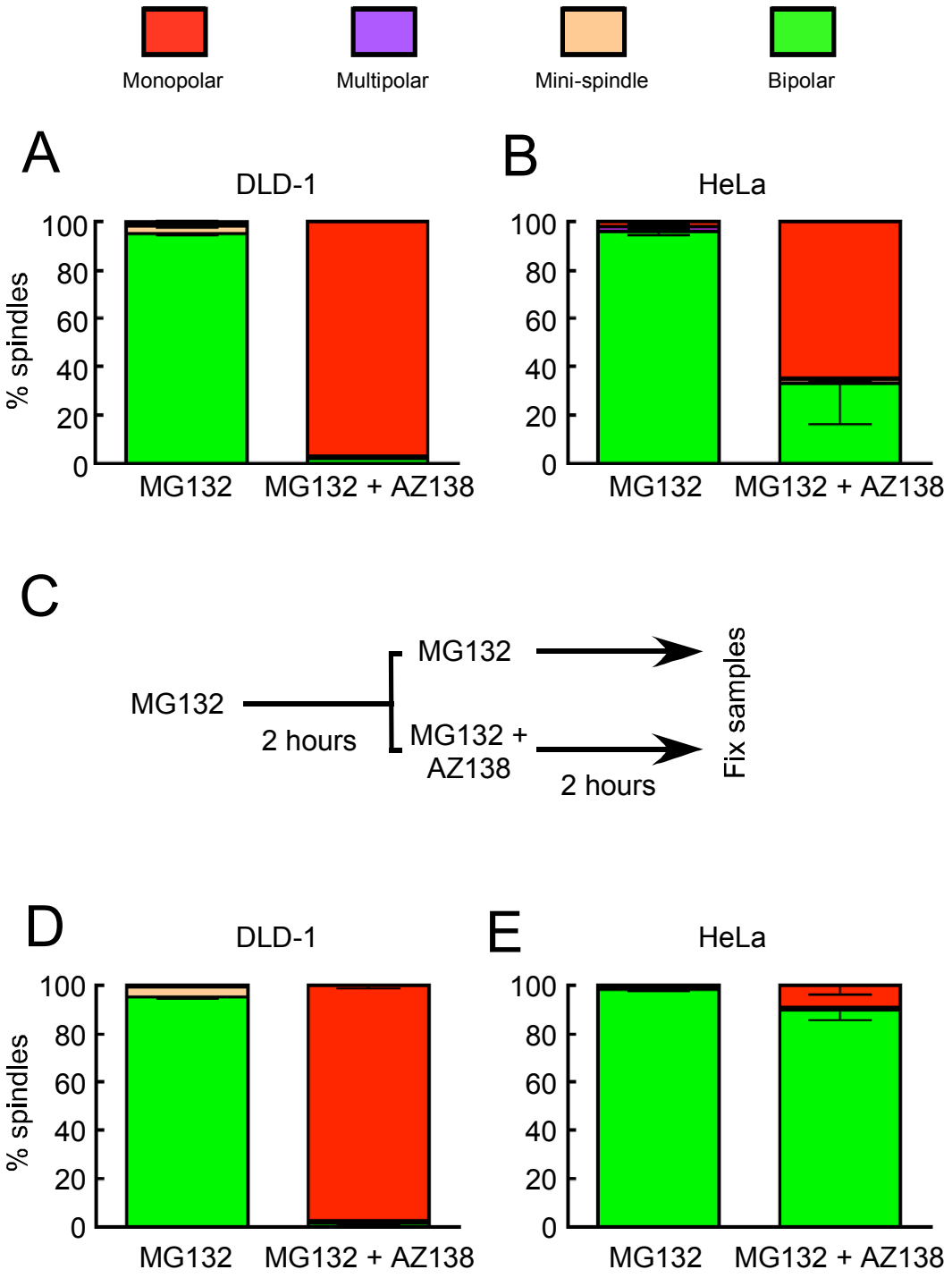

Figure 4.4 Eg5 activity is required for the maintenance of spindle bipolarity in DLD-1 but not in HeLa cells

DLD-1 (A) and HeLa cells (B) were treated with MG132 or MG132 and the Eg5 inhibitor AZ138 for 2 hours. The cells were then fixed and stained with Hoechst to observe DNA and antibodies against Aurora A to determine the resulting spindle phenotype, which were then plotted on in the form of histograms. (C) Diagram representing the protocol used to observe the effect of Eg5 inhibition on the maintenance of bipolar spindles. Cells were treated as indicated in protocol (C), then fixed and stained with Hoechst and antibodies against Aurora A. The resulting spindle phenotypes seen in DLD-1 and HeLa cells were displayed in graphs (D) and (E) respectively. In the graphs (A), (B), (D) and (E) 300 cells were counted for each condition from 3 independent experiments. Error bars represent s.e.m.

Eg5 activity was clearly required in the majority of cells analysed for the creation of spindle polarity.

To test whether Eg5 activity was also required to maintain a bipolar spindle, I first collected cells with assembled bipolar spindles by treating cell samples with the proteosome inhibitor MG132. This has been shown to inhibit the degradation of Securin and other target proteins of the anaphase promoting complex, consequently preventing the onset of anaphase and stopping any additional cells from entering mitosis, thus resulting in an accumulation of cells with bipolar spindles (Sherwood et al., 1993, Wojcik et al., 1996). These cells were then exposed to an Eg5 inhibitor to determine whether they required the activity of Eg5 to maintain their bipolarity (Figure 4.4C). DLD-1 bipolar cells collapsed following the treatment, confirming that Eg5 activity was required to maintain bipolar spindles (Figure 4.4 D). Surprisingly however, the equivalent treatment in HeLa cells showed that 90% were able to maintain separated centrosomes (Figure 4.4E). This unexpected divergence in results therefore revealed some important differences that exist between HeLa and DLD-1 cells, which will be discussed in the summary in more detail, as well as highlighting the danger of forming universal assumptions based on a single cell line.

Despite this unexpected result, the experimental test showed that the protocol could be used to investigate the capacity of cells to maintain their bipolar spindles in response to drug treatment. Therefore, following an adapted version of the protocol, I treated bipolar DLD-1 cells with MLN8054 to determine if they were dependent on Aurora A activity for the maintenance of their bipolarity (Figure 4.5A). The quantitation of the cells revealed that MLN8054 treatment caused only 11% of the bipolar spindles to collapse into monopolar or mini-spindles, while the majority (76%) were able to maintain their bipolarity (Figure 4.5B). This result appeared to indicate that most cells did not require Aurora A activity to maintain their bipolar spindles. However, a similar method of spindle phenotype characterisation, employed in the previous chapter, was found to be misleading resulting in the underestimation of the full effects of Aurora A inhibition (Figure 3.4). Therefore, to explore whether the bipolar cells were truly unaffected by the Aurora A inhibition, I measured their inter-pole distances. The analysis of control bipolar inter-pole distances showed they were maintained at an average length of 12.6 μm, while MLN8054 treatment significantly reduced the bipolar inter-pole distance to 9.4 μm (Figure 4.5C). Therefore, although it did not cause the complete collapse of centrosomes, Aurora A activity was required for the maintenance of normal spindle length in DLD-1 cells.

To determine whether HeLa cells were also dependent on Aurora A activity for the maintenance of normal spindle bipolarity, the same protocol was used (Figure 4.6A). The analysis of the resulting effects showed that, similar to as observed in DLD-1 cells, only a small proportion (8%) of HeLa

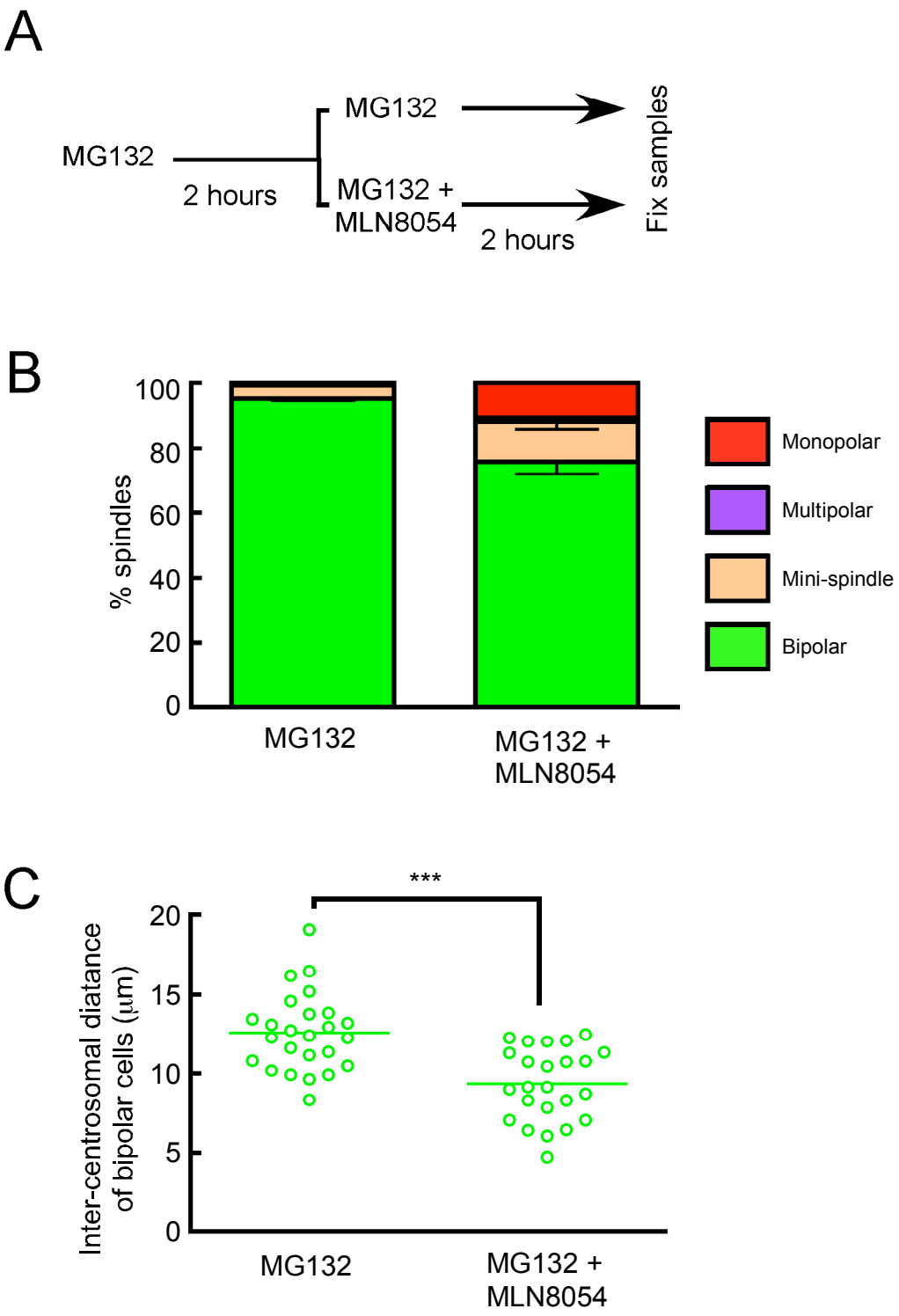

Figure 4.5 Aurora A activity is required to maintain centrosome separation in DLD-1 cells

DLD-1 cells were treated as indicated in (A) before being fixed and stained with Hoescht to visualise DNA and immunostained with antibodies against Aurora A to determine the spindle polarity. Mitotic cells were then categorised as having monopolar, bipolar, multipolar or mini-spindles (for example pictures see figure 3.4A), and plotted on graph (B). A minimum of 300 cells were counted for each condition as part of 3 independent experiments. (C) The inter-centrosomal distances of cells classified as having bipolar spindles were measured and plotted in the dot plot in. Over 25 inter-centrosomal distances were measured for each condition from 3 independent experiments. Error bars represent s.e.m. *** = P<0.001 two-tailed t test.

bipolar cells collapsed following Aurora A inhibition, while the majority of cells were analysed as remaining bipolar. To determine whether these bipolar spindles were reduced in length, similar to observed in DLD-1 cells, they were also measured. The measurement of the bipolar spindles revealed that while the average inter-pole distance of control bipolar HeLa cells was 10.4 μm, MLN8054 treatment caused a significant reduction in the spindle length of bipolar cells to 6.9 μm. Therefore the combined HeLa and DLD-1 data demonstrates that Aurora A activity is required to maintain the normal inter-pole separation in both HeLa and DLD-1 cells.

4.6 Aurora A activity is required for the maturation of the centrosome

Aurora A activity is clearly involved in both the efficient formation and maintenance of mitotic spindles, however to explore how the kinase is involved in these processes, I analysed changes in the cellular localisation of particular spindle components in response to Aurora A inhibition. Through the detailed analysis of changes in the localisation of various cellular components I hoped to identify regulatory pathways that may be dependent on Aurora A activity.

The Aurora A protein itself has been found to be rapidly exchanged between the centrosome and a cytoplasmic pool (Stenoien et al., 2003, Berdnik and Knoblich, 2002), although in *Xenopus*, its centrosomal localisation has been reported to be stabilised by substrate interaction (Giet and Prigent, 2001, Roghi et al., 1998, Stenoien et al., 2003). Furthermore, the application of VX680 to DLD-1 cells has also been shown to cause a reduction of the proportion of Aurora A protein that is localised to centrosomes (Tyler et al., 2007). These findings suggest that Aurora A localisation may be effected by its activity, however previously discussed evidence suggests that VX680 may have off-target effects (Figure 3.4). Furthermore, both a kinase-dead mutant and small molecule inhibited Aurora A have been shown to still be able to localise to the centrosomes, indicating that its activity may not be required for its localisation (Girdler et al., 2006, Manfredi et al., 2007). To resolve these discrepancies in the current literature and determine if Aurora A localisation is indeed dependent on its activity, I analysed images of fixed cells that had been immunostained with antibodies against Aurora A to determine any changes in response to MLN8054 treatment (Figure 4.7A). In control cells, Aurora A was strongly localised to the centrosomes and along the spindle microtubules. Cells exposed to MLN8054 however, showed reduced Aurora A antibody staining on the spindle, although no apparent reduction could be seen in Aurora A centrosome localisation. Intriguingly however, if the protocol was altered so as not to extract the cells before their fixation, no change could be seen in the localisation of Aurora A antibody to either the centrosomes or the spindle after MLN8054 treatment when compared to control cells (data not shown). This therefore suggests that Aurora A activity may not be required for the localisation of Aurora A protein to the

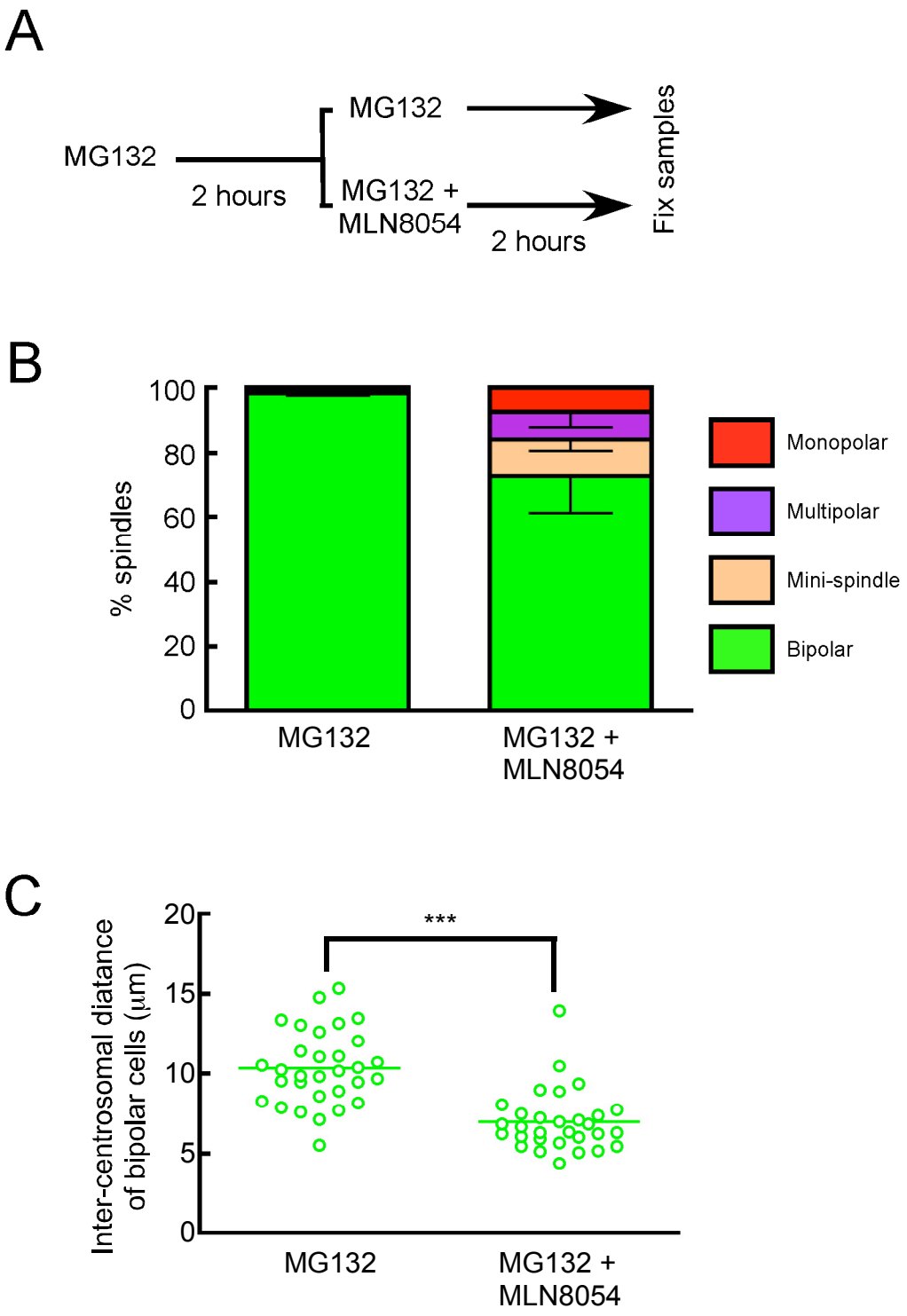

Figure 4.6 Aurora A activity is required to maintain centrosome separation in HeLa cells

HeLa cells were treated as indicated in (A) before being fixed and stained with Hoescht to visualise DNA and immunostained with antibodies against Aurora A to determine the spindle polarity. Mitotic cells were then categorised as having monopolar, bipolar, multipolar or mini-spindles (for example pictures see figure 3.4A), and plotted on graph (B). A minimum of 300 cells were counted for each condition as part of 3 independent experiments. (C) The inter-centrosomal distances of cells classified as having bipolar spindles were measured and plotted in the dot plot in. Over 25 inter-centrosomal distances were measured for each condition from 3 independent experiments. Error bars represent s.e.m. *** = P<0.001 two-tailed t test.

centrosome and spindle. However, because Aurora A protein could be partially 'washed away' from the spindle after MLN8054 treatment by extracting them before fixation, the kinase activity may be just one of the cellular features required for the stable localisation of the protein to the spindle microtubules.

Aurora A activity has also been reported to be required for the localisation of various other spindle and centrosomal components during the process of centrosome maturation (Berdnik and Knoblich, 2002, Hannak et al., 2001, Mori et al., 2007, Terada et al., 2003). In particular, the phosphorylation of TACC by Aurora A has been reported to be required for the localisation of TACC protein to the spindle and centrosome (Giet et al., 2002, Kinoshita et al., 2005, LeRoy et al., 2007). Contrary to these findings however, a mutant form of TACC, which cannot be phosphorylated on a Aurora A consensus phosphorylation site, has been shown in *Drosophila* embryos to partially localise to centrosomes (Barros et al., 2005). To determine any differences in TACC localisation after Aurora A inhibition in my experimental set up, I once again used immunofluorescence microscopy to observe changes in protein localisation after MLN8054 treatment. The observation of immunostained cells enabled me to confirm that TACC3 was localised along the spindle microtubules and on the centrosomes of control cells (Figure 4.7B). The inhibition of Aurora A activity with MLN8054 treatment however, caused TACC3 to be completely mislocalised from the centrosomes and spindle, thus confirming that Aurora A activity was indeed required for TACC3 localisation.

A demonstrated role for TACC at the centrosome and spindle, is to load chTOG/XMAP215 onto the minus-ends of the microtubules and cause an increase to its microtubule stabilising activity (Peset et al., 2005, Lee et al., 2001, Gergely et al., 2003, Kinoshita et al., 2005). I therefore wanted to determine if the mislocalisation of TACC3 from the centrosome and spindle, which was seen after Aurora A inhibition, also caused the mislocalisation of chTOG. Immunofluorescence microscopy revealed that chTOG was clearly localised to the centrosomal area of control cells. This localisation was reduced in some of the cell population after treatment with MLN8054, however a proportion of cells seemed not be effected (Figure 4.7C). Therefore to determine whether Aurora A inhibition caused an overall mislocalisation of chTOG from centrosomes, I quantified antibody intensity at the centrosomes of monopolar cell samples, and then divided this by its level in the rest of the cell. This latter step was employed to normalise the antibody intensity readings and give a value of chTOG 'centrosome/cell'. By inhibiting Eg5 activity, I was able to induce the monopolarity and allow efficient and comparable analysis of the centrosomal and cellular chTOG levels. Following this methodology, it was revealed that cells exposed to just Eg5 inhibition showed average levels of chTOG at 9.3 centrosome/cell, whereas MLN8054 treatment combined with Eg5 inhibition caused the antibody intensity to be reduced to 7.5 centrosome/cell

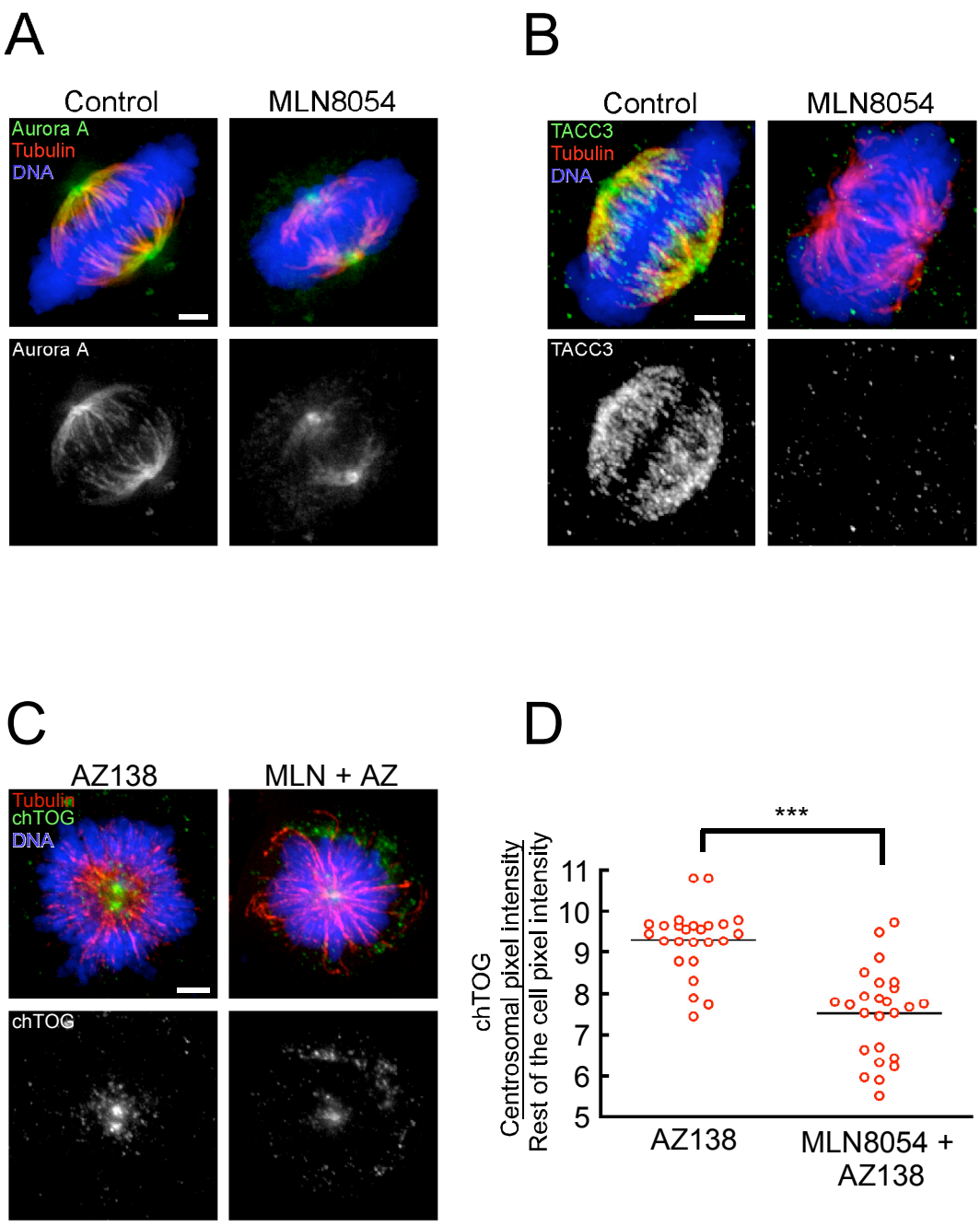

Figure 4.7 Aurora A activity is required for the proper localisation of Aurora A, TACC3 and chTOG in HeLa cells
HeLa cells were treated with 1 μM MLN8054, AZ138 or a combination of the two treatments for 2 hours. The samples were fixed and stained with Hoescht to visualise DNA and immunostained with Tubulin in combination with Aurora A (A), TACC3 (B) or chTOG(C) to observe changes in protein localisation (scale bars = 3 μM). All images shown were created from projected z-section images. The localisation of chTOG was quantitated in (D) by determining antibody intensity at the centrosome then normalising it by dividing the value at the centrosome by the antibody intensity from the whole cell. The graph represents data taken from 3 independent experiments. *** = P<0.001 two-tail t test.

(Figure 4.7D). This therefore confirmed that Aurora A activity was involved in localising chTOG to the centrosome.

The localisation of Kif2a and MCAK have also been shown to be regulated by Aurora A activity (Jang et al., 2009, Zhang et al., 2008). Interestingly both have also been shown to effect microtubule stability and are required to maintain normal steady state microtubule length, microtubule nucleation and spindle bipolarity (Ganem and Compton, 2004, Jang et al., 2008, Manning and Compton, 2007, Moore and Wordeman, 2004, Walczak et al., 1996, Zhang et al., 2008). I could not however, detect any changes in the localisation of either Kif2a or MCAK after MLN8054 treatment (data not shown).

4.7 Summary

In this chapter I described how Aurora A activity is involved in the separation of centrosomes and the determination of spindle length, a role which is likely to be at least partially dependent on the control of K-fibre length. Aurora A inhibition did not however, have as a profound effect on spindle bipolarity as inhibiting Eg5 activity, or produce effects as potent as described in previous publications (Roghi et al., 1998, Glover et al., 1995, Liu and Ruderman, 2006, Girdler et al., 2006). Despite this, the detailed examination of spindles formed in the absence of Aurora A activity nonetheless indicated that Aurora A activity plays an important role in spindle construction.

The spindle abnormalities seen after MLN8054 treatment indicate that the inactivated Aurora A kinase is unable to perform its usual roles in the phosphorylation and organisation of various spindle and centrosomal components. Indeed, the stable localisation of a proportion of Aurora A protein to the spindle was found to be dependent on its activity. Furthermore, consistent with previous reports, Aurora A activity was also found to be required for the proper localisation of TACC3 to the centrosomes and spindle (Giet et al., 2002, Kinoshita et al., 2005, LeRoy et al., 2007). This relationship between TACC3 and Aurora A activity may be responsible for the determination of K-fibre length, as shortened microtubules were also seen in *Drosophila* embryos expressing non-phosphorylateable TACC3 (Barros et al., 2005). Similar to TACC3, chTOG was also dependent on Aurora A activity to achieve normal levels of localisation to the centrosome. This reduction of chTOG at centrosomes after Aurora A inhibition may have been the result of TACC3 mislocalisation, as TACC is proposed to load chTOG/XMAP215 onto the minus-ends of the microtubules (Peset et al., 2005, Lee et al., 2001, Gergely et al., 2003, Kinoshita et al., 2005). The reduction in the levels of chTOG on the centrosome may have contributed to the disorganisation of spindles after MLN8054 treatment, as similar spindle abnormalities have also

been seen after the depletion of chTOG/XMAP215 (Cassimeris and Morabito, 2004, Gergely et al., 2003).

In addition to Aurora A activity being involved in spindle formation and maintenance, consistent with published reports, data presented during this chapter demonstrated that Eg5 activity also played a part in spindle development (Blangy et al., 1995). Interestingly however, inter-cell line differences were observed in response to the requirement for Eg5 activity in the formation and maintenance of spindle bipolarity. Indeed, while DLD-1 cells were prevented from forming bipolar spindles following Eg5 inhibition (Figure 4.4A), 33% of HeLa cells were found to exhibit bipolar spindles after the same treatment (Figure 4.4B). Furthermore, bipolar spindles which had been previously formed in DLD-1 cells were unable to be maintained after Eg5 inhibition, causing them to collapse into monopolars (Figure 4.4D). In contrast, 90% of HeLa cells were able to maintain previously formed bipolar spindles once Eg5 activity was inhibited (Figure 4.4E).

These contrasting responses to Eg5 inhibition may be explained by the different methods employed by the cell lines to separate and maintain the separation of centrosomes. HeLa cells are capable of separating their centrosomes before nuclear envelope breakdown through the nucleation of astral microtubules and their associations with cortical actin and the actin cytoskeleton (Whitehead et al., 1996, Buttrick et al., 2008, Robinson et al., 1999, Cytrynbaum et al., 2005, Cao et al., 2010). Conversely, DLD-1 cells exhibit late centrosome separation, which is dependent on astral microtubules connections with the cell cortex following nuclear envelope breakdown and the nucleation of the spindle microtubules (see (Morrow et al., 2005) for schematic). These observations may highlight integral differences in spindle construction between the two cell lines which may therefore explain their divergent dependencies on Eg5 activity.

A further explanation for the differences in response to Eg5 inhibition may be that HeLa cells could be able to maintain centrosome separation early in mitosis due to the dependence on astral microtubules before nuclear envelope breakdown. Therefore when Eg5 activity is inhibited, HeLa cells may rely on astral microtubule connections to separate and maintain the separation of their centrosomes before the formation of spindles, which may represent the 33% of bipolar spindles observed after MLN8054 treatment. HeLa cells may also have increased astral microtubule attachments after the formation of a spindle, which may offer support the bipolar spindle structure when Eg5 activity is inhibited. Immature HeLa spindles, which are unable to form appropriate levels of support to stabilise their bipolarity may however collapse on inhibition of Eg5, possibly explaining why 90% and not 100% of HeLa cells are able to maintain their bipolarity after Eg5 inhibition (Figure 4.4B). Consistent with the dependency of HeLa cells on astral microtubules, previous reports have highlighted the presence of astral microtubules as acting as spindle tethers in HeLa cells (Thery et al., 2007). In contrast, DLD-1 cells may not have an equivalent supportive

spindle mechanism and this may explain why they are not capable of maintaining previously formed bipolar spindles on Eg5 inhibition. This is however a speculative hypothesis, and there are other potential explanations for the inter-cell line variation, such as the differing sensitivities of the two cell lines to the Eg5 inhibitor.

Despite the inter-cell line differences observed in response to Eg5 inhibition and whatever their cause may be, the main focus of this chapter was the demonstration that Aurora A activity is involved in the process of spindle formation and maintenance. In the next chapter, I will describe whether this role for Aurora A activity in spindle assembly extends to the ability to efficiently align chromosomes.

5 Investigating the importance of Aurora A activity in chromosome alignment

5.1 Introduction

The major function of the mitotic spindle is to properly align chromosomes to form a metaphase plate, it is therefore unsurprising to learn that conditions which disrupt the proper functioning of the spindle, also cause chromosomal misalignment (Mayer et al., 1999, Gascoigne and Taylor, 2008, Schneider et al., 2007). Thus, as Aurora A activity has been shown to have an important role in spindle assembly (Figures 4.1 to 4.7) it was apparent that it may have an additional function in the alignment of chromosomes. Aurora A has even been shown to interact with the kinetochore protein CENP-A, which has been implicated in the recruitment of various proteins to the kinetochore, thus the proper functioning of the kinetochore (Kunitoku et al., 2003). Indeed, consistent with a role for Aurora A activity in chromosome alignment, previous investigations have shown that the inactivation of Aurora A kinase to causes severe chromosomal misalignments (Glover et al., 1995, Mori et al., 2007, Girdler et al., 2006, Liu and Ruderman, 2006). The chromosome misalignments described in these publications, were however reported to occur as a result of the high occurrence of monopolar spindles. Such potent monopolar effects were not seen after Aurora A inhibition in my investigation, nor were they in a number of similar investigations (Berdnik and Knoblich, 2002, Peset et al., 2005, Hoar et al., 2007). I therefore set about to determine whether the less acute spindle defects produced by the Aurora A inhibition during my investigation would also cause problems with chromosome alignment during mitosis. This investigation would consequently facilitate both a better understanding of the role Aurora A activity in the regulation of spindle assembly and determine whether this role extends to facilitating proper spindle function.

5.2 Aurora A activity is required for efficient chromosome alignment

To test the efficiency of cells to align chromosomes, I first induced a consistent degree of chromosomal misalignments by treating HeLa cells with an Eg5 inhibitor. I then washed away the inhibitor, and allowed the cells to recover from the induced alignment defects in a series of different conditions (Figure 5.1A). After this recovery period, the cells were fixed and immunostained to observe any problems in chromosome realignment.

Analysis of the fixed cell samples revealed that maintaining exposure to the Eg5 inhibitor produced severe chromosome misalignment in 100% of cells (Figure 5.1B), with 97% also displaying monopolar spindles, a phenotype that is consistent with the published literature (Figure 5.1C) (Mayer et al., 1999, Kapoor et al., 2000). The high occurrence of monopolar spindles after Eg5 inhibition indicates that the chromosomal misalignments were a consequence of the acute spindle defects. In contrast to sustained Eg5 inhibition, the removal of the Eg5 inhibitor and its replacement with DMSO, allowed 97% of cells to form bipolar spindles, most of which were able to align their chromosomes. In fact, only 9% of cells allowed to recover from the Eg5 inhibition in DMSO showed signs of severe chromosomal misalignment. Conversely, when an Aurora B inhibitor was applied to cells after the removal of the Eg5 inhibitor, severe chromosome misalignments were seen in 96% of cells analysed. This inability to align chromosomes in the absence of Aurora B activity is consistent with previous reports implicating Aurora B activity in the process (Kapoor et al., 2000). However, unlike the sustained Eg5 treatment, which produced a similar severity in chromosome alignment defects, 94% of cells treated with the Aurora B inhibitor were seen to have bipolar spindles after Aurora B inhibition.

To determine whether Aurora A activity, like that of Aurora B, was required for efficient chromosome alignment in the presence of a bipolar spindle, I analysed the fixed cell samples to assess their ability to recover from Eg5 inhibitor-induced alignment defects in the presence of 1 μM MLN8054 (Figure 5.1A). The quantitation of the treated cells revealed that most showed signs of chromosomal misalignment after the treatment with only 23% of MLN8054-treated cells displayed neatly organised metaphase plates (Figure 5.1B). This level of chromosome misalignment therefore demonstrates that Aurora A activity was indeed required for efficient chromosome alignment in cells. Interestingly however, similar to the effects of Aurora B inhibition, the majority (78%) of MLN8054-treated cells exhibited bipolar spindles (Figure 5.1C). This finding is comparable to data derived from the depletion of Aurora A or the use of micro-injected antibody to disrupt its function, both of which caused misalignment defects despite the formation of a bipolar spindle (Marumoto et al., 2003, Kunitoku et al., 2003).

The inability to align chromosomes therefore suggests that either, similar to the effects of Aurora B inhibition, the chromosome alignment defects were not a result of inadequately formed spindle structures, or conversely, the complete effects of the Aurora A inhibition on spindles were not detected. To investigate the latter possibility, I measured the inter-pole distances of the cells to highlight any potentially undetected defects in the spindles (Figure 5.1D). Unsurprisingly, the measurement of the inter-pole distance of cells exposed to sustained Eg5 inhibition, revealed an average measurement of only 1.6 μm, reflecting the monopolarity of the spindles. In comparison, washing the effects of Eg5 inhibition from cells and replacing it with either DMSO or the Aurora B

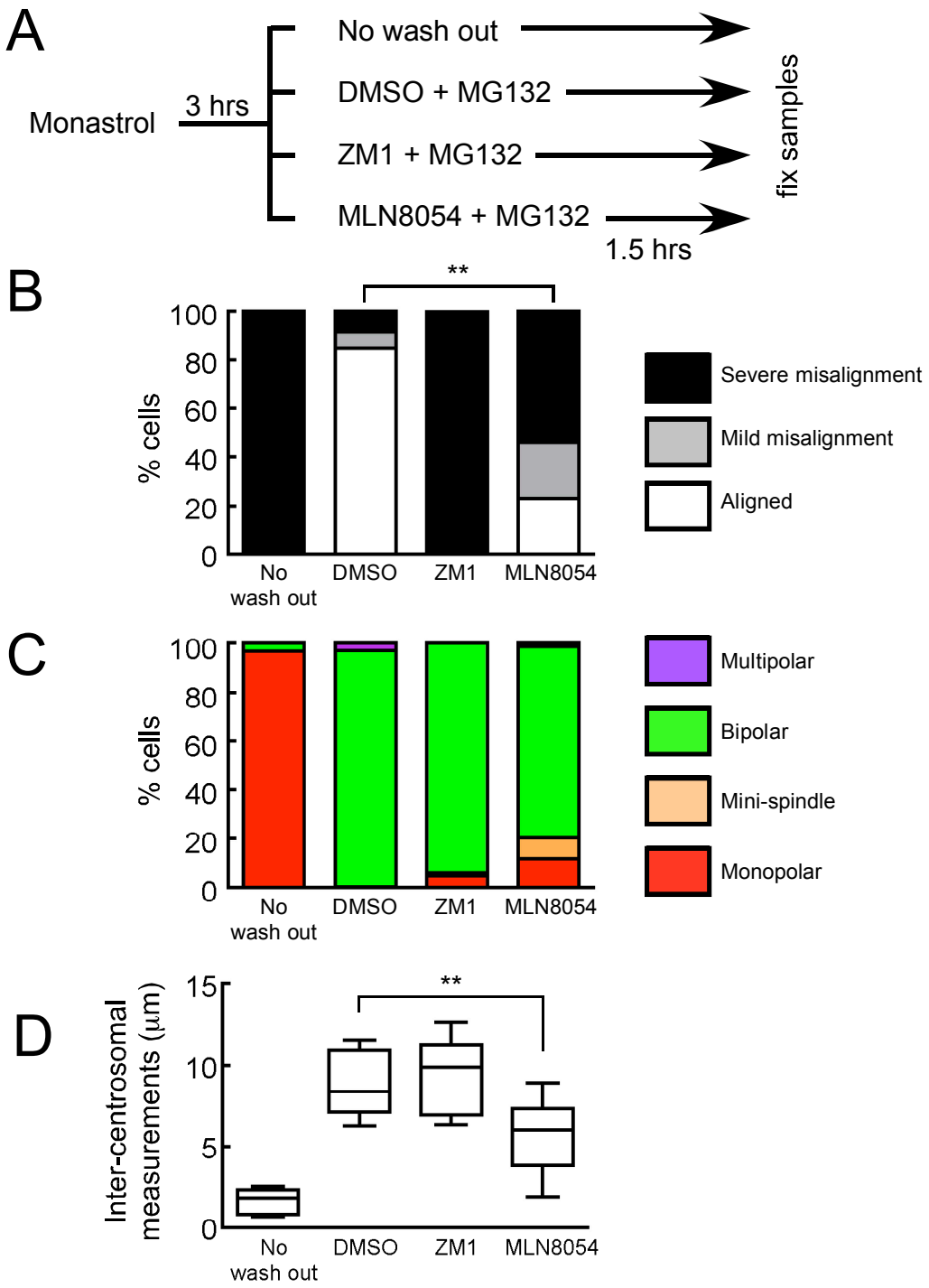

Figure 5.1 Aurora A activity is required for the efficient alignment of chromosomes in HeLa cells

(A) The protocol used to determine whether Aurora A activity has a role in chromosome alignment. After treating HeLa cells according to the protocol outlined in (A), cells were then fixed and immunostained with antibodies against Pericentrin and Tubulin to determine the spindle phenotype and Aurora B together with Hoerscht to allow determination of chromosomal alignment. (B) Quantitation of chromosomal alignment. Cells were classified as having 'mild' misalignment if up to four of their chromosomes were unaligned, or if there were more than four, they were classified as having 'severe' misalignment. Cells were classified as being 'aligned' if all their chromosomes were in the form of a neat metaphase plate. Cell samples treated with 1 μM MLN8054 were found to have significantly fewer bipolar spindles than those threated with DMSO. (C) Histogram to show spindle phenotypes of the samples treated as indicated in (A). (D) Measurement of spindle lengths of samples treated as indicated in (A), calculated from z-stacked images. All graphs represent data taken from three independent experiments. ** = P<0.01 two-tailed t test.

inhibitor, showed average spindle lengths of 8.9 μm and 9.5 μm respectively. Importantly however, if cells were instead treated with MLN8054 after removing the Eg5 inhibitor, an average spindle length of only 5.7 μm was detected. Some cells were even recorded to have spindle lengths as low as 1.9 μm. Therefore, although MLN8054 did not have such a dramatic an effect on spindle polarity as Eg5 inhibition, the observed shortened spindle lengths after MLN8054 treatment showed that Aurora A activity was required in the process of spindle reassembly after induced monopolarity. This role for Aurora A activity in spindle formation is consistent with previous observations (Figure 4.2), and may provide an explanation as to why cells are unable to realign their chromosomes after Aurora A inhibition.

5.3 Aurora A activity is required for the regulation of microtubule nucleation and organisation

Having clearly shown that Aurora A activity functions in the regulation of the assembly of spindle structures (Figure 4.1, 4.2 and 5.1), I next sought to identify how the kinase controls spindle formation and function. Many reports, including those in the previous chapter, have linked the presence of the Aurora A protein or its kinase activity to the maturation of centrosomes, thus implicating a role for the kinase in the regulation of microtubule nucleation from the centrosome (Figure 4.7) (Mori et al., 2007, Terada et al., 2003, Hannak et al., 2001, Berdnik and Knoblich, 2002, Hachet et al., 2007, Barros et al., 2005, Piehl et al., 2004). Aurora A activity has also been associated with the regulation of spindle formation in the absence of centrosomes in *Xenopus* egg extracts (Koffa et al., 2006, Liu and Ruderman, 2006, Zhang et al., 2008). In support of this finding, chromosome/kinetochore microtubule nucleation has also been linked to the interaction of Aurora A with its activator, TPX2, in human cells (Bird and Hyman, 2008). These studies all indicate that Aurora A kinase activity serves to regulate the nucleation of microtubules in order to create normal spindle structures, which could explain why reduced K-fibre lengths and inter-pole measurements are seen after Aurora A inhibition (Figure 4.2 and 4.3).

To confirm whether the role described for Aurora A activity in the regulation of microtubule nucleation would explain the abnormal spindle structures seen after MLN8054 treatment, I analysed the effect of the treatment on microtubule repolymerisation. To effectively observe microtubule repolymerisation, I first induced the depolymerisation of all cellular microtubules through a combination of cold treatment and the depolymerising effects of Nocodazole. The cells were subsequently incubated in warmed Nocodazole to ensure that they were able to regain their function after the cold treatment. When released from the Nocodazole, the cells were allowed to repolymerise in a range of different conditions, before being fixed and immunostained to observe

their tubulin polymers and various kinetochore components (Figure 5.2). As a comparative control for the experiment, I maintained the exposure of some cells to Nocodazole, to enable the examination of cells that were completely unable to reform spindles (Figures 5.2 A(i) and 5.3 A). Interestingly, unlike cells analysed immediately after the Nocodazole and cold treatment (data not shown), immunofluorescence microscopy revealed that a large number of short tubulin polymer stubs were present over the DNA of the Nocodazole treated cells. This suggested that a limited amount of centrosome-independent microtubule nucleation may have been possible, although the tiny microtubule polymers were clearly unable to form any kind of spindle. The ability to carry out chromosome/kinetochore microtubule nucleation has been previously reported to be dependent on the interaction of Aurora A with its activator, TPX2 (Bird and Hyman, 2008). This observation thus indicates that the process of chromosome/kinetochore nucleation may be reliant on active Aurora A. To test this theory, preceding the cold and Nocodazole treatments, I treated cells with MLN8054 in combination with Nocodazole, and looked for evidence of similar microtubule polymers over the DNA (Figure 5.2B(i)). In contrast to the effects seen after preventing Aurora A and TPX2 interaction however, the inhibition of Aurora A activity did not appear to cause a reduction in the level of the small tubulin polymers, suggesting that Aurora A activity may not be required for the initiation of chromosome directed microtubule nucleation (Figure 5.3B) (Bird and Hyman, 2008).

To determine whether Aurora A activity was required for the control of more general microtubule polymerisation, I observed spindles which were permitted to form in the absence of Nocodazole (Figure 5.2Aii). Unsurprisingly, in cells which had not been treated with either Nocodazole or MLN8054, microtubules were able to polymerise and form bipolar spindles with robust K-fibres, which emanated out from the centrosomal area and attached to the kinetochores of chromosomes (Figure 5.3C). These K-fibre attachments were highlighted by low Mad2 levels at kinetochores and the neat organisation of chromosomes within cells (Waters et al., 1998, Chen et al., 1996, Waters et al., 1996). Importantly however, microtubules nucleated in the presence of MLN8054 were noticeably disorganised compared with those within control spindles (Figures 5.2B(ii) and 5.3B). In many cases, the spindles of MLN8054-treated cells did not appear to have any clear canonical structure, and were made up of unfocused and often crooked microtubules of varying thickness and length. Some microtubules could even be seen to extend outside of the spindle bundle, with their unattached ends stretching out into the cell with no apparent function. Despite the disorganised and chaotic state of the spindles formed in the absence of Aurora A activity, the spindle fibres appeared to be attached to kinetochores, as judged by low levels of Mad2 at kinetochores. Unsurprisingly however, the chromosomes within the cells were not generally aligned on the metaphase plate. From the immunofluorescent images of the various samples it can therefore be deduced that Aurora A activity is involved in the regulation of microtubule nucleation, and is necessary for their organisation and the formation of a functional spindle.

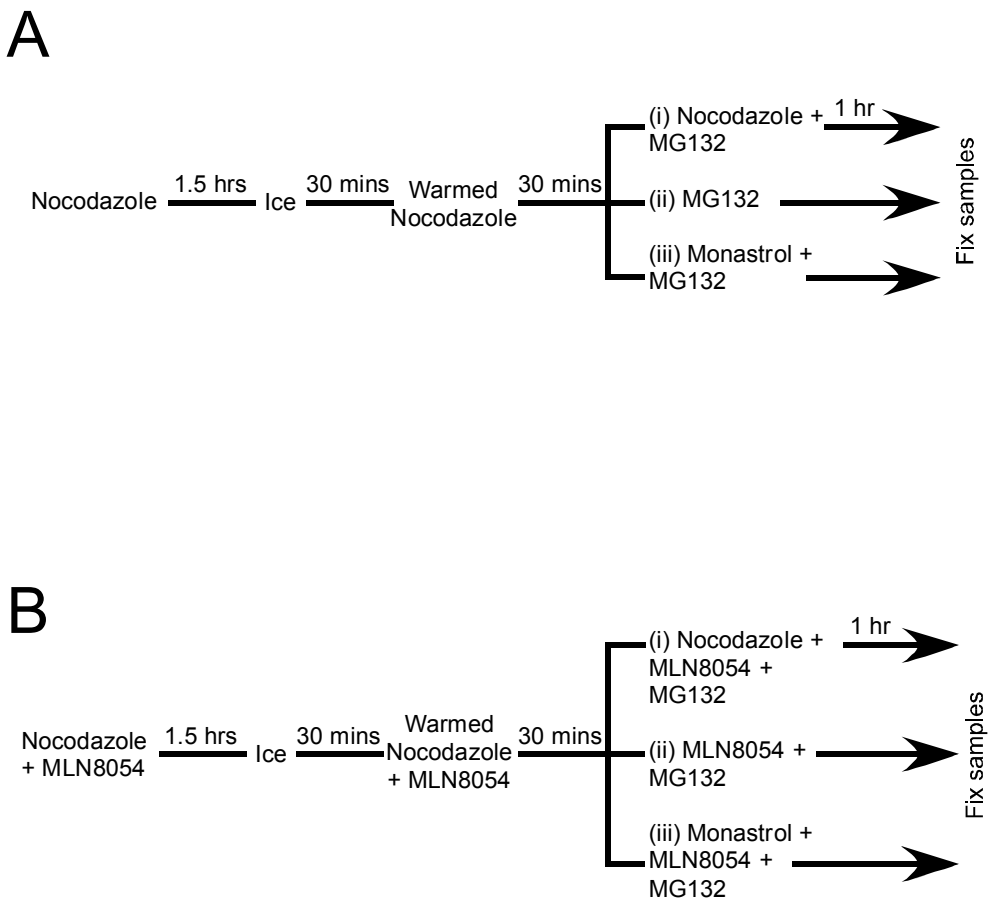

Figure 5.2 Protocols to determine whether Aurora A activity is involved in the regulation of microtubule nucleation and organisation
(A(i))-A(iii)) Protocols used to examine the regrowth of microtubules in the absence of MLN8054 treatment. (B(i)-B(iii)) Protocols used to examine the regrowth of microtubules following MLN8054 treatment.

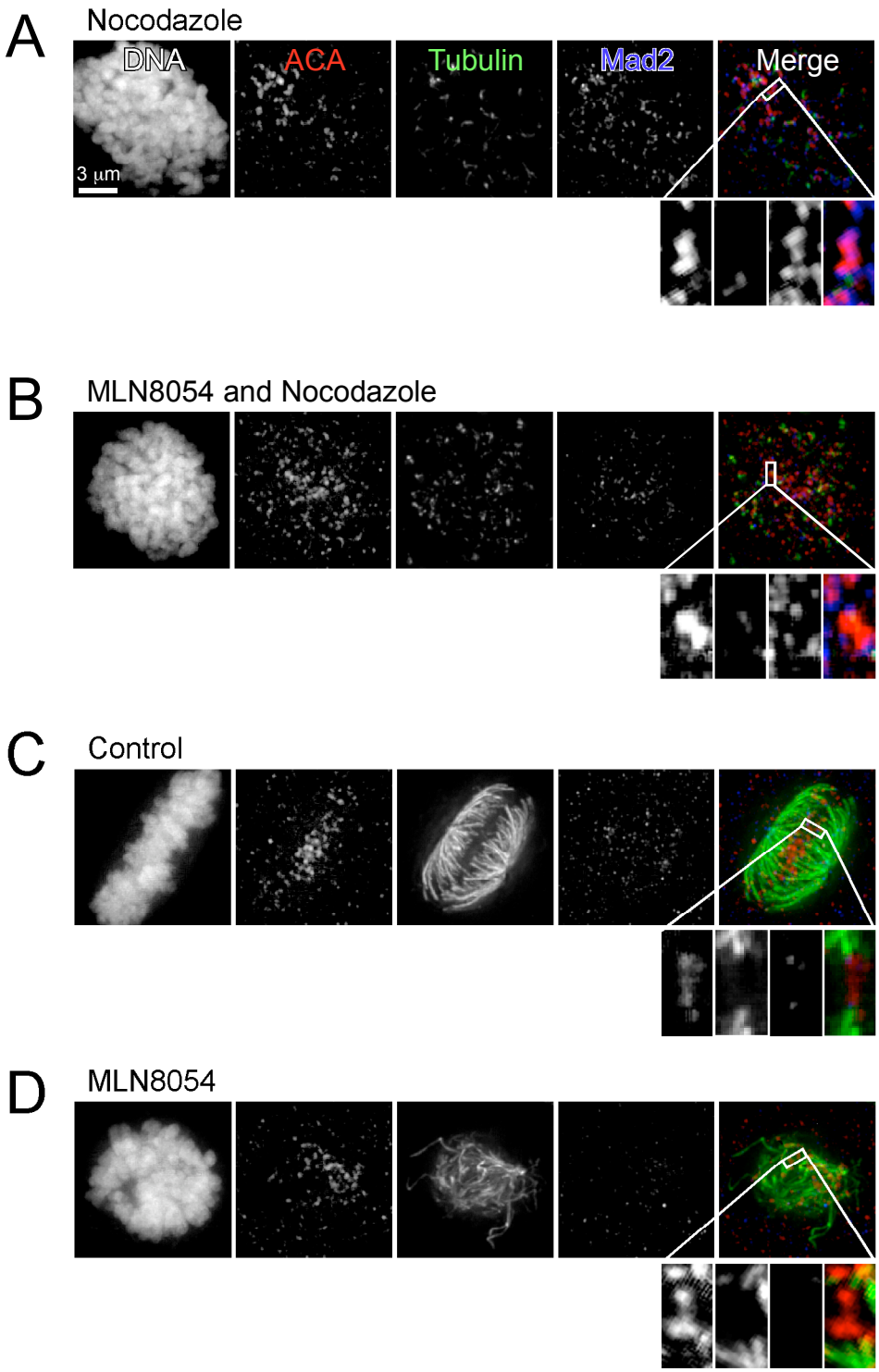

Figure 5.3 Aurora A activity regulates the formation and organisation of spindle microtubules
Immunofluorescence images of HeLa cells treated according to the microtubule regrowth protocols shown in Figure 5.2:
(A) Nocodazole: Figure 5.2A(i)
(B) MLN8054 and Nocodazole: Figure 5.2B(i)
(C) Control: Figure 5.2A(ii)
(D) MLN8054: Figure 5.2B(ii)
The cells were fixed and immunostained with antibodies against ACA (red), Tubulin (green) and Mad2 (blue), Hoescht was use to visualise the DNA (white). Z-sectioned images were taken of the samples, which were then projected to produce the images shown. Blow-ups were used to highlight microtubule-kinetochore attachments.

5.4 Aurora A activity is required for the formation of a functional spindle

The chromosome misalignments observed in cells with spindles nucleated in the absence of Aurora A activity indicated that the kinase activity is required for the regulation of spindle function as well as the formation of its structure. An alternative hypothesis however, is that Aurora A activity may be required for the proper attachment of kinetochores to the spindle microtubules. In support of this hypothesis, the activity of Ipl1, the single yeast Aurora kinase, has been shown to be involved in the regulation of chromosome attachment to the spindle, indicating that mammalian Aurora A may also have a similar role (Biggins and Murray, 1999, Li et al., 2002, Biggins and Murray, 2001). Furthermore, I have been able to faintly detect Aurora A antibody at the centromeres of immunostained cells, which may indicate a proportion of cellular Aurora A which may carry out its potential function in regulating microtubule attachments (Figure 5.4A). Aurora A over-expression has also been shown to cause an increase in unattached chromosomes, thus potentially strengthening the association between the kinase and the regulation of microtubule-kinetochore attachments (Anand et al., 2003). The link between Aurora A activity and chromosomal attachment could therefore provide an alternate explanation as to why cells are not able to efficiently align their chromosomes after Aurora A inhibition.

To explore this alternative hypothesis, I carried out the close examination of kinetochore microtubule occupancy in cells with spindles formed in the absence of Aurora A activity. Immunofluorescent images of these spindles appeared to indicate that microtubules were however attached to kinetochores (Figure 5.3), although to gain a more definitive understanding of chromosome attachment, I employed a quantifiable approach. By using the pixel intensity of Mad2-directed antibody at kinetochores of images of fixed and immunostained cells, I was able to quantify the level of microtubule occupancy at kinetochores (Waters et al., 1998, Chen et al., 1996). This level of Mad2 at kinetochores was normalised by dividing the pixel intensity with that of ACA on adjacent centromeres to give a value of Mad2/ACA. As a negative control for the experiment, cells were prevented from forming microtubules through their treatment with Nocodazole. These cells showed high levels of Mad2 at kinetochores (average 3.7 Mad2/ACA), which is consistent with the notion that microtubule occupation at kinetochores causes a reduction in Mad2 levels (Figures 5.2A(i), 5.3A and 5.4B). The encouragingly high Mad2 level at kinetochores is also consistent with the observation that no long microtubules were formed after the Nocodazole treatment (Figure 5.3A). When MLN8054 was added in conjunction with Nocodazole, cells were observed to have similarly high levels of Mad2 at their kinetochores (average of 3.6 Mad2/ACA) (Figures 5.2 B(i), 5.3B and 5.4B). This similarity in Mad2 levels at kinetochores after the two treatments was vital to the integrity of the experiment, as it indicated that the MLN8054

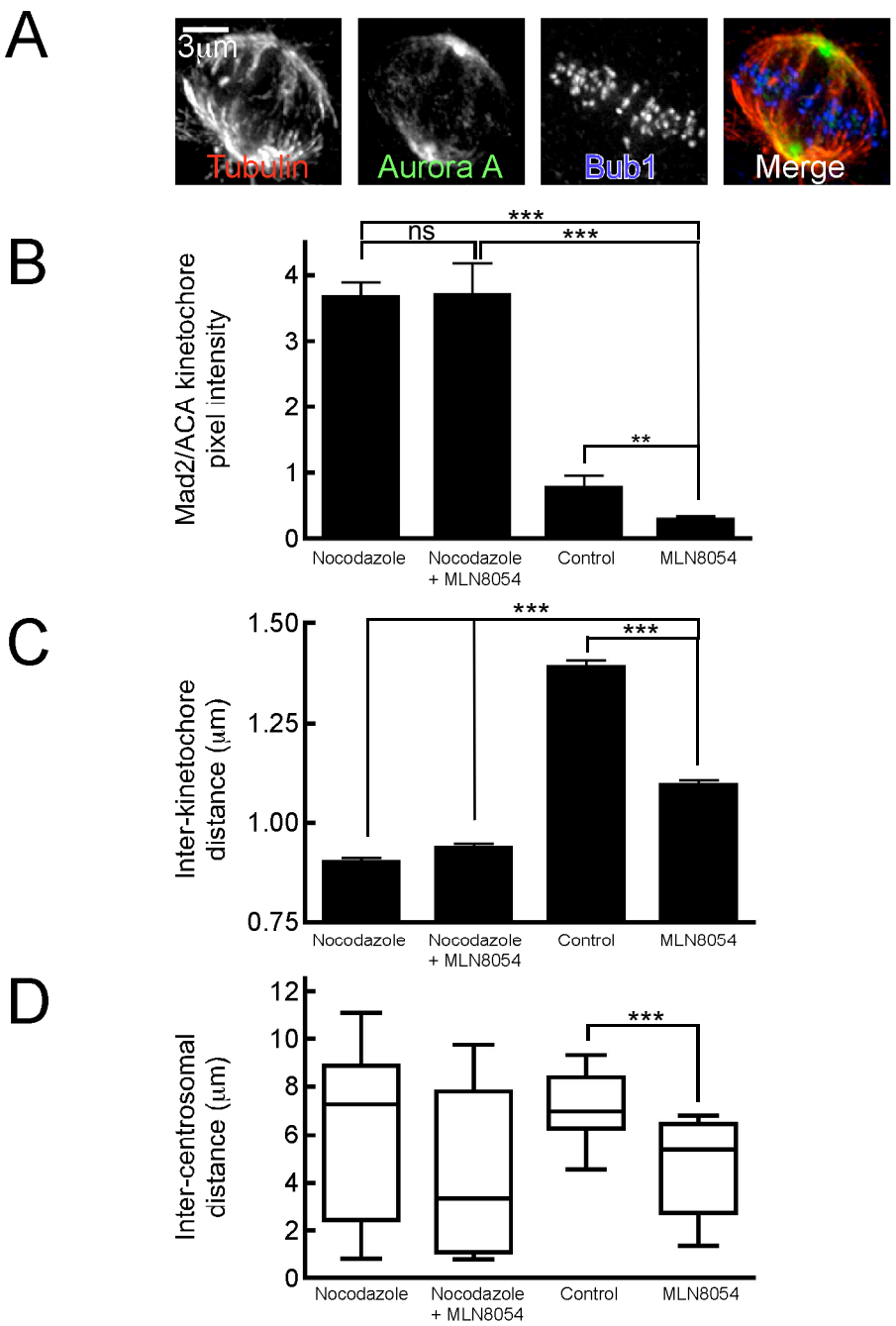

Figure 5.4 Aurora A activity is not required for the attachment of K-fibres to kinetochores but is needed for the generation of normal inter-kinetochore stretch
(A) Immunofluorescence images of an untreated HeLa cell, which was fixed and immunostained with antibodies against Tubulin (red), Aurora A (green) and Bub1 (blue). (B-D) HeLa cells were treated according to the microtubule regrowth protocols shown in Figure 5.2: Nocodazole = Figure 5.2A(i); MLN8054 and Nocodazole = Figure 5.2B(i); control = Figure 5.2A(ii); MLN8054 = Figure 5.2B(ii). (B) Histograms to show kinetochore localisation of Mad2. The pixel intensity of both Mad2 and ACA antibodies at kinetochores was measured from projected z-stacked images. Mad2 levels were then normalised by dividing the Mad2 pixel intensity at individual kinetochores with that of ACA at corresponding centromeres. Between 305 and 399 kinetochores were analysed for each condition from, three independent experiments. (C) Measurements of the distance between sister kinetochores, a minimum of 417 inter-kinetochore distances were measured from 3 independent experiments. Both graphs in (B and C) display the mean and the s.e.m. (D) Inter-centrosomal measurements from a minimum of 16 cells for each condition taken from 3 independent experiments, shown in the form of box and whisker graphs. Two-tail t-tests used to analyse the significance of differences observed.
ns = P>0.01; ** = P<0.01; *** = P<0.001.

treatment did not cause a reduction in Mad2 at kinetochores independent of microtubule attachment.

Control cells that had been permitted to nucleate their microtubules unperturbed, exhibited an average Mad2 level of only 0.8 Mad2/ACA at kinetochores (range of -10.5 to 27.6) (Figures 5.2A(ii) and 5.4B). This relatively low value indicated the presence of high numbers of microtubule connections with kinetochores. Similarly, when MLN8054 was used to inhibit Aurora A activity in cells, low levels of Mad2 at kinetochores were also seen. Interestingly however, MLN8054-treated cells showed slightly lower levels of Mad2 than seen in the control cell samples, with cells showing on average 0.3 Mad2/ACA (range of -2.3 to 8.2). This finding demonstrated that Aurora A activity is not required for the attachment of spindle fibres to kinetochores. Therefore, the chromosomal misalignments seen after Aurora A inhibition were not produced by the failure of chromosomes to attach to the spindle.

To further examine how Aurora A activity is involved in the alignment of chromosomes, I next looked at the requirement for the kinase's activity in the generation of spindle mechanical force. If the spindle structural abnormalities observed after inhibiting Aurora A activity were sufficiently severe enough to prevent the generation of adequate spindle force on chromosomes, the cell would therefore be unable of efficiently align its DNA (Skibbens and Salmon, 1997, Skibbens et al., 1993). This could therefore explain the existence of chromosome misalignments after Aurora A inhibition. To test this theory, I measured the inter-kinetochore distances of cells exposed to a variety of different treatments, to determine whether appropriate tension was being generated by the spindle (Chen et al., 1996, Waters et al., 1996). Analysis of cells exposed to sustained Nocodazole treatment, whether combined with MLN8054 treatment or not, revealed that they displayed relatively small inter-kinetochore distances, averaging at 0.9 μm (Figures 5.2A(i), 5.2B(i) and 5.4C). This small inter-kinetochore measurement indicated a lack of tension across the kinetochore pairs, which is consistent with the treatment of Nocodazole preventing the formation of a spindle capable of generating force to chromosomes (Figures 5.3A and 5.3B). In contrast to this, cells which were permitted to re-nucleate microtubules without their treatment with Nocodazole or MLN8054, exhibited kinetochore pairs that were held on average 1.4 μm from each other (Figures 5.2A(ii) and 5.4C). This larger inter-kinetochore distance is indicative of force generated across the centrosome by a functional spindle (Figure 5.3C). In comparison however, cells with spindles formed in the presence of MLN8054 were only able to produce an average kinetochore separation of 1.1 μm (Figure 5.4C). This suggests that although MLN8054 treatment did permit the formation of a spindle which was able to produce some mechanical force across kinetochore pairs, the tension generated was not as great as that seen in the control cells. Therefore, Aurora A activity may be required for the formation of spindles capable of applying adequate force

to chromosomes, and consequently enabling their efficient alignment. Interestingly however, the greater inter-kinetochore distances seen in MLN8054-treated cells compared to the Nocodazole treated samples, suggested that Aurora A activity was not required for the generation of all the mechanical force exerted on the chromosomes. This observation therefore indicates the presence of additional factors other than Aurora A activity work to control spindle function, thus possibly explaining how some cells were able to correct severe chromosomal misalignments even in the absence of Aurora A activity (Figure 5.1B).

Despite the ability of cells to generate some tension across kinetochore pairs after Aurora A inhibition, Aurora A activity was clearly required to produce normal levels of force on the chromosomes. To test whether these observations could be related to inadequacies in the spindle structure, I measured the inter-pole distances of the spindles to assess defects in spindle structure (Figures 5.2 and 5.4D). Cells that had been exposed to Nocodazole, either with or without MLN8054, exhibited a large range of inter-pole distances, possibly reflecting the inability to affect centrosomal positioning after nuclear envelope breakdown due to the lack of spindle microtubules (Figures 5.3A and 5.3B). Intriguingly however, most cells which had been exclusively treated with Nocodazole showed a notably larger degree of centrosome separation than cells that had been additionally exposed to MLN8054. In comparison to Nocodazole treated cells, control cells with undisrupted microtubule re-growth were observed to have a relatively small range of spindle lengths, with an average inter-pole distance of 7.1 μm. Importantly through, the average spindle length reformed in after MLN8054 treatment was only 4.7 μm. The shortened spindle lengths observed after MLN8054 treatment is consistent with previous findings (Figure 5.1D), which together with the observation that the spindles are also disorganised (Figure 5.3D), further highlight the existence of abnormal spindles after Aurora A inhibition. These spindle abnormalities could be used to explain how the cells are unable to produce normal levels of inter-kinetochore tension and consequently efficiently align their chromosomes following Aurora A inhibition.

5.5 Chromosome alignment defects seen after MLN8054 treatment are produced by Aurora A inhibition and not Aurora B inhibition

Despite the evidence suggesting that Aurora A inhibition causes chromosome alignment defects, it could be argued that the effects could be caused by off-target Aurora B inhibition by the treatment with MLN8054 (Figure 5.1B) (Adams et al., 2001b, Ditchfield et al., 2003, Hauf et al., 2003). I have previously shown MLN8054 to have a high degree of specificity towards Aurora A activity at the 1 μM concentration employed throughout the investigation however, at certain concentrations it can also inhibit Aurora B activity (Figure 3.3). Therefore to confirm that the MLN8054 treatment

does not inhibit Aurora B when used at 1 μM, I employed a further method of testing for Aurora B activity. This method relied on exploiting the role of Aurora B activity in the correction of improper microtubule/kinetochore attachments as a way of identifying whether Aurora B activity was being inhibited. Key to this method was the creation of monopolar spindles and the consequential prevention of normal levels of spindle-induced tension across kinetochore pairs. The production of monopolar spindles should therefore only allow monotelic attachments to exist in the presence of Aurora B activity to lack of the centrosomal stretch generated by amphitelic kinetochore attachment (see Figure 1.4) (Hauf et al., 2003, Cassimeris et al., 1994, Cimini et al., 2006, Kapoor et al., 2000). Therefore the existence of any kinetochore pairs that were attached to the spindle in any way other than through a monotelic connection, would be evidence of inactivated Aurora B kinase. Consistent with this theory, only monotelic attachments were detected in spindles treated with an Eg5 inhibitor, highlighting the presence of active Aurora B (Figures 5.2A(iii) and 5.5A). Importantly, cells treated with MLN8054 in combination with the Eg5 inhibitor, also appeared to exclusively demonstrate monotelic attachments (Figures 5.2B(iii) and 5.5B). The short K-fibres produced by the MLN8054 treatment however, made analysis of microtubule attachments difficult. Therefore, as a further method of detecting microtubule occupancy, Mad2 immunostaining was used to detect the single disconnected sisters of monotelically attached kinetochore pairs. Using this method of detection, single unattached kinetochores were observed to occur in MLN8054-treated cells, thus indicating the presence of monotelic attachments, and supporting the finding that Aurora B activity was not inhibited by the MLN8054 treatment. Furthermore, the observation that Mad2 levels were not reduced after the MLN8054 treatment also indicated the presence of active Aurora B after the MLN8054 treatment, as Aurora B inhibition has been shown to cause the reduction in Mad2 levels at kinetochores (Ditchfield et al., 2003). This collective evidence therefore demonstrates that Aurora B must have been active after the MLN8054 treatment, further validating my previously described Western blot results (Figure 3.3). Consequently, the chromosomal misalignments seen after the MLN8054 treatment must have been a product of Aurora A inhibition, thus demonstrating the requirement for Aurora A activity in the regulation of the development and organisation of structurally and functionally sound spindles (Figures 5.1B and 5.3D).

5.6 Summary

Through observing the effects of MLN8054 on the ability of a cell to recover from severe spindle disruption, I have shown that Aurora A activity was required for the efficient alignment of chromosomes. The alignment defects seen after Aurora A inhibition were not caused by problems with kinetochore/spindle attachments. Instead, reduced levels of inter-kinetochore tension and

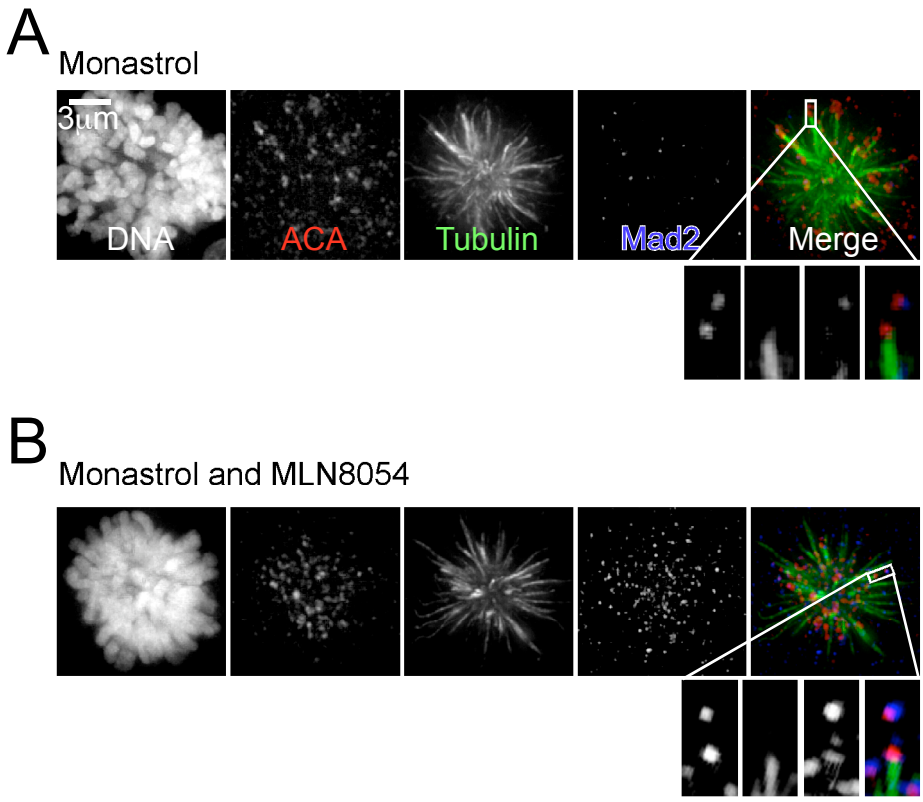

Figure 5.5 Aurora B activity is not inhibited by 1 μM MLN8054 in HeLa cells
Immunofluorescence images of HeLa cells treated according to the microtubule regrowth protocols shown in Figure 5.2:
(A) Monastrol: Figure 5.2A(iii)
(B) Monastrol and MLN8054: Figure 5.2B(iii)
The cells were fixed and immunostained with antibodies against ACA (red), Tubulin (green) and Mad2 (blue), Hoescht was use to visualise the DNA (white). Z-sectioned images were taken of the cells with blow-ups to highlight microtubule-kinetochore attachments. Images levels were scaled identically.

shortened spindle lengths and levels of organisation seen after the MLN8054 treatment indicated that the role for Aurora A kinase activity in the alignment of chromosomes was derived from its function in spindle assembly.

The roles for Aurora A activity in spindle assembly and chromosome alignment potentially have important implications in mitotic progression. In the next chapter, I will discuss whether the chromosome misalignments observed after Aurora A inhibition are capable of extending the mitotic period and examine their effect on long-term cell viability.

6 Investigating the role of Aurora A activity in mitotic progression and the preservation of cell viability

6.1 Introduction

In the previous chapter I described the role played by Aurora A activity in the efficient alignment of chromosomes. An inability of cells to align their chromosomes such as that described after Aurora A inhibition has previously been shown to maintain the activation of the spindle assembly checkpoint, delaying a cell's progression into anaphase and consequently extending the time spent in mitosis (Mayer et al., 1999, Musacchio and Hardwick, 2002, Nicklas and Koch, 1969, Li and Nicklas, 1995, Rieder et al., 1995). One could therefore infer that the inhibition of Aurora A activity would also increase the time cells spent in mitosis. Consistent with this theory, Aurora A inhibition has been shown to cause an increase in the proportion of mitotic cells, thus indicating that cells were delayed in mitosis (Manfredi et al., 2007). Additionally, the micro-injection of anti-Aurora A antibodies was even shown to often prevent the completion of mitosis (Marumoto et al., 2003). Furthermore, during the course of this investigation, Hoar and colleagues also reported that the inhibition of Aurora A caused a delay to the progression from prophase to anaphase, with the time spent in mitosis more than doubling (Hoar et al., 2007). These reports of mitotic delays indicate that the spindle assembly checkpoint is activated by Aurora A inactivation, presumably in response to the dependency on Aurora A to efficiently align chromosomes (Figure 5.1B).

Intriguingly, although cells without active Aurora A were often observed to divide after extended mitotic periods, many displayed segregation defects and the progeny was detected as being aneuploid (Marumoto et al., 2003, Hoar et al., 2007, Katayama et al., 2001, Glover et al., 1995). The observation of segregation defects after Aurora A inhibition suggested that not all of the chromosomes had been properly aligned before the commitment to anaphase. Interestingly, two reports published after this investigation linked Aurora A activity with the ability to maintain spindle checkpoint activation, which may explain the occurrence of segregation defects after the inactivation of the kinase (Wysong et al., 2009, Kaestner et al., 2009). Alternatively, the apparent ability of the cells to exit mitosis without aligning chromosomes, could be attributed to the cells 'slipping' from mitosis after the extended delays, as in similar reports of the effects of spindle disruption cells have been shown to exit mitosis after extended delays to produce aneuploid progeny (for review see [Musacchio and Hardwick, 2002, Musacchio and Salmon, 2007, Rieder and Maiato, 2004, Weaver and Cleveland, 2005]; (Mayer et al., 1999, Li and Nicklas, 1995, Rieder et al., 1995, Gascoigne and Taylor, 2008)). Importantly however, the spindle checkpoint has been

shown to be functional in these cells, with the segregation errors being attributed to merotelic attachments that had gone undetected by the cell (Gascoigne and Taylor, 2008).

Therefore to better understand the role of Aurora A activity in mitotic progression, I set out to examine whether Aurora A inhibition in my experimental set up caused delays to mitosis, and whether cells were capable of dividing after the delay. I also examined whether Aurora A activity was required for the maintenance of the overall viability of cells, a line of investigation that would have important implications for targeting of Aurora A activity as an anti-cancer therapy.

6.2 Aurora A activity is required for normal mitotic progression

To test whether cells were dependent on Aurora A activity for their timely progression through mitosis, I treated DLD-1 cells with MLN8054, using phase contrast time-lapse microscopy to track the progression of individual cells through the cell cycle. By distinguishing the breakdown of the nuclear envelope and subsequent decondensation of chromatin within the cells, I was able to ascertain the duration of mitosis. I also assessed the ability of cells to perform cytokinesis and divide into two cells following their exit from mitosis. The results of this observation would provide an indication of whether it was eventually possible to adequately correct the spindle abnormalities and chromosome misalignments previously observed after Aurora A inhibition (Figures 3.4, 5.1 and 5.3).

The analysis of time-lapse microscopy movies revealed that control cells spent an average of 47 minutes in mitosis, before dividing into two daughter cells (Figure 6.1). In comparison, treating cells with MLN8054 caused cells to spend an average of 71 minutes in mitosis, 54% longer than control cells. An increase in mitotic period in response to Aurora A inhibition is consistent with reports of similar spindle defect-inducing treatments, indicating the sustained activation of the spindle assembly checkpoint activation in response to the misaligned chromosomes (Mayer et al., 1999, Kapoor et al., 2000). Interestingly however, cells exposed to MLN8054 were capable of dividing into two daughter cells after their extended mitotic period, although the nature of the phase-contrast imaging made it difficult to identify any possible chromosome segregation defects.

Similar to the MLN8054 treatment, treating cells with an Eg5 inhibitor also caused a mitotic delay compared to control cells, however the extension to the time in mitosis experienced by cells was even more pronounced. Cells treated with the Eg5 inhibitor took an average of 689 minutes to exit mitosis, after which they were observed to exit mitosis without dividing, similar to recent reports of the effects of the treatment in the literature (Gascoigne and Taylor, 2008). This extended mitotic delay followed by the exit without division indicated that cells were unable to produce a spindle

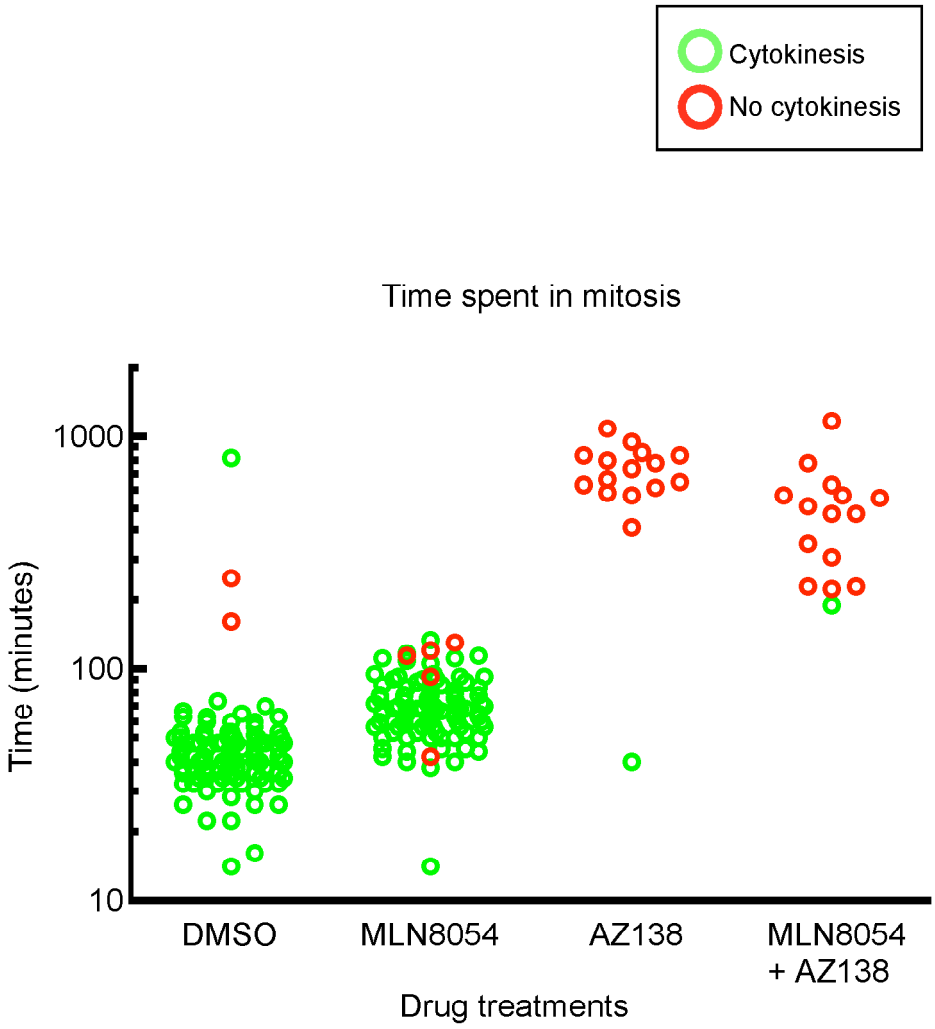

Figure 6.1 The inhibition of Aurora A activity causes cells to delay in mitosis
DLD-1 cells were treated with DMSO, 1 μM MLN8054, the Eg5 inhibitor AZ138 or a combination of 1 μM MLN8054 and AZ138. Time-lapse phase contrast images were taken every 2 minutes to enable the analysis of mitosis (determined as being between nuclear envelope breakdown and chromosome decondensation). Green circles represent cells that performed cytokinesis, while red circles indicate cells that were unable to do so.

capable of aligning chromosomes, suggesting that cells were only permitted to exit mitosis by mitotic slippage (Rieder and Maiato, 2004, Brito and Rieder, 2006, Weaver and Cleveland, 2005, Gascoigne and Taylor, 2008). The difference observed in how cells are affected by Eg5 and Aurora A inhibition is consistent with the differing severity of spindle effects produced by the two treatments (Figures 4.2 and 5.1).

The large mitotic delay seen after Eg5 inhibition not only indicated the presence of severe chromosome misalignments, but also highlighted the existence of a functional checkpoint, which was capable of detecting them. Similarly, after Aurora A inhibition, the mitotic delay prior to division also suggested the activation of the spindle assembly checkpoint consequently preventing anaphase onset. Interestingly however, Aurora A activity has been implicated in the proper functioning of the spindle assembly checkpoint (Wysong et al., 2009, Kaestner et al., 2009, Kunitoku et al., 2003). Furthermore, Aurora A over-expression has also been reported to cause the override of the spindle assembly checkpoint (Anand et al., 2003, Dutertre and Prigent, 2003, Jiang et al., 2003a, Rong et al., 2007). The role of Aurora A kinase activity in the checkpoint override following Aurora A over-expression is however up for debate, as some studies have found the activity to be required for the override of the checkpoint (Anand et al., 2003), while others have found it not to be (Jiang et al., 2003a, Littlepage and Ruderman, 2002, Meraldi et al., 2002). This large range of varying reports implicating Aurora A in the spindle checkpoint provoked the examination of the integrity of the spindle assembly checkpoint following MLN8054 treatment, in order to resolve the role of Aurora A activity in the process.

Therefore, to explore the role of Aurora A activity in the spindle assembly checkpoint, I exposed cells to both Eg5 and Aurora A inhibition. If the checkpoint was indeed dependent on Aurora A activity, the extended delay seen after Eg5 inhibition should no longer be seen after the combined treatment, as the cells would not have a functional checkpoint to alert them to the severely unaligned chromosomes caused by inhibiting Eg5 activity. In a similar experiment, Aurora B inhibition has been previously shown to cause the override of a Taxol induced mitotic arrest, driving cells out of mitosis and highlighting a compromised checkpoint in the absence of active Aurora B (Ditchfield et al., 2003). Surprisingly however, although a decrease in average mitotic timings was seen after the dual inhibition of Eg5 and Aurora A, the cells were not driven straight out of mitosis. Instead, cells were held in mitosis for an average period of 481 minutes. This result is similar to two recently published papers, which also used MLN8054 to show that spindle toxin induced mitotic arrest could be slightly reduced by inhibiting Aurora A activity (Wysong et al., 2009, Kaestner et al., 2009).

6.3 The extended mitotic period observed after Aurora A inhibition is due to the cells inability to efficiently align their chromosomes

Similar to DLD-1 cells, HeLa cells also suffered mitotic delays after MLN8054 treatment (Figure 6.2A). The induced delay was however more substantial than that described in DLD-1 cells, as after MLN8054 treatment the average HeLa mitotic timing was extended to 292.7 minutes, 289% longer than the mitotic period of control cells (Figure 6.4A). Also like DLD-1 cells, the majority of HeLa cells were capable of dividing following MLN8054 treatment, although a small number of cells suffered from cytokinesis defects, while others were observed to start blebbing during mitosis, indicating the instigation of cell death (Charras, 2008).

The long mitotic delays produced in both DLD-1 and HeLa cells, indicated that Aurora A inhibition caused chromosome alignment defects, thus preventing the onset of anaphase (for review see [Taylor et al., 2004]). Consistent with this idea, I previously described how Aurora A inhibition prevented the efficient alignment of chromosomes (Figure 5.1). Although to determine whether the mitotic delay following MLN8054 treatment was in direct response to chromosome misalignments caused by Aurora A inhibition, I analysed live HeLa cells which had been transfected with GFP-Histone H2B. Analysis of this cell line with the use of time-lapse fluorescent microscopy enabled the examination of chromosome alignments in response to different treatments. The analysis of control cells showed that their chromosomes were able to efficiently align after nuclear envelope breakdown to form a metaphase plate, before evenly dividing (Figure 6.2B(i)). Cells treated with MLN8054 however, were not able to align their chromosomes as efficiently as control cells (Figures 6.3(ii) and 6.3(iii)). Instead, chromosomes moved around each other in a disorganised bundle for extended periods. In some instances, the MLN8054 treatment even prevented chromosomes from forming a metaphase plate at all, causing cells to decondense their chromosomes without dividing (Figure 6.2B(ii)). Many cells were however observed to form metaphase plates and divide after the MLN8054 treatment, although segregation errors were detected (Figure 6.2B(iii)). The observation that cells treated with MLN8054 were able to progress to anaphase despite the presence of misaligned chromosomes or exit mitosis without dividing was predicted to have a detrimental effect on the long-term viability of cells.

6.4 Aurora A inhibition causes aneuploidy and cell death

The purpose of mitosis is to produce two genetically identical daughter cells. The inhibition of Aurora A however, appeared to cause problems with the ability of cells to evenly divide, indicating that many of the treated cells' progeny may be aneuploid. To test this possibility and investigate whether Aurora A activity has a role in preserving the genetic stability and thus the viability of

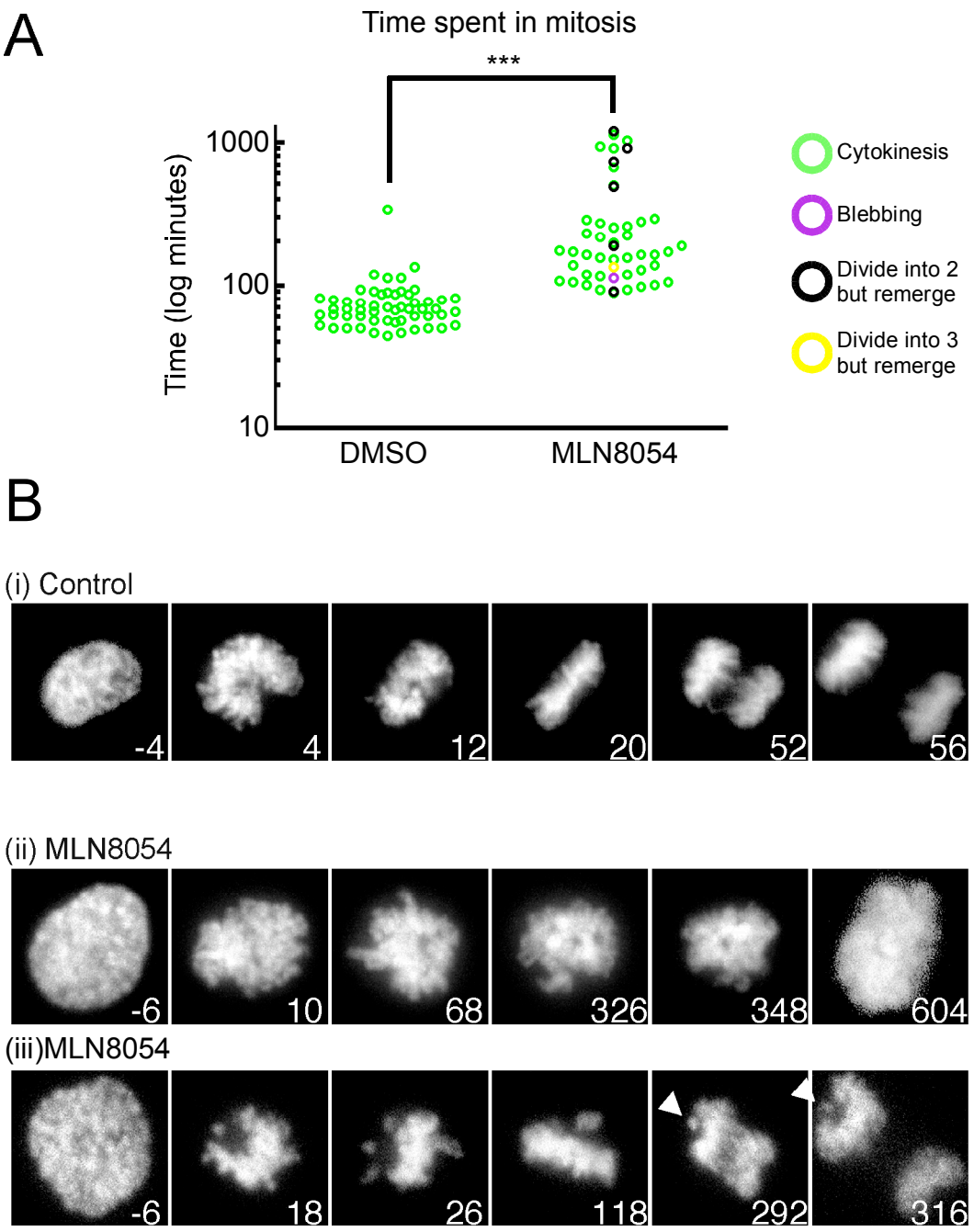

Figure 6.2 The inhibition of Aurora A activity causes HeLa cells to delay in mitosis by preventing efficient chromosome alignment
(A) HeLa cells were treated with DMSO or 1 μM MLN8054 and analysed using time-lapse phase contrast images, which were taken every 2 minutes. The images were transformed into movies, which were analysed, and the time taken for cells to go through mitosis (determined as being between nuclear envelope break down and chromosome decondensation) was plotted. Green circles represent cells that performed cytokinesis; purple circles represent cells that began blebbing during mitosis; black circles represent cells that appeared to divide into two daughter cells but remerged into one cell; yellow cells indicate cells that appeared to divide into three cells but refomed into two daughters.
(B) Projected z-section images from the time-lapse movies of GFP histone H2B HeLa cells treated with DMSO (i) or 1 μM MLN8054 (ii) and (iii). Numbers shown in the bottom right-hand corner of each image indicate time elapsed in minutes after nuclear envelope breakdown. Arrowheads highlight a pair of sister chromatids that were not properly aligned before the initiation of anaphase. *** = P<0.001 two-tailed t test.

cells, I used flow cytometry to analyse the DNA content of the cells that had been treated with MLN8054.

As a positive control for the experiment, I observed what effect long-term Eg5 inhibition had on the DNA content of cells. I had previously observed Eg5 inhibition to produce severe spindle assembly defects, chromosome misalignments and long mitotic delays, and I therefore expected the cell cycle profiles to reflect this severity of the treatment (Figures 5.1 and 6.1). Consistent with this prediction, the flow cytometry profiles showed that the exposure of the cells to an Eg5 inhibitor caused a huge increase in the proportion of cells with 4N DNA (Figure 6.3A(i)). A massive 44% of cells were shown to accumulate in mitosis after 24 hours of treatment, compared with only 3% of mitotic cells in control samples. Additionally, after 24 hours of treatment only a minimal peak representing cells with 2N DNA could be seen. This is consistent with the live cell imaging data, as it indicates that cells were unable to divide into two genetically identical daughter cells. A large sub-G1 peak could also be seen after only 24 hours of Eg5 inhibition, with near total genetic disintegration seen after 48 hours of inhibitor treatment. The occurrence of such profound sub-G1 population indicated that cell death was induced by the Eg5 inhibitor, which is consistent with data from a recent publication (Gascoigne and Taylor, 2008).

Similar to the effects of Eg5 inhibition, the flow cytometry profiles of MLN8054-treated cells also revealed an accumulation of cells in mitosis (Figure 6.3(ii)). The proportion of cells accumulated in mitosis after MLN8054 treatment was not however as profound as that seen after Eg5 inhibition, with only 19% of cells being identified as being mitotic after 24 hours of treatment. Interestingly, a clear peak could still be seen representing cells with 2N DNA even after 48 hours of MLN8054 treatment, which consistent with the live-cell imaging, suggested that cell division was possible (Figure 6.2A). As the treatment period increased however, the peaks representing both 2N and 4N DNA were notably diminished. A large sub-G1 population could be detected after 72 hours of MLN8054 treatment, 24 hours longer than it had taken Eg5 inhibition to cause roughly equivalent cellular effects. Eventually, after 96 hours of MLN8054 treatment only 1% of cells were recorded as being in mitosis. This finding, together with the considerable sub-G1 population, indicated that like Eg5 inhibition, sustained MLN8054 treatment caused profound cell death.

To test whether the increase in the proportion of cells in the sub-G1 population seen after extended MLN8054 treatment, did actually represent the loss of cell viability, I tested the growth and survival of cells exposed to MLN8054 using a colony formation assay (Figures 6.3B and 6.3C). The crystal violet staining of control colony formation samples illustrated the occurrence of many colonies after 14 days of growth. In comparison, sustained treatment with the positive control inhibitor, ZM1, prevented the occurrence of cell colonies after the same time period. This is consistent with published data, which described ZM1 treatment as negatively effecting the

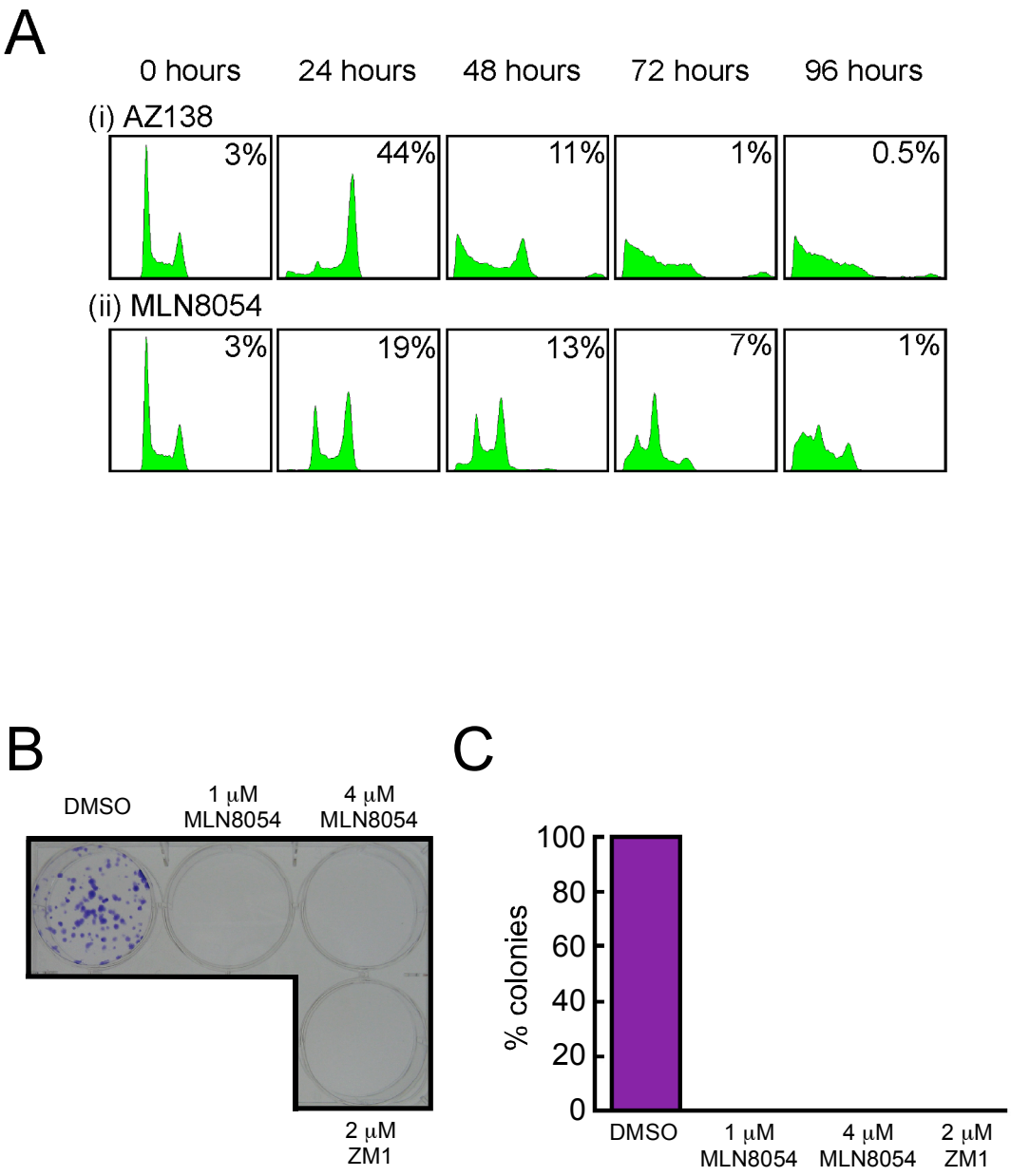

Figure 6.3 Aurora A inhibition causes an accumulation of HeLa cells in mitosis, and eventually lose viability
(A) Flow cytometry PI cell cycle profiles of HeLa cells treated with the Eg5 inhibitor AZ138 (i) or 1 μM MLN8054 (ii) for the indicated time periods. The numbers shown in the top right-hand corner of the graphs refer to the percentage of the cell population in mitosis as determined by MPM-2 staining. (B) HeLa cells were treated with DMSO, 1 μM MLN8054, 4 μM MLN8054 or 2 μM ZM1 for 14 days before fixing and then staining with crystal violet. (C) Qunatitation of the colony formation assay shown in (B).

proliferation and viability of cells (Girdler et al., 2006). Similar to this result, the treatment of cells with either 1 μM or 4 μM MLN8054, also prevented the formation of colonies. This observation therefore indicates that Aurora A activity is required for the preservation of the long-term viability of cells presumably through the maintenance of cells genetic integrity (Hoar et al., 2007). In agreement with these findings, reports have shown Aurora A inhibition or depletion to bring about apoptosis (Huck et al., 2010, Dar et al., 2008, Kaestner et al., 2009, Yang et al., 2010a). Indeed, such as activation if the DNA damage checkpoint may occur as a result of the production of the aneuploidy, which was shown to be caused by the MLN8054 treatment

6.5 Summary

In this chapter I described how the inhibition of Aurora A activity caused a marked delay of cells in mitosis. This delay was found to be related to the diminished ability of cells to align their chromosomes after Aurora A inhibition, indicating the presence of a functional spindle assembly checkpoint. Cells were capable of dividing after the mitotic delays induced by Aurora A inhibition, however segregation errors were observed. Long–term Aurora A inhibition caused the development of aneuploidy and the occurrence of sub-G1 peak with eventual loss of cell viability, demonstrating the degenerative effect of the segregation errors.

The analysis of the effects of Aurora A inhibition on mitosis therefore presented an interesting paradox, in which the spindle assembly checkpoint was activated causing a delay to anaphase, although the segregation defects and aneuploidy produced by the treatment indicated that cells were capable of dividing with misaligned chromosomes. The occurrence of such a phenomenon has been attributed to the dependency on Aurora A activity to maintain the activation of the spindle assembly checkpoint (Wysong et al., 2009, Kaestner et al., 2009). To explore this theory, I used the spindle disrupting power of an Eg5 inhibitor to cause the prolonged activation of the spindle assembly checkpoint, but found that the co-inhibition of Aurora A activity did not compromise the spindle checkpoint to the same extent as seen after inhibiting Aurora B (Ditchfield et al., 2003). The combined Eg5 and Aurora A inhibition did however cause cells to spend less time in mitosis than cells treated with just Eg5 inhibitor, although evidence for a direct role for Aurora A activity in the maintenance of the spindle assembly checkpoint is yet to be presented (Wysong et al., 2009, Kaestner et al., 2009). The simplest explanation for the reduction in mitotic delay after the combined treatment is instead that the two drugs have an antagonistic effect when used together, a phenomenon that has been noted to occur in the past with the combination of treatments (Lee et al., 2008, Borisy et al., 2003, Hata et al., 2005). This increases potency on the combination may be derived from increased off-target effects. Thus drugs which individually have minimal effects on

off-target kinases may cause the augmentation of additional effects, providing an explanation of the accelerated of the mitotic slippage experienced by cells (Manfredi et al., 2007, Mayer et al., 1999).

Despite the occurrence of a slight acceleration to mitotic slippage, the observation of considerable mitotic delays following combined Eg5 and Aurora A inhibition, suggests that Aurora A activity does not play a prominent role in the spindle assembly checkpoint. This proposition is supported by the extended mitotic delays seen when inhibiting just Aurora A activity, as well as the maintenance of the localisation of checkpoint protein when treating cells with Nocodazole in combination with MLN8054. This combined evidence therefore indicates that cells exposed to an Aurora A inhibitor must therefore have been able to satisfy the spindle assembly checkpoint in order to progress to anaphase and divide. The existence of segregation defects after Aurora A inhibition is therefore not likely to be a general loss of checkpoint function, but may instead be indicative of the altered ability of cells to identify chromosomal misalignments. This phenomenon may be derived from the disorganised nature of the spindles formed after Aurora A inhibition which, despite their abnormal appearance, are capable of connecting to kinetochores. The abnormal spindles formed after Aurora A inhibition may be able to produce adequate tension to kinetochores regardless of their unaligned state, thus enabling the satisfaction of the checkpoint, consequentially allowing cells to progress to anaphase with segregation errors (Li and Nicklas, 1995, Nicklas et al., 1998, Nicklas et al., 1995, Skoufias et al., 2001, Biggins and Murray, 2001, Stern and Murray, 2001). Consistent with this idea, I have described the incidence of multipolar spindles and spindle deformities after Aurora A inhibition, which may be capable of applying sufficient degrees of tension to kinetochores irrespective of their alignment on the metaphase plate (Figures 3.4 and 5.3D). Furthermore, in the previous chapter, I showed that spindles grown in the absence of Aurora A activity were able to attach chromosomes to a high degree, despite their low inter-kinetochore stretch and misalignment. This high level of K-fibre attachment therefore reduced the levels of Mad2 on the kinetochores. The reduction level of Mad2 has been previously shown to allow cells to prematurely exit mitosis without first arranging chromosomes on metaphase plates (Orr et al., 2007). Therefore, the ability of spindles formed in the absence of Aurora A to sufficiently reduce the levels of kinetochore Mad2 even on misaligned chromosomes, may permit the progression through to anaphase without first properly aligning chromosomes. Therefore, although it is not completely clear how cells can progress through to anaphase despite the presence of misaligned chromosomes, a number of interesting possibilities must first be considered instead of simply assuming Aurora A activity has a role in the spindle assembly checkpoint. In the next chapter, I will discuss my investigation into increasing the potency of MLN8054 towards Aurora A activity, to determine whether it is possible to increase the severity to the effects to mitotic spindles and mitotic progression.

7 Increasing the potency of Aurora A inhibition

7.1 Introduction

I have previously described how the inhibition of Aurora A activity caused the development of shortened and disorganised spindles, preventing the efficient alignment of chromosomes and consequently delaying mitosis. Previous studies had however found the inactivation of Aurora A to have more potent effects on mitotic spindles than I had observed, describing instead the high occurrence of monopolar spindles (Glover et al., 1995, Mori et al., 2007, Girdler et al., 2006). The spindle phenotypes described in these reports are highly similar to those seen after the inhibition of Eg5 activity, which also produces monopolar spindles, as well as causing cytokinesis defects and the loss of cell viability (Figures 4.1, 6.1 and 6.3) (Mayer et al., 1999, Walczak et al., 1998). In this chapter, I examine whether Aurora A can be inhibited more potently to produce phenotypes more similar those seen after Eg5 inhibition, than the relatively mild spindle effects I have described so far in the investigation (Figures 3.4 and 4.2).

Throughout the investigation I employed MLN8054 at a concentration of 1 μM, which was determined by Western blotting to have a high degree of potency towards Aurora A kinase activity (Figure 3.3). The accuracy of techniques such as Western blotting however, is limited by the sensitivity of the experimental components, such as the antibodies used to immunoblot the samples. Indeed, the phospho-Aurora antibody used for the Western blots was incapable of detecting signal below a certain concentration of protein, showing no signal when less than 5% of total control cell lysate was present on the membrane. Therefore, although no signal was seen to indicate the presence of phospho-Aurora after 1 μM MLN8054 treatment of HeLa cells, the antibody limitations may mean that complete kinase inactivation is not actually achieved at this concentration (Figure 3.3C). Therefore, it may be possible to increase the potency of Aurora A inhibition, which may enable a more accurate characterisation of the role of Aurora A kinase activity within cells.

7.2 Increasing the concentration of MLN8054 causes more potent Aurora A inhibitory effects but also inhibits Aurora B activity

To increase the potency of Aurora A inhibition achieved by MLN8054, the obvious solution would be to simply increase the concentration of the MLN8054 applied to cells. Indeed, analysing the polarity of fixed cell samples indicated that increasing the concentration of MLN8054 did cause a

reduction in the proportion of bipolar spindles (Figure 7.1A). As Aurora A activity is required for bipolar spindle assembly, the reduction in the bipolar index therefore suggested that MLN8054 could be used at higher concentrations to inhibit Aurora A activity more potently (Glover et al., 1995, Girdler et al., 2006, Mori et al., 2007). Furthermore, cell cycle profiles revealed that while 1 μM MLN8054 caused an increase of cells with 4N DNA, treatment with 3 μM MLN8054 caused a further accumulation of cells with 4N DNA. This accumulation of cells with 4N DNA may represent an extended mitotic delay caused by the higher MLN8054 concentration, which is comparable to that seen after Eg5 inhibition (Figure 6.3A).

MLN8054 has been used in the investigation so far at 1 μM, as I found that higher concentrations of MLN8054 caused the inhibition of Aurora B activity as well as Aurora A, as judged by the analysis of Western blots (Figure 3.3). To further investigate the effect of high dose MLN8054 treatment on Aurora B activity however, I observed the ability of the cells to divide, as cytokinesis failure is a hallmark of Aurora B inhibition (Bischoff and Plowman, 1999, Adams et al., 2001a, Nigg, 2001b). The investigation into Aurora B activity in response to MLN8054 was carried out using phase contrast time-lapse imaging of live cells (Figure 7.1C). Interestingly, both control cells and the majority of cells treated with 1 μM MLN8054 were observed to divide. Increasing the concentration of MLN8054 however, caused a dramatic increase in cells which were unable to perform cytokinesis, which was similar to the effects of the Aurora B inhibitor ZM1 that also produced cytokinesis failures. Therefore, the analysis of the ability of cells to perform cytokinesis supported the previously described Western blot data that increasing the concentration of MLN8054 lead to the dual inhibition of Aurora B as well as Aurora A activity. Importantly, it was not possible to identify the effects of Aurora B inhibition from the flow cytometry profiles, as it was not possible to distinguish between cells which had overridden the spindle assembly checkpoint and failed to divide, from those which were held in mitosis (Figure 7.1B). This therefore highlights the danger of using flow cytometry profiles to draw conclusions regarding specific cell cycle phases.

In addition to inhibiting Aurora B activity and causing cytokinesis failure, increasing the concentration of MLN8054 from 1 μM to 4 μM also increased the time by which cells were delayed in mitosis (Figure 7.1C). On average, exposure of cells to 1 μM MLN8054, caused them to spend 117 minutes in mitosis (a 156% increase compared to control samples), while treatment with 4 μM MLN8054 caused cells to spend an average of 158 minutes in mitosis (a 244% increase compared to control cells). This increased mitotic delay seen after treatment with the higher concentration of MLN8054, together with the reduction in cell bipolarity, suggested that it may indeed be possible to increase the potency of Aurora A inhibition by increasing the concentration of MLN8054.

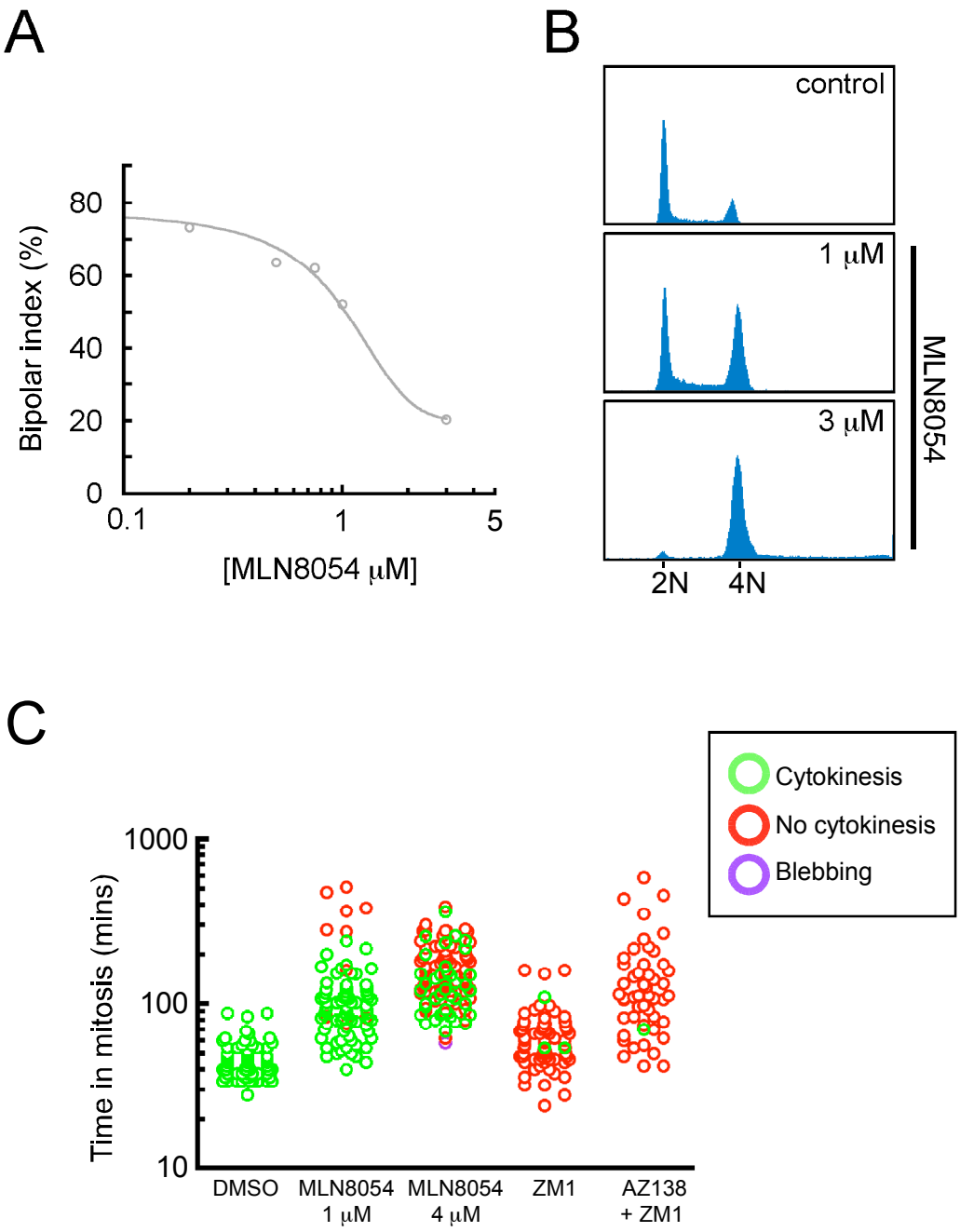

Figure 7.1 Increasing the MLN8054 concentration appears to increase the potency of Aurora A inhibition, however Aurora B inhibitory phenotypes are also detected
(A) HeLa cells were treated with a range of MLN8054 concentrations for 2 hours before fixing. The cells were then immunostained with antibodies against Aurora A to observe centrosomes and enable the identification of bipolar spindles. (B) Flow cytometry PI profiles of HeLa cells treated with 0 μM, 1 μM and 3 μM MLN8054 for 24 hours. (C) DLD-1 cells were treated with the indicated drugs while time-lapse phase contrast microscopy images were taken every 2 minutes. The resulting movies were analysed and the time taken for cells to go through mitosis was plotted (determined as being between nuclear envelope break down and chromosome decondensation). Green circles represent cells that performed cytokinesis, red circles indicate cells that were unable to do so, and purple circles denote cells that started blebbing while in mitosis.

The reduction in bipolar index after MLN8054 treatment, suggested that by increasing the concentration of MLN8054 it may be possible to produce spindle defects more comparable to those seen after Eg5 inhibition (Figure 4.4). The mitotic delay experienced by the cells treated with 4 μM MLN8054 however, was not as pronounced as that reported to occur after Eg5 inhibition (Figure 6.1). Thus to determine whether the mitotic effects produced my 4 μM MLN8054 were comparable to those seen after Eg5 inhibition, with combined Aurora B inhibition, I applied both ZM1 and an Eg5 inhibitor to cells and observed the affect on mitotic progression. Interestingly, the average mitotic timing of cells treated with both ZM1 and the Eg5 inhibitor 145 minutes, which was highly comparable to that seen after 4 μM MLN8054 treatment. This therefore indicated that increasing the concentration of MLN8054 resulted in an increased affect on the spindles of cells, which was bore close resemblance to those seen after Eg5 inhibition as well as the previously described effects of removing Aurora A activity from cells (Mori et al., 2007, Glover et al., 1995, Girdler et al., 2006). Therefore, it appeared to be possible to increase the potency of Aurora A inhibition by increasing the concentration of MLN8054 above 1 μM, although above 1 μM MLN8054, Aurora B activity was also affected. Therefore to effectively probe the role of Aurora A activity I required a method by which I could increase the concentration of MLN8054 to more potently inhibit Aurora A activity, but identify a method by which Aurora B activity was not also inhibited at this higher concentration of MLN8054.

7.3 A drug-resistant cell line can be used to revert the high-dose effects of MLN8054 on Aurora B activity

To enable to use of MLN8054 at higher concentrations without inhibiting Aurora B activity in addition to Aurora A, I investigated the use of drug-resistant mutants. A variety of different drug-resistant cell lines have previously been created in the lab by continuously exposing the colon cancer cell line HCT116 to the Aurora B inhibitor ZM1 (Girdler et al., 2008). HCT116 cells were chosen as they have defective mismatch repair machinery, which has been shown to cause higher levels of spontaneous mutations (Glaab and Tindall, 1997). This hyper-mutation rate enabled Girdler and colleagues to produce a greater number of genetic variants within the culture population, thus increasing the possibility of selecting for a drug-resistant mutant. Furthermore, HCT116 cells have been shown to express a relatively low level of p-glycoprotein, which is involved in removing a broad range of harmful substances from the cell (Teraishi et al., 2005). Reduced expression of p-glycoprotein therefore reduces the chance of resistancy to ZM1 through the expression of drug pumps, and increases the possibility of drug-resistant mutant cells developing.

The continued exposure of the HCT116 cells to ZM1 produced a variety of resistant mutants, some of which were shown by the examination of flow cytometry profiles to revert the Aurora B inhibitory effects of MLN8054 (Girdler et al., 2008). To confirm whether this data reflected the ability of the mutated cell lines to maintain the activity of Aurora B in the presence of presence of high doses of MLN8054, I used Western blotting to observe the activity of Aurora B after MLN8054 treatments.

Before testing the drug-resistancy of the mutant HCT116 cells however, the effect of MLN8054 in wild-type HCT116 cells was assessed to determine whether Aurora A activity could be inhibited and any effects on Aurora B activity in the wild-type cells. The wild-type HCT116 cells were exposed to a range of MLN8054 concentrations, and the analysis of the Western blotted samples revealed that both Aurora A and B activity could be inhibited by MLN8054. Interestingly, both kinases were inhibited at roughly equivalent concentrations as those in HeLa and DLD-1 cells (Figure 3.3). At 0.25 μM MLN8054, Aurora A activity was markedly reduced in the wild-type HCT116 cells, as indicated by low phospho-Aurora A (Thr288) and phospho-TACC3 (Ser558) signal. Increasing the concentration of MLN8054 to 1 μM, caused the removal of all visible phospho-Aurora A signal from the blotted samples, indicating the potent inhibition of the kinase. Similarly, a reduction in phospho-Histone H3 (Ser10) indicated a reduction of Aurora B activity when samples were treated with only 0.75 μM MLN8054. Phospho-Aurora B (Thr232) signal was also reduced in samples treated with 2 μM MLN8054. Therefore, although Aurora A can be inhibited by MLN8054 in wild-type HCT116 cells, Aurora B activity is also inhibited at similar concentrations.

To determine whether the mutations produced after the extended exposure to ZM1 were capable of inferring resistance to the Aurora B inhibitory effects of MLN8054, I treated the mutant HCT116 cell lines with a range of MLN8054 concentrations and analysed the effect. One of the HCT116 cell lines, found to have a Tyrosine residue substituted for a histadine at position 156 in Aurora B (referred to as R12 – (Girdler et al., 2008)), showed a high level of resistance against high dose MLN8054 effects on Aurora B activity (Figure 7.2B). Indeed, the analysis of the phospho-Histone H3 (Ser10) and phospho-Aurora B (Thr232) signal in MLN8054-treated R12 samples, showed no reduction even after their treatment with 10 μM MLN8054. Importantly however, Aurora A activity was seen to be inhibited by 1 μM MLN8054 in the R12 HCT116 cell line, as determined by phospho-Aurora A (Thr288) and phospho-TACC3 (Ser558) signal.

The Western blot analysis therefore revealed that the mutant R12 HCT116 cell line was resistant to the effects of MLN8054 on Aurora B, however to confirm whether this resistance would be capable to reverting the cytokinesis defects observed after high does MLN8054 treatments, I compared the

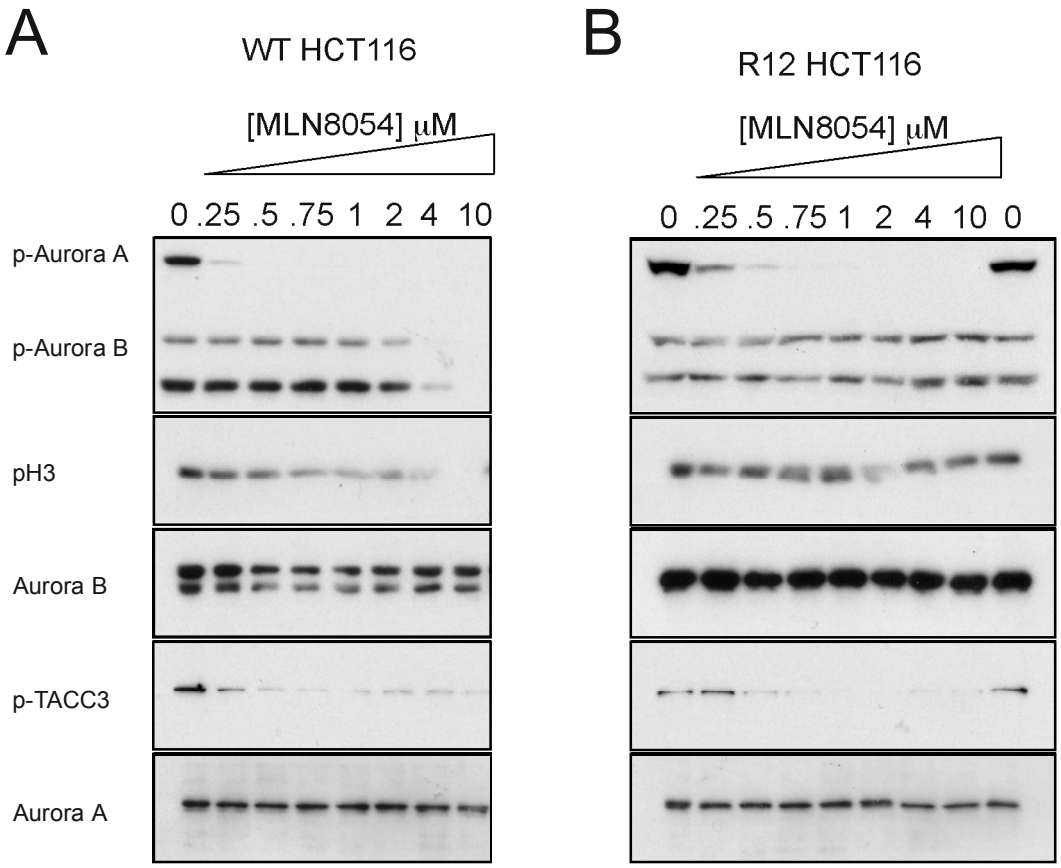

Figure 7.2 R12 HCT116 cells are resistant to high-dose MLN8054 Aurora B inhibition
Wild-type (A) and R12 (B) HCT116 cells were treated with Nocodazole for 16 hours before being treated with MG132 and a range of MLN8054 concentrations for a further 2 hours. The samples were then harvested and assayed for Aurora A and B activity by Western blot, using antibodies against phospho-Aurora A (Thr288), phopho-TACC3(Ser558), phospho-Aurora B (Thr232) and phopho-Histone H3 (Ser10). The images displayed represent results that were consistent over three independent experiments.

cell cycle flow-cytometry profiles of wild-type and R12 HCT116 cell lines, which had both been treated with a range of MLN8054 concentrations for 24 hours (Figure 7.3). Consistent with previously reported HeLa data (Figure 6.3A), 1 μM MLN8054 caused the reduction in the proportion of cells with 2N DNA in both the wild-type and the mutant cell line and increased the proportion of R12 cells with 4N DNA. This 4N DNA increase most probably represents an extension to the time taken for the R12 cells to progress through mitosis after the 1 μM MLN8054 treatment (Figures 6.1 and 6.2A). Wild-type cells however, did not show a similar increase in the population of cells with 4N DNA, and instead exhibited a shortening and widening of the 4N peak. The inability of wild-type cells to show the same accumulation of cells with 4N DNA, may be indicative of the combined inhibition of Aurora A and B activity, which may be experienced by wild-type cells.

Increasing the concentration of MLN8054 applied to wild-type HCT116 cells to 4 μM and 10 μM, produced effects that were very typical of Aurora B inhibition, with most cells observed as having either 4N or 8N DNA following the treatments. This finding is indicative of the treated cells being unable to perform cytokinesis and separate their genetic material into two cells, a phenomenon that has previously been noted following Aurora B inhibition (Ditchfield et al., 2003). In contrast, the cycle profiles of R12 HCT116 cells treated 4 μM and 10 μM, were strikingly similar to those of 1 μM MLN8054-treated R12 cells, indicating that, unlike wild-type cells, the three treatments produced very similar cell cycle effects. All three MLN8054 concentrations caused the R12 cell line to accumulate a greater population of cells with 4N DNA and decrease the proportion of cells with 2N DNA. This phenotype may either demonstrate a delay in mitosis, or indicate the inability of cells to perform cytokinesis following the treatments. The presence of only a very small 8N DNA population indicates that any cytokinesis defects caused by the MLN8054 treatments are unlikely be the result of profound Aurora B inhibition, although mild Aurora B inhibition cannot be ruled out, particularly after the treatment of the cells with 10 μM MLN8054, which caused the largest 8N DNA peak. Despite the possibility of mild cytokinesis defects at 10 μM MLN8054, the argument for the existence of profound cytokinesis failure is further weakened by the observation of a relatively large cell population with 2N DNA after MLN8054 treatment in the R12 cell line. This population indicates that, unlike wild-type cells, the R12 cells were still able to divide even after the high doses of MLN8054 treatment.

Therefore R12 HCT115 reversion of the Aurora B inhibitory phenotype seen in wild-type cells after high dose MLN8054 treatment indicates the possibility of potentially increasing the potency of Aurora A inhibition. Whether it is actually possible to more potently increase Aurora A inhibition in this way remains to be seen. It is interesting to observe however, that no dramatic changes occurred in the cell cycle profiles of R12 HCT116 in response to increases in MLN8054

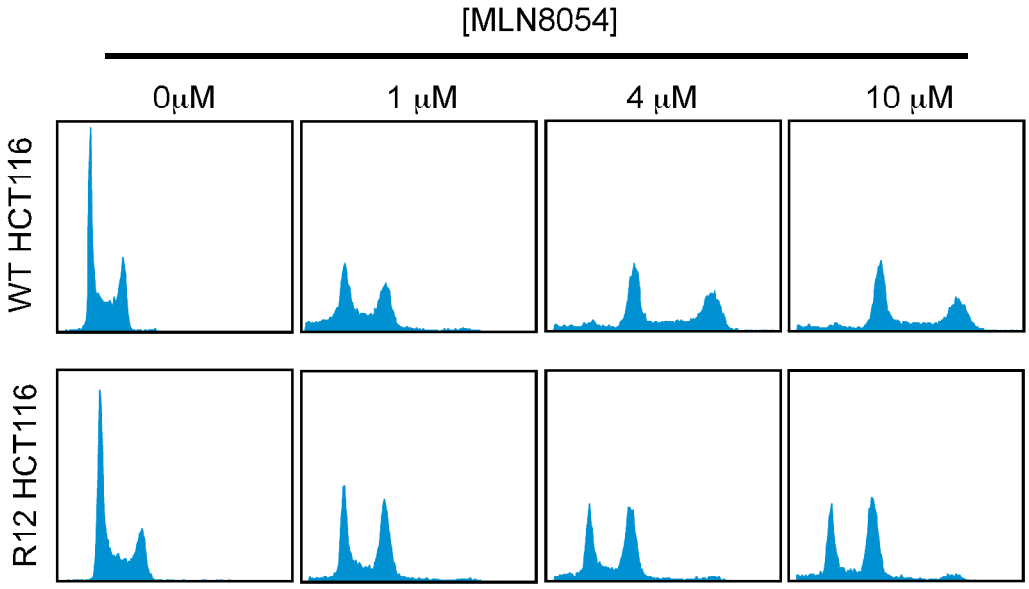

Figure 7.3 Potent and specific Aurora A inhibition causes an accumulation of cells with 4N DNA, but does not appear to block cell cycle progression
Flow cytometry PI cell cycle profiles of wild-type and R12 HCT116 cells treated for 24 hours with the indicated concentrations of MLN8054.

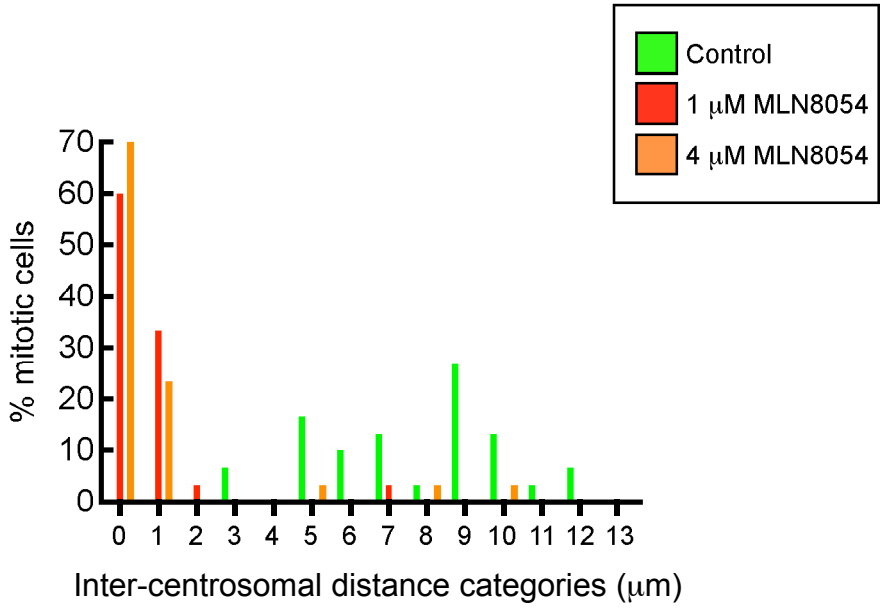

Figure 7.4 Treating R12 HCT116 cells with MLN8054 causes the profound occurrence of monopolar spindles
R12 HCT116 cells were treated with 0 μM, 1 μM or 4 μM MLN8054 for 2 hours and then fixed. The fixed samples were then immunostained with antibodies against Aurora A to observe centrosomes and phospho-Histone H3 to determine the cell cycle stage.
Z-sectioned images were taken of cells during the period after nuclear envelope breakdown and before anaphase, and inter-pole distances were measured. The graph shows data from three independent experiments, with 30 cells analysed for each condition. The stated values for inter-centrosomal measurements represent categories of grouped measurements, which include cells with inter-centrosomal distances of up to 0.99 μm more than the indicated value.

concentration, indicating that potent Aurora A inhibition may be already achieved at 1 μM MLN8054.

7.4 Increasing the potency of Aurora A inhibition in HCT116 cells decreases the inter-pole distance

The revelation that increasing the concentration of MLN8054 did not largely appear to cause large changes to cell cycle profiles of R12 HCT116 cells or further affect their ability to divide, suggested that Aurora A activity may not have been optimally inactivated at 1 μM MLN8054. To gain a more accurate understanding of the level of Aurora A inhibition achieved however, I next examined the degree of centrosome separation seen in cells treated with a range of MLN8054 concentrations.

I have previously shown that 1 μM MLN8054 treatment of HeLa cells caused a shortening of inter-pole measurements (Figure 4.2). Despite this, the majority of cells were capable of separating their centrosomes to form bipolar spindles (Figure 3.4), which is inconsistent with a number of reports which described the occurrence of large numbers of monopolar spindles after Aurora A inactivation (Glover et al., 1995, Mori et al., 2007, Girdler et al., 2006). I have also described the reduction in HeLa cell spindle bipolarity with increasing concentrations of MLN8054 (Figure 7.1A). Therefore, using fixed and immunostained R12 HCT116 cells, I tested whether these cells also experienced a reduction in spindle polarity, in relation to increasing the concentration of MLN8054 (Figure 7.4).

Measurements of control cells R12 HCT116 showed a range of different spindle lengths, presumably reflecting the different stages of mitotic progression (Figure 4.2). The most common inter-pole length category of the control R12 HCT116 cells was observed to be 9-9.99 microns, and the largest inter-pole distance recorded distance as 12.51 microns, both of which are highly comparable with the HeLa cell data. Treatment of the R12 cells with 1 μM of MLN8054 however, severely shortened the inter-pole distances of the R12 cells, with most recorded as having centrosomes less than 1.99 microns apart reflecting the profound monopolarity of the population. This finding was in stark contrast to the same treatment in HeLa cells, where the equivalent MLN8054 concentration only caused a slight shortening to the majority of spindle lengths (Figure 4.2). These clear differences in the effects of MLN8054 in HCT116 cells compared to HeLa or DLD-1 cells is surprising, as Western blotting showed the cells lines exhibited relatively equivalent levels of Aurora A inhibition (Figures 3.3 and 7.2). The observed differences in spindle polarity after MLN8054 treatment, may therefore be indicative of cellular differences in response to Aurora

A inhibition, or could either be indicative of off-target effects in the HCT116 cells. Interestingly, a similar in the differing abilities to form bipolar spindles between HCT116 cells and HeLa cells has also seen after Aurora A was down regulated in previous reports in the literature (Marumoto et al., 2003).

Although the Rl2 HCT116 cells experienced profound monopolarity after their treatment with 1 μM MLN8054, a range of larger inter-pole distances were recorded, with the longest spindle length being 7.68 microns. Increasing the concentration of MLN8054 to 4 μM caused a slightly higher proportion of R12 HCT116 cells to have inter-pole distances of less than 1 micron. Importantly however, as with the 1 μM treatment, a proportion of the population were also observed to have a range of larger inter-pole distances of up to 10.06 microns.

Despite occurrence of a large range of spindle lengths after MLN8054 treatment, the occurrence of high numbers of monopolar spindles after Aurora A inhibition is more consistent with several reports in the literature, which describe a prominent role for Aurora A activity in bipolar spindle formation (Glover et al., 1995, Mori et al., 2007, Girdler et al., 2006). The production of a high proportion of monopolar spindles is similar to the effect of inhibiting Eg5 activity, which has also been shown to cause extended mitotic delays, after which cells were not capable of dividing (Figures 4.4A, 4.4B, 6.1 and 6.3A). Despite the similarity in respect to both treatments causing profound monopolarity, the cell cycle profiles of R12 HCT116 cells treated with MLN8054 indicated that they did not experience similar extreme mitotic delays, and were still able to divide (Figure 7.3). This interesting discrepancy between the two treatments, despite apparently causing the same spindle defects, therefore required further investigation.

7.5 Increasing the potency of Aurora A inhibition extends the delay in mitosis, although cells can still divide

To address whether the cell cycle profiles of R12 HCT116 cells were correct in indicating that cells were still able to divide after potent Aurora A inhibition, despite the prominent presence of monopolar spindles, I used phase contrast microscopy and time-lapse imaging to follow the mitotic progression of individual cells (Figure 7.5).

To determine the time cells spent in mitosis in response to various treatments, the duration between nuclear envelope breakdown and chromosome decondensation was recorded, as well as whether cell division had taken place. Using this methodology, I found untreated wild-type cells to take an average of 41 minutes to progress through mitosis, after which they all divided into two daughter

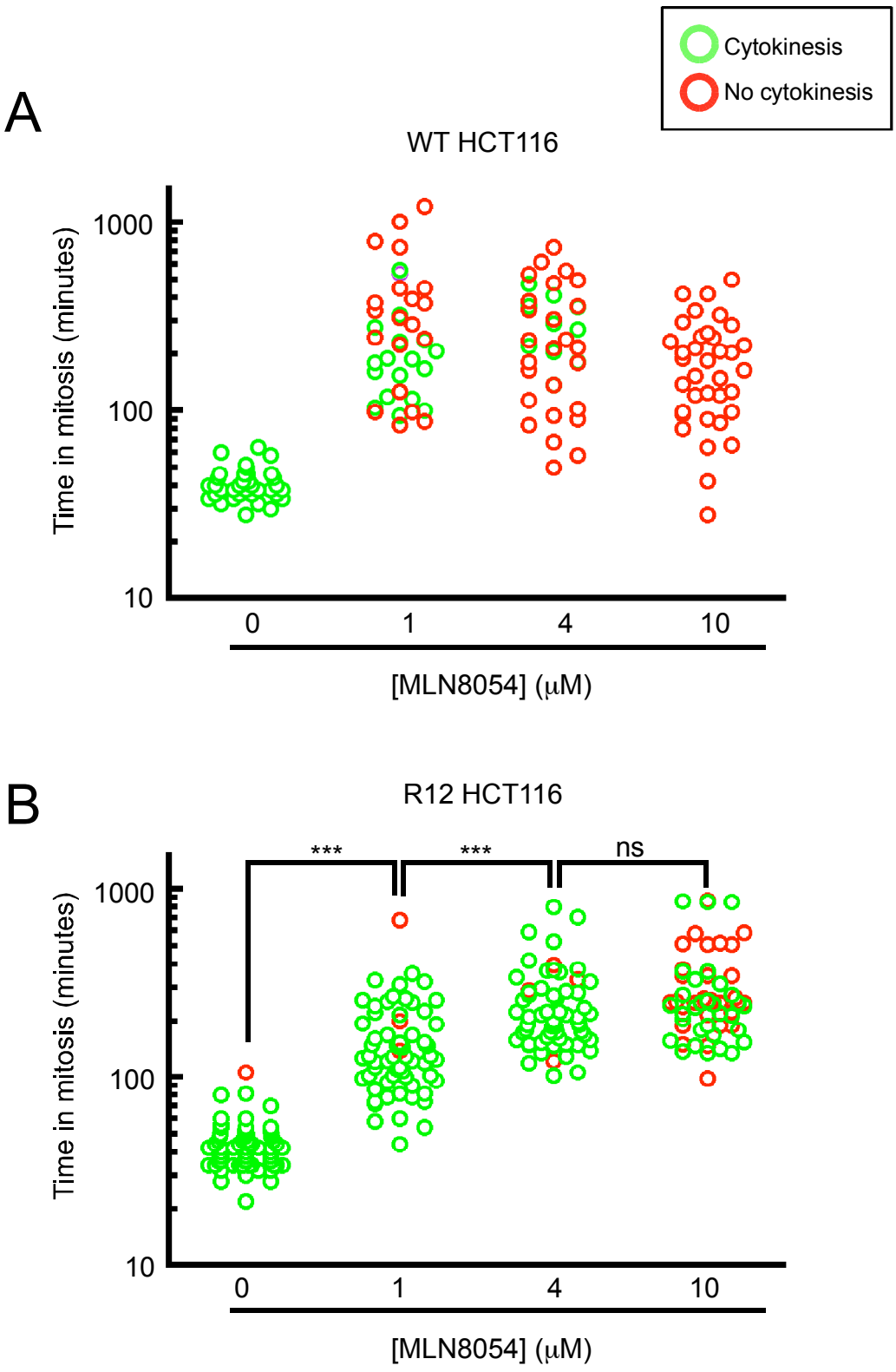

Figure 7.5 Increasing the concentration of MLN8054 up to 4 μM increases the time R12 HCT116 cells spent in mitosis, although they are eventually able to divide
Wild-type (A) and R12 (B) HCT116 cells were treated with the indicated concentrations of MLN8054, and time-lapse phase contrast microscopy images were taken every 2 minutes. The resulting movies were analysed and the time taken for cells to go through mitosis was plotted (determined as between nuclear envelope break down and chromosome decondensation). Green circles represent cells that performed cytokinesis and red circles indicate cells that were unable to do so. *** = P<0.001; ns = P>0.01 two-tailed t test.

cells (Figure 7.5A). In contrast, 1 μM MLN8054 caused the wild-type cells to exhibit a large range of different mitotic periods, although all cells spent longer in mitosis than untreated cells, with an average mitotic period of 312 minutes. Therefore, consistent with data from both MLN8054-treated HeLa and DLD-1 cells, MLN8054 treatment caused a mitotic delay in wild-type HCT116 cells (Figures 6.1 and 6.2A). Unlike HeLa or DLD-1 cells however, a large proportion of wild-type HCT116 cells were unable to perform cytokinesis after treatment with 1 μM MLN8054 (Figures 6.1 and 7.1). This could reflect the apparently more severe effect of MLN8054 treatment in the HCT116 cells, or alternatively it may be indicative of mild Aurora B inhibition being caused by MLN8054 at this concentration. Interestingly, increasing the concentration of MLN8054 to 4 μM, did not significantly change the mitotic period compared to cells treated with 1 μM MLN8054, however the majority of cells were also prevented from dividing (Figure 7.5A). At 4 μM MLN8054 however, the level of phospho-Aurora B had previously been shown to be greatly decreased, suggesting that MLN8054 was inhibiting Aurora B activity at this concentration (Figure 7.2). This could therefore indicate a compromised spindle assembly checkpoint, which may explain the increase in cytokinesis defects and the slight decrease in mitotic timings. Increasing the concentration of MLN8054 to 10 μM, further reduced the time wild-type HCT116 cells spent in mitosis, with an average mitotic period of 189 minutes (Figure 7.5A). Furthermore, all cells were prevented from performing cytokinesis, which is consistent with previously described data and reports in the published literature which show the Aurora B activity is inhibited following treatment with high concentrations of MLN8054 (Manfredi et al., 2007, Hoar et al., 2007).

To test whether the effects of high dose MLN8054 could be reverted by using the drug-resistant cell line, I applied the same range of MLN8054 concentrations to R12 HCT116 cells. Following the progression of individual cells through mitosis revealed that, similar to wild-type cells, control R12 HCT116 cells spent 42 minutes in mitosis before dividing (Figure 7.5B). Furthermore, treating the R12 HCT116 cells with 1 μM MLN8054 extended the time cells spent in mitosis, with an average mitotic period being recorded as lasting 160 minutes. Interestingly however, this increase was not as profound as that seen in wild-type cells, and unlike wild-type cells, R12 HCT116 cells were still able to divide. The ability of the R12 HCT116 cells to revert the cytokinesis defects and even reduce the degree of mitotic delay, suggests these phenomena may be derived from Aurora B inhibitory effects. The finding that 1 μM MLN8054 may cause Aurora B inhibition in HCT116 cells has important implications, particularly for the findings of some reports in the literature, which use 1 μM MLN8054 to make assumptions regarding Aurora A activity in HCT116 cells (Kaestner et al., 2009).

Increasing the concentration of MLN8054 further to 4 μM in R12 HCT116 cells, prolonged mitosis still further, causing the average cell to take 248 minutes to exit mitosis. In agreement with the HCT116 flow cytometry profiles however, many cells were still able to divide after the extended mitotic periods (Figure 7.3). Cells treated with 10 μM though, experienced no significant further mitotic delay when compared with cells treated with 4 μM MLN8054. A great increase was however observed in the proportion of cells that were unable to divide following treatment with 10 μM MLN8054. Consequently, in the previously discussed flow cytometry profiles, the slight increase to the population of the 4N DNA population of cells treated with 10 μM MLN8054 may therefore have been caused by cells that were unable to perform cytokinesis rather than by a great extension to the mitotic delay (Figure 7.2).

Therefore, although it may be possible that a mild Aurora B inhibition is seen in R12 HCT116 cells after treatment with 10 μM MLN8054, it was largely possible to revert the Aurora B inhibition seen after lower concentrations using the drug-resistant cell line. By exploiting this resistancy for MLN8054 effects to Aurora B activity, it was possible to increase the concentration of MLN8054 without also inhibiting Aurora B, to reveal the effect of potently inhibiting Aurora A activity. Interestingly, increasing the concentration of MLN8054 of 1 μM to 4 μM, caused an extension to the time R12 cells spent in mitosis, however the vast majority were still able to divide. Although this supports the findings of the flow cytometry graphs (Figure 7.3), it is surprising when considering that most cells appear to have monopolar spindles after MLN8054 treatment (Figure 7.4). The ability of cells to divide following Aurora A inhibition suggests that they are eventually able to form functional spindles after the extended mitotic delays. Indeed, as well as the prominent occurrence of monopolar spindles after both 1 μM and 4 μM MLN8054 treatments, a range of longer spindle lengths were also recorded, supporting the assumption that cells are eventually capable of forming bipolar spindles.

An alternative explanation for the ability of cells to exit mitosis despite the profound occurrence of monopolar spindles may however be that Aurora A inhibition causes the override of the spindle assembly checkpoint. This latter theory would therefore imply that cells were capable of exiting mitosis without rectifying their monopolar spindles and satisfying the spindle assembly checkpoint. I have previously been unable to identify a prominent role for Aurora A activity in the spindle assembly checkpoint, however it was not clear whether such a role could be detected when more potently inhibiting Aurora A activity.

7.6 Aurora A activity does not have a prominent role in the spindle assembly checkpoint

The ability of cells to arrest after Aurora A inhibition, and effectively localise spindle checkpoint proteins in the absence of Aurora A activity after Nocodazole treatment, supports the idea that Aurora A activity does not have a role in the spindle assembly checkpoint (Figures 6.1, 6.2, 5.3B and 5.4B). In addition, increasing the potency of Aurora A inhibition still permitted cells to stay in mitosis and divide after extended mitotic delays, indicating the presence of an active spindle checkpoint in cells after Aurora A inhibition (Figure 7.7B). However, two recently published papers have described a role for Aurora A activity in the proper functioning of the spindle assembly checkpoint (Wysong et al., 2009, Kaestner et al., 2009).

Therefore, to examine whether potent Aurora A inhibition was capable of overriding the spindle assembly checkpoint, I inhibited Eg5 activity to cause chromosome misalignments to maintain checkpoint activation (Figure 4.4) (Kapoor et al., 2000, Mayer et al., 1999). Consistent with sustained checkpoint activation, the flow cytometry profiles of both wild-type and R12 HCT116 cells treated with an Eg5 inhibitor for 24 hours showed an accumulation of cells with 4N DNA (Figure 7.6). By co-treating the cells with MLN8054, I hoped to determine whether Aurora A inhibition could cause cells to be driven out of mitosis, despite the chromosome alignment defects produced by inhibiting Eg5 activity. If such an effect was seen to occur, it would imply a role for Aurora A activity in the spindle assembly checkpoint.

By following this experimental deign, the resulting flow cytometry profiles of wild-type cells co-treated with 1 μM MLN8054 for 24 hours showed a slight reduction in the proportion of cells with 4N DNA when compared to cells treated with just the Eg5 inhibitor. Increasing the concentration of MLN8054 to 4 μM and 10 μM however, greatly reduced the 4N population of cells and introduced a prominent 8N peak. In comparison to wild-type cells however, the majority of R12 HCT116 cells were able to maintain 4N DNA after combined Eg5 and potent Aurora A inhibition. A small 8N peak could however be detected after the treatment of the cells with 10 μM MLN8054 and Eg5 inhibitor. This clear difference observed after the co-treatment of MLN8054 in the wild-type and the drug-resistant HCT116 cells, is likely to result from the ability of MLN8054 to inhibit Aurora B activity in wild-type cells, but not in the drug-resistant cell line. Therefore, the inhibition of Aurora B by MLN8054 in wild-type cells provides an example of a checkpoint override, where the 8N cell population is representative of cells which have been driven out of mitosis (Ditchfield et al., 2003, Kapoor et al., 2000). The absence of such a prominent 8N cell population after the equivalent treatment in R12 cells indicates that cells were not driven out of mitosis when cells were able to resist the Aurora B inhibitory effect of MLN8054. Therefore, the potent inhibition of

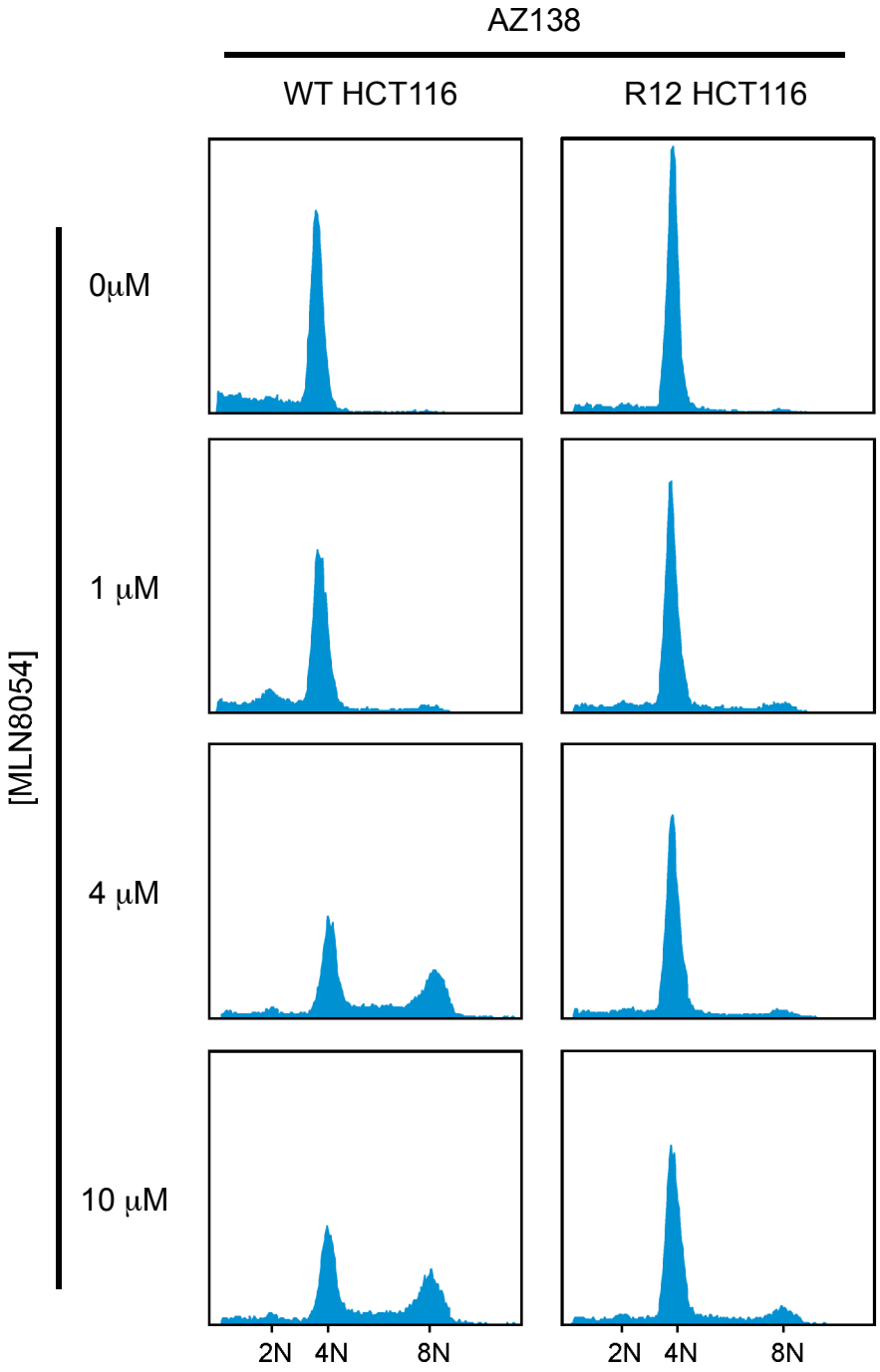

Figure 7.6 Potent Aurora A inhibition does not noticeably override the spindle assembly checkpoint
Flow cytometry PI profiles of wild-type and R12 HCT116 cells, which were harvested after 24 hours of treatment with the indicated concentrations of MLN8054 in combination with AZ138.

Aurora A activity did not noticeably compromise the spindle assembly checkpoint, indicating that Aurora A activity does not play a prominent role in it the checkpoint.

Whether the Aurora A kinase activity is involved in the maintenance of the checkpoint is however still up for debate, as the cell cycle profiles do not allow one to distinguish between cells that were held in mitosis, from those which were prematurely forced into G1 after an extended delay (as seen in DLD-1 cells in Figure 6.1). Despite this limitation, Aurora A activity clearly is not prominently involved in the spindle assembly checkpoint.

7.7 Summary

In the previous chapters I inhibited Aurora A activity demonstrating its roles in efficient spindle assembly, chromosome alignment, timely mitotic progression and normal cell division. However, inconsistencies with the published literature suggested that it may be possible to inhibit Aurora A to a greater degree to produce more profound effects on spindle assembly and mitotic progression (Glover et al., 1995, Mori et al., 2007, Girdler et al., 2006). While investigating this hypothesis, I found that simply increasing its concentration also caused the inhibition of Aurora B activity. Despite this, the observation of decreased spindle bipolarity together with the extension of mitotic timings, suggested that it might indeed be possible to increase the potency of Aurora A inhibition by raising the concentration of MLN8054.

Through using a drug-resistant HCT116 cell line, I was able to revert the effects of MLN8054 on Aurora B activity, thus enabling the characterisation of the effects of potent Aurora A inhibition without also inhibiting Aurora B activity. Using this cell line I was able to increase the concentration of MLN8054, which produced profound monopolarity in the spindles of mitotic cells and extended the time cells spent in mitosis. Despite the monopolarity of their spindles, the cells were still able to divide even after the extended mitotic delays. This phenomenon was not considered to be derived from a prominent role for Aurora A activity in the functioning of the spindle assembly checkpoint, but rather cells were predicted to be capable of forming bipolar spindles after extended mitotic periods. Therefore the potent inhibition of Aurora A activity confirmed that Aurora A activity was required for the formation of normal bipolar spindles and for the timely progression through mitosis. Aurora A activity however was not required for the process of cell division as cells were eventually able to form adequate spindles without the kinase activity. The accuracy of the cell division in the absence of Aurora A activity is however as yet unknown, although cell cycle profiles indicated the existence of a relatively narrow 2N DNA peak after 24 hours of MLN8054 treatment in HCT116 cells, possibly suggesting even segregation.

So far in the investigation, the role of Aurora A activity has only been investigated in cancer cell lines. Therefore, in the next chapter I will describe data in which non-transformed cells were tested for their dependency on Aurora A activity. This line of investigation may consequently have important connotations in developing a more universal understanding of the role of Aurora A activity in cells, as well as understanding the effects of Aurora A-directed anti-cancer therapies to healthy cells in the human body.

8 Investigating the role of Aurora A kinase activity in a non-transformed cell line

8.1 Introduction

Cancerous cell lines are commonly used for research in laboratories as they are typically fast growing, have been immortalised, and are relatively robust to life in culture conditions. These qualities have meant that many cancer cell lines, such as the ones used in this investigation, have been well characterised, making them ideal model systems. It was important however, to examine the role of Aurora A activity within non-tumour cells to develop a better understanding of the role of Aurora A kinase activity within the normally functioning human body. Furthermore, if any differences were observed between the roles of Aurora A activity in non-tumour cell lines and in cancer cells, this may have important implications for the effectiveness of the targeting of Aurora A kinase activity as an anti-cancer therapy. For these reasons, I therefore decided to expand the investigation to examine the role of Aurora A activity in non-transformed cells.

To enable the practical use of non-transformed cells, it was necessary to select a cell line that had been immortalised to prevent the induction of scenescence, in response to telomere shortening resulting from a limited proliferation capacity. Despite being immortalised, the cell line could still be used as a model to exemplify the role of Aurora A activity in non-transfomed cells as they, unlike transformed cells, would not form a tumour if injected into mice and were subject to contact inhibition induced senescence (data not shown). It must be noted however, that although non-transformed cells were used to model the role of Aurora A activity in non-cancer cells, the process of isolating and culturing cells has been shown to cause adaptation to culture conditions, and thus cultures cells cannot be used synonymously with healthy tissue samples (Engelmann and Valtink, 2004). To truly investigate the role of Aurora A kinase activity in healthy tissues, *in vivo* methods would have to be emplyed, however in this investigation such techniques were not feasable, and non-transformed culture cells were used as an adequate alternative.

Differences between transformed and non-transformed cell lines in their response to various anti-mitotic drug treatments have been previously reported (Orth et al., 2008, Brito et al., 2008, Brito and Rieder, 2009, Gascoigne and Taylor, 2008). In all these cases, a greater proportion of cell death was observed in the cancer cell lines compared to the non-transformed lines when exposed to anti-mitotic drugs. In light of this data, I set out to determine whether non-transformed cell lines were

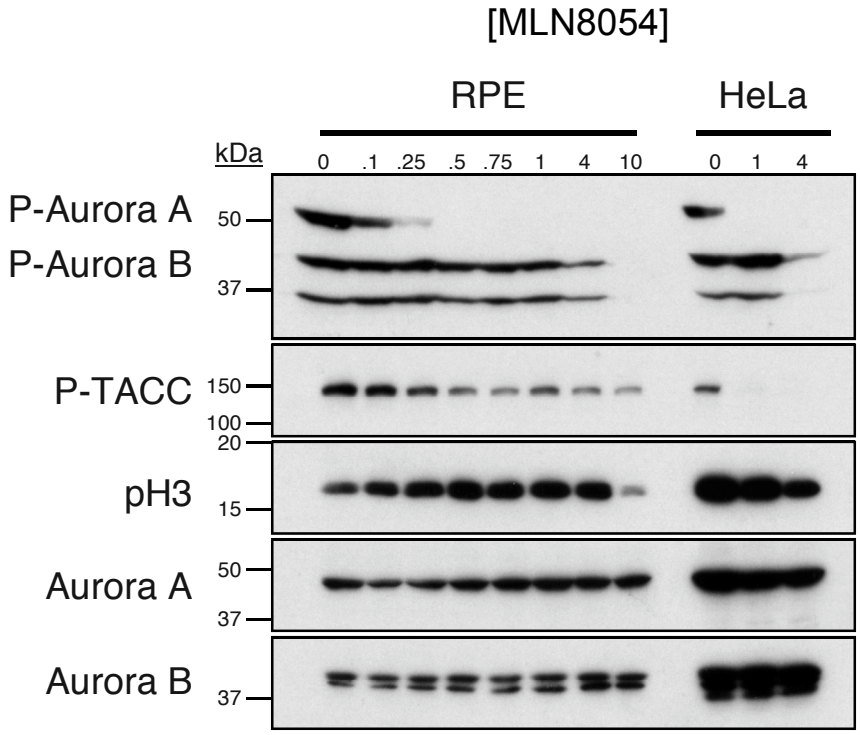

Figure 8.1 MLN8054 can be used to specifically inhibit Aurora A activity in RPE cells
RPE cells were mitotically enriched with Nocodazole for 16 hours before being treated with MG132 and a range of concentrations of MLN8054 for a further 2 hours. The samples were then harvested and lysed, and whole cell lysates were separated using SDS PAGE. Immunoblotting was used to detect the presence of: Aurora A, Phospho-Aurora A (Thr288), phospho-Aurora B (Thr232), phospho-TACC (Ser558), Aurora B and phospho-histone H3 (Ser10). HeLa cells were treated identically with 0 μM, 1 μM and 4 μM MLN8054 and run on the same blot to compare inhibition levels. The images displayed represent results that were consistent over three independent experiments.

also more resilient to Aurora A inhibition, as well as addressing whether Aurora A activity played an equivalent role in non-transformed cells to that observed in the cancer cell lines tested.

8.2 Aurora A activity can be inhibited in RPE cells

Immortalised human retinal pigment epithelial (RPE) cells expressing telomerase reverse transcriptase (hTERT) were selected as the non-transformed cell line to be investigated in this chapter. The expression of hTERT prolongs cell life by reconstituting telomerase activity, thus preventing senescence occurring as a result of telomere shortening. Importantly however, neither aneuploidy nor transformation is induced by the expression of hTERT, making it an ideal method of immortalisation for the investigation into non-transformed cells (Jiang et al., 2003a, Gascoigne and Taylor, 2008, Jiang et al., 2003b).

After selecting the non-transformed cell line, I next set out determine whether they were responsive to the Aurora A inhibitory effects of MLN8054. To do this, I treated the RPE cells with a range of MLN8054 concentrations, separated the samples on a SDS PAGE gel, and then used Western blotting to observe any changes in activity (Figure 8.1). Encouragingly, the disappearance of bands representing phospho-Aurora A indicated that Aurora A activity could indeed be inhibited in RPE cells with MLN8054. Furthermore, the level of Aurora A inhibition produced appeared to be equivalent to that observed in cancer cells samples (Figures 3.3 and 7.2), where the complete disappearance of phospho-Aurora A signal was observed to occur in samples treated with 1 μM MLN8054. Interestingly however, in RPE cell samples the phospho-TACC3 signal was not reduced to equivalent levels as in HeLa cell samples shown on the same blot. Despite these differences, the RPE Western blot data demonstrated that Aurora A activity could be potently be inhibited at 1 μM MLN8054.

As well as inhibiting Aurora A activity however, MLN8054 could also inhibit Aurora B activity in the RPE cell line. RPE samples treated with 4 μM MLN8054 showed a reduction in phospho-Aurora B signal when compared with a control sample, however treatment with 10 μM MLN8054 appeared to abolish the signal completely. This level of inhibition was consistent with that seen in HeLa cells on the same Western blot, however the RPE phospho-histone H3 signal was only reduced when RPE cells were treated with 10 μM MLN8054. This differs from the cancer cell lines tested, as the level of phospho-Histone H3 signal in HeLa, DLD-1 and HCT116 cells was affected by lower concentrations of MLN8054 (Figures 3.3, 7.2 and 8.1). Therefore, although differences could be observed between the effects of MLN8054 on the downstream targets of Aurora A and Aurora B, the analysis of the phosphorylated forms of Aurora A and B in the Western blots

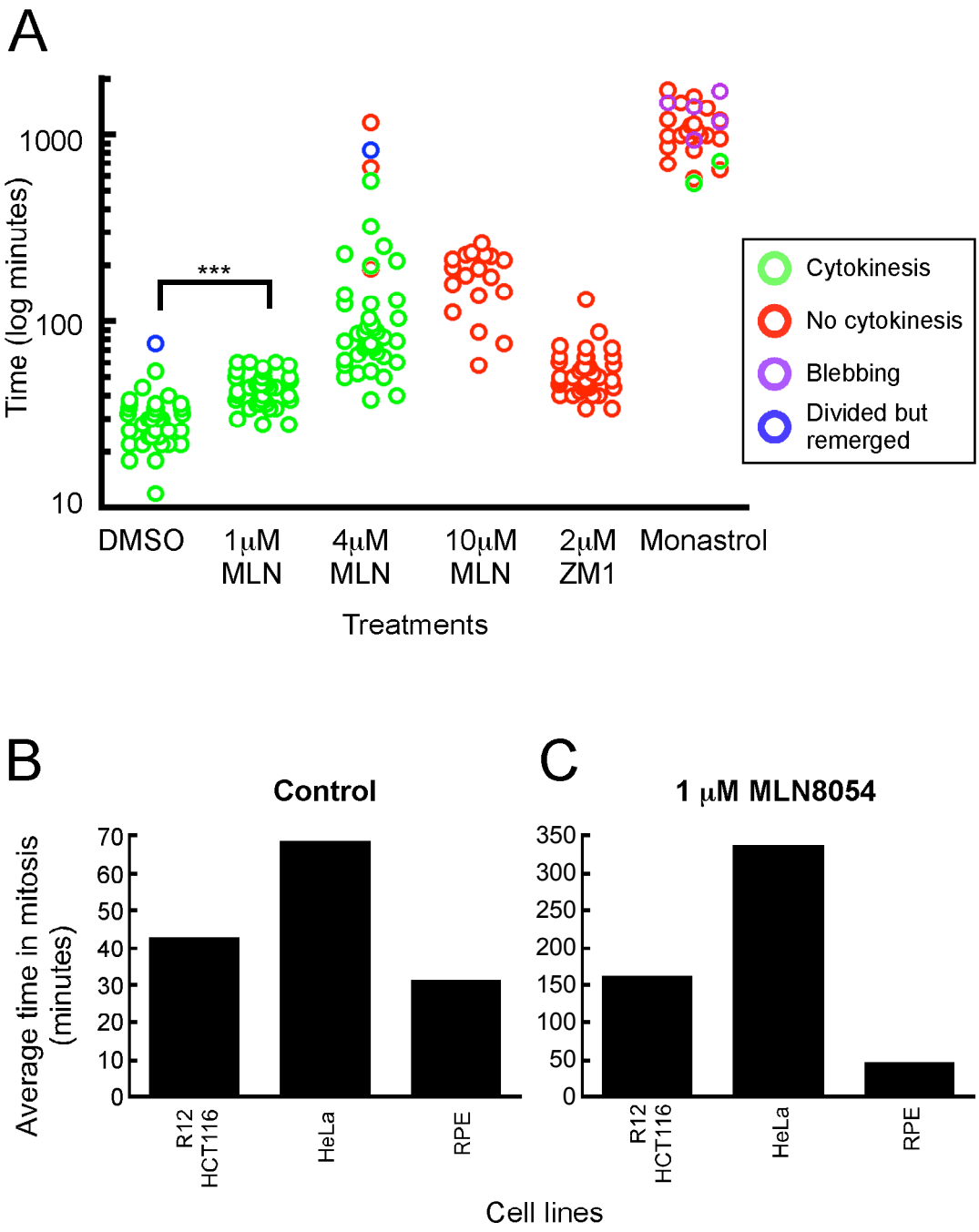

Figure 8.2 Inhibiting Aurora A activity increases the time RPE cells spend in mitosis, but not to the extent seen in transformed cell lines
(A) RPE cells were treated with the indicated drugs, and time-lapse phase contrast images were taken every 2 minutes. The images were made into movies, which were analysed, and the time taken for cells to go through mitosis was plotted (determined as between nuclear envelope break-down and chromosome decondensation). Green circles represent cells that performed cytokinesis, red circles indicate cells that were unable to do so, purple circles denote cells that started blebbing while in mitosis and blue circles show cells which appeared to divide into 2 cells but then remerged back into one. (B and C) Histograms showing the average time R12 HCT116, HeLa and RPE cells spent in mitosis after treatment with DMSO (B) and 1 μM MLN8054 (C). The graphs contain data from at least three independent phase contrast time-lapse experiments. *** = P<0.001 two-tailed t test.

demonstrated that MLN8054 could be used at 1 μM in non-transformed cells to potently and specifically inhibit Aurora A activity.

8.3 Inhibiting Aurora A activity causes mitotic delays in RPE cells, although not to the extent seen in the cancer cell lines

After confirming that it was possible to inhibit Aurora A activity in RPE cells, I next wanted to establish if, as seen in cancer cells, Aurora A activity was required for efficient mitotic progression. To address this question I used phase-contrast time-lapse microscopy to observe the length of time individual cells took to go from nuclear envelope break-down to chromosome decondensation. The quantitation of the mitotic timings revealed that control RPE cells took on average 31 minutes to go through mitosis before dividing into two cells (Figure 8.2A). Interestingly, this mitotic period is slightly shorter than those previously described for the cancer cell lines, with HeLa and R12 HCT116 cells observed to take an average of 68 and 42 minutes to complete mitosis respectively (Figure 8.2B). Treatment of the RPE cells with 1 μM MLN8054 caused them to be retained in mitosis for 44 minutes, a 42% increase in mitotic period compared to control cells. This increase is strikingly shorter than those seen in HeLa and R12 HCT116 cells after the equivalent treatment, which saw average mitotic periods increase by 289% and 281% respectively (Figure 8.2C). Despite the differences in severity of MLN8054 treatment on transformed and non-transformed mitoses, as with cancer cell lines the majority of the RPE cells were able to divide following their treatment with 1 μM MLN8054 (Figures 6.1, 6.2A, 7.1C and 7.5B).

Raising the concentration of MLN8054 to 4 μM, caused a further extension to the RPE mitotic period, with cells taking an average of 168 minutes to exit mitosis, a 443% increase to the mitotic timing compared with control cells. Interestingly, unlike previously reported cancer cell data, the vast majority of RPE cells were still able to perform cytokinesis after treatment with 4 μM MLN8054 (Figures 7.1C and 7.5A). This difference may be due to RPE cells being more resilient to the effects of 4 μM MLN8054 on down stream components of the Aurora B signalling pathway compared to the cancer cell lines tested, as determined by phospho-Histone H3 levels (Figure 8.1). Treating RPE cells with 10 μM MLN8054, caused cells to spend an average of 173 minutes in mitosis, a 458% increase compared to control cells. This extended mitotic period suggested that 10 μM MLN8054 was capable of causing more profound chromosomal alignment defects, possibly through an increased potency towards Aurora A activity. Importantly however, all cells analysed were incapable of dividing, demonstrating the effects of Aurora B inhibition seen at this MLN8054 concentration and confirming that MLN8054 cannot be used at 10 μM to specifically inhibit Aurora A activity (Figure 8.1). The lower concentrations of MLN8054 however, were capable of

specifically inhibiting Aurora A activity, and clearly showed that Aurora A inhibition extended the time RPE cells spent in mitosis. Interestingly though, Aurora A inhibition did not produce such profound increases to mitotic periods in RPE cells as was seen in cancer cells after the equivalent treatments, indicating the existence of integral differences between the cell types.

8.4 In RPE cells the processes of chromosome alignment and spindle assembly are not as severely affected by Aurora A inhibition as in cancer cells

To explore the reason as for why RPE cells did not experience as acute effects to mitotic progression as cancer cell lines, despite apparently equivalent levels of Aurora A inhibition, I examined the effect of Aurora A inhibition on the ability of RPE cells to form bipolar spindles and align their chromosomes. I had previously shown Aurora A inhibition to reduce the efficiency of bipolar spindle assembly and chromosome alignment in cancer cells, consequentially causing extended mitotic delays (Figures 5.1 and 6.1). Therefore, consistent with the previously outlined identical experiment in HeLa cells, I treated RPE cells with an Eg5 inhibitor to create monopolar spindles, then allowed the cells to reorganise their spindles and align their chromosomes in the presence of various treatments (Figure 8.3A). The analysis of the fixed cell samples revealed that as seen in HeLa cells, maintaining the Eg5 inhibition produced severe chromosomal alignment defects in 100% of cells (Figure 8.3B). This treatment also produced monopolar spindles in the majority of cells analysed, explaining the source of the chromosome misalignments (Figure 8.3C). In contrast, washing out the effects of the Eg5 inhibitor produced the occurrence of bipolar spindles and properly aligned chromosomes (Figures 8.3B DMSO and 8.3C DMSO). Replacing the Eg5 inhibitor with an Aurora B inhibitor however, prevented the re-alignment of the chromosomes in 98% of cells, despite the reformation of their bipolar spindles (Figures 8.3B-ZM1and 8.3C-ZM1). Importantly, all three of the treatments produced phenotype profiles which were consistent with previously described HeLa data as well as published reports of similar treatments (Figure 5.1) (Mayer et al., 1999, Kapoor et al., 2000, Adams et al., 2001b, Ditchfield et al., 2003).

Interestingly however, the replacement of the Eg5 inhibitor with 1 μM MLN8054 in the RPE cells produced strikingly less severe cellular phenotypes from those described in the cancer cell line. Only 23% of HeLa cells were previously reported to be capable of realigning their chromosomes in the absence of Aurora A inhibition in the allocated time period (Figure 5.1A), whereas 90% of RPE cells were recorded as having completely aligned chromosomes after the same treatment, and 100% of cells were observed as having bipolar spindles (Figures 8.3B-MLN8054 and 8.3C-MLN8054). Although I had previously also described the majority of HeLa cells to be capable of forming bipolar spindles after MLN8054 treatment, the spindles were found to be shorter than in control

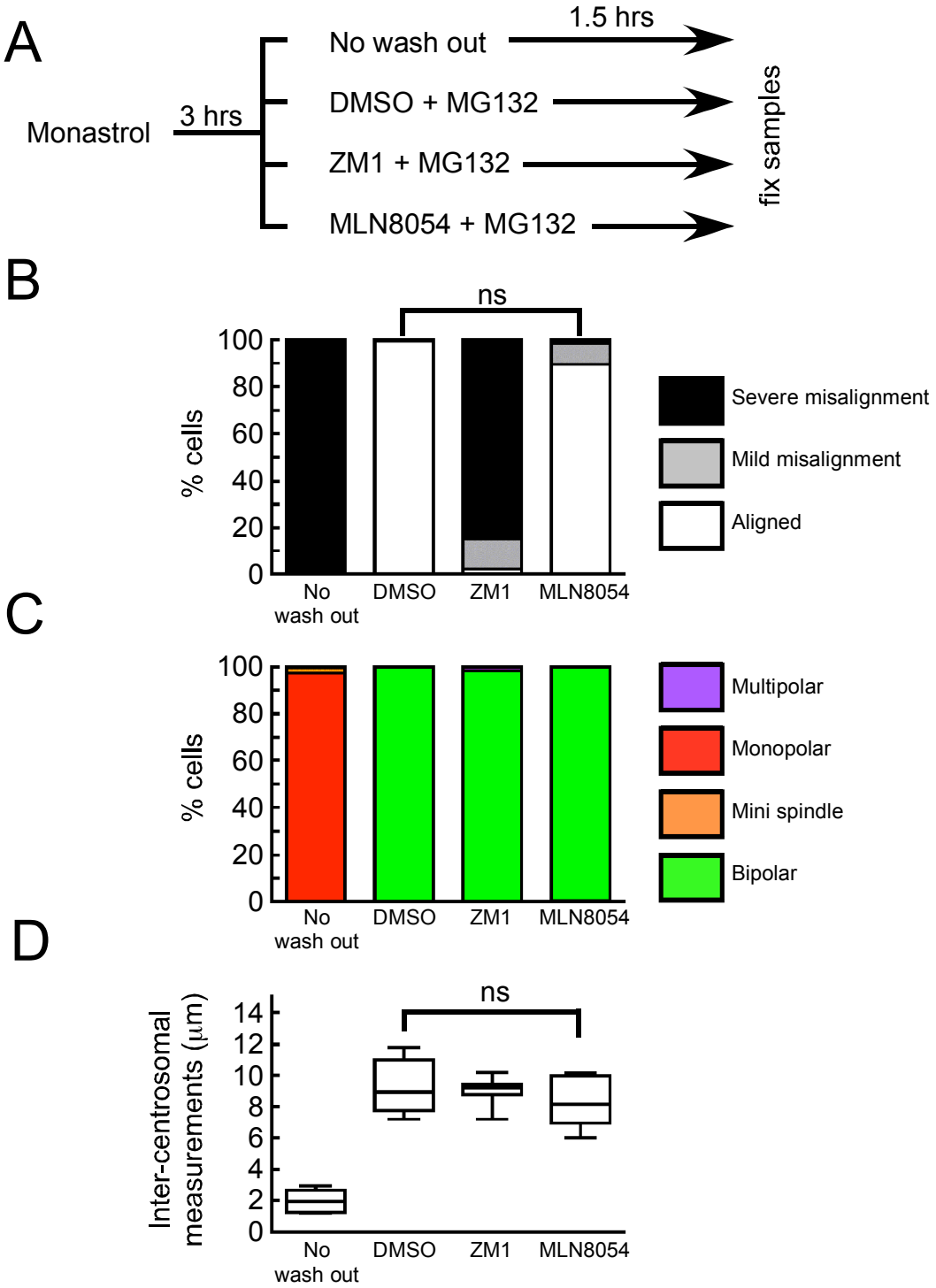

Figure 8.3 Aurora A inhibition only minimally reduces the efficiency of chromosome alignment in RPE cells and does not significantly reduce centrosome separation
(A) Protocol used to determine whether Aurora A activity has a role in chromosome alignment. After following the protocol outlined in (A), RPE cells were fixed and stained with antibodies against Pericentrin and Tubulin to determine the spindle phenotype, and Aurora B together with Hoesht to allow the observation of chromosomal alignment.
(B) Quantitation of efficiency of chromosomal alignment. Cells were classified as having 'mild' misalignment if up to 4 of their chromosomes were unaligned or, if there were more than 4, the cell was termed as having 'severe' misalignment. Cells treated with 1 μM MLN8054 were found to show no significant difference in in their ability to align their chromosomes (two-tailed t test). (C) Histogram to show spindle phenotypes of the samples. (D) Measurement of spindle lengths from z-stacked images. The graphs represent data taken from three independent experiments. ns = P>0.01 two-tailed t-test.

cells (Figures 5.1C and 5.1D). Therefore to determine whether RPE cells also suffered from shortened spindles after Aurora A inhibition, I measured the inter-pole distances of the cells exposed to each of the previously described treatments (Figure 8.3A). The measurement of fixed cell spindles revealed that sustained Eg5 inhibition produced severely shortened spindle lengths an average of 1.9 microns long, reflecting the monopolarity of the spindles (Figure 8.3D). Comparatively, cells exposed to DMSO or ZM1 after the removal of the Eg5 inhibitor, showed longer average spindle lengths of 9.3 and 9.0 microns respectively. Interestingly, RPE spindles formed in the presence of MLN8054, showed similar spindle lengths that were not significantly different from those of control cells, with an average spindle length measurement of 8.3 microns. This data therefore indicated that RPE cells were more efficiently able to form spindles of a normal length and align their chromosomes in the absence of Aurora A inhibition, than the cancer cell line tested. This distinction may therefore provide an explanation as to why the cancer cell lines were delayed in mitosis for longer periods than the RPE cells.

To test whether the relatively short mitotic delay caused in RPE cells by Aurora A inhibition was related to their superior ability to align their chromosomes compared to cancer cells, I analysed live RPE cells which had been transfected with GFP-histone to observe their DNA (Gascoigne and Taylor, 2008). The analysis of cellular DNA by fluorescence microscopy time-lapse imaging showed that, as with HeLa cell samples (Figure 6.2B(i)), control RPE cells experienced nuclear envelope breakdown, before organising their chromosomes into a metaphase plate and proceeded into anaphase and dividing into two cells (Figure 8.4A). Consistent with previously described live cell data (Figure 8.2A), when treated with MLN8054, RPE cells were delayed in mitosis (Figure 8.4B). RPE cells were however able to align chromosomes to form a metaphase plate in a shorter time period that seen in HeLa cell samples exposed to Aurora A inhibition (Figure 6.2B). Furthermore, unlike HeLa cells, no segregation defects were observed in the RPE cells analysed, demonstrating the presence of correctly aligned chromosomes (Figures 6.2B (ii) and 6.2B(iii)). The ability of RPE cells to more efficiently form bipolar spindles and align their chromosomes after Aurora A inhibition can therefore be used to explain why RPE cells only suffer comparatively short mitotic delays after the treatment. Alternatively however, Aurora A activity may not be as potently inhibited as in the cancer cell lines tested, despite evidence to the contrary (Figure 8.1). Without the creation of mutant RPE cells that able to revert the high dose MLN8054 effects on Aurora B activity however, it would not be possible to increase the concentration of MLN8054 while maintaining specificity towards Aurora A activity.

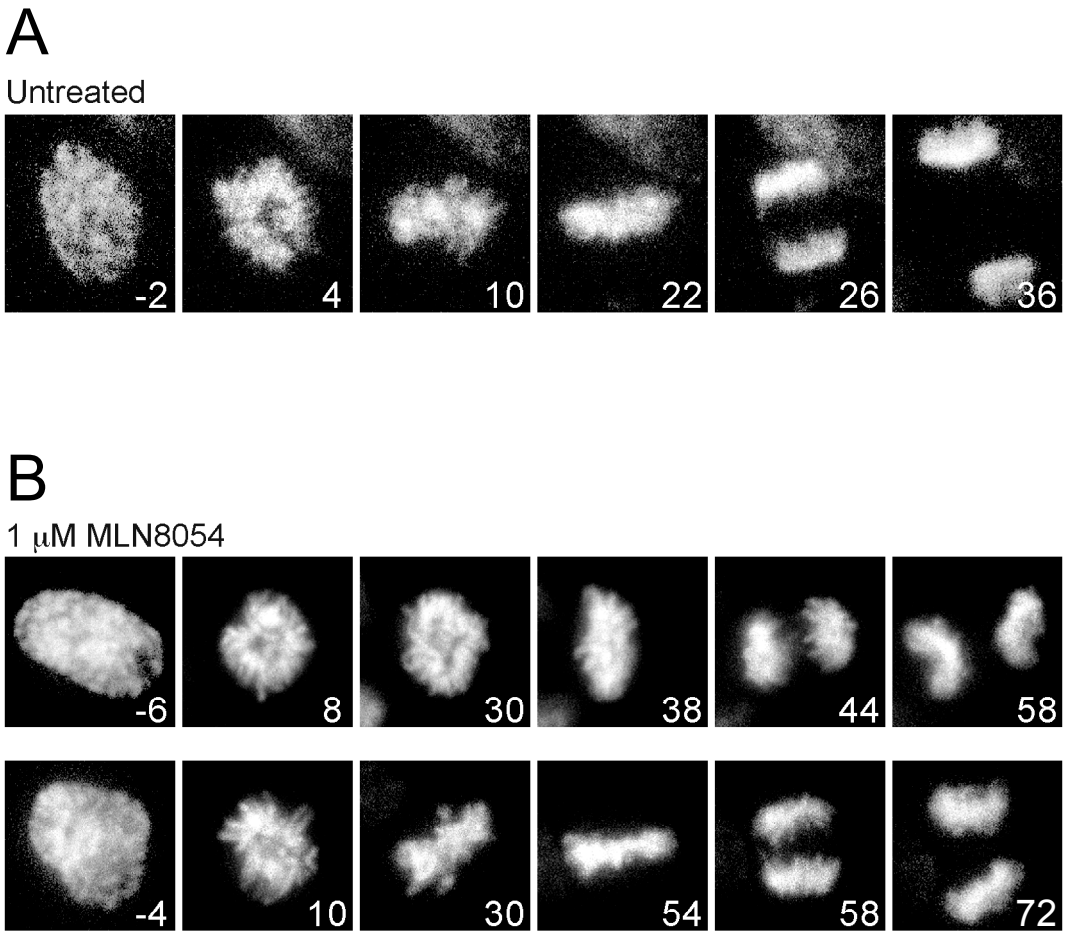

Figure 8.4 Aurora A inhibition in RPE cells causes the extension of prometaphase, however they are eventually able to divide after only a relatively short delay
Projected z-section time-lapse images of GFP histone H2B RPE cells treated with 0 μM MLN8054 (A) and 1 μM MLN8054 (B). Numbers shown in the bottom right-hand corner of each image indicate the time elapsed in minutes after nuclear envelope breakdown.

8.5 Long-term Aurora A inhibition causes RPE cells to accumulate with 2N DNA and permits the survival of a number of sparsely populated colonies

The ability of non-transformed cells to more efficiently align their chromosomes compared to cancer cells in the absence of Aurora A activity, indicates that the cells may be more resilient to the degenerative effects of Aurora A inhibition compared to the cancer cell lines tested. Differences between the long-term viability of transformed and non-transformed cells may have important implications for the targeting of Aurora A activity as an anti-cancer therapy, since an important consideration in determining the effectiveness of a therapeutic agent is whether healthy tissue is also damaged during the targeting of the disease.

I previously described how prolonged exposure to 1 μM MLN8054 caused HeLa cells to experience severe chromosomal misalignment, prolonged delay in mitosis, aneuploidy and eventually cell death, indicating the toxicity of the treatment (Figures 6.3A(ii), 6.3B and 6.3C). In comparison, flow cytometry profiles of RPE cells treated with 1 μM MLN8054 for 24 hours, showed only a very slight increase in the proportion of cells with 4N DNA, demonstrating the comparatively short mitotic delay experienced by the cells (Figure 8.5A). Interestingly, treatment with 1 μM MLN8054 for more than 48 hours caused the proportion of RPE cells with 4N DNA to be reduced, suggesting that cells were prevented from progressing normally through the cell cycle. Furthermore, the development of a sub-G1 peak after 72 hours of treatment indicated that cells were dead or dieing as a result of the inhibitor treatment. Interestingly however, after 96 hours of sustained Aurora A inhibition, only a minimal sub-G1 population was detected, with most cells instead forming a prominent 2N DNA peak (Figure 8.5A).

Treating RPE cells with 4 μM MLN8054 produced similar cell cycle profiles to those seen after the 1 μM treatment, although a slightly larger 4N peak and smaller 2N DNA peak were detected, indicating of the longer mitotic delay experienced by the cells (Figure 8.2A). Despite this, both 1 μM and 4 μM MLN8054 produced distinct effects to RPE cells than those observed in HeLa cells, with the flow cytometry profiles appearing to indicate that RPE cells only experienced a comparatively low amount of cell death after extended Aurora A inhibition.

To assess the long-term viability of the RPE cells exposed sustained Aurora A inhibition, and address whether they did indeed suffer only relatively low amounts of cell death, I tested their ability to form colonies after prolonged Aurora A inhibition. Following 14 days of the exposure of the RPE cells to a range of different treatments, I fixed and stained the samples with crystal violet and analysed the samples to detect cell colonies (Figure 8.5B). Previously, HeLa cells had been shown to be unable to form colonies after sustained treatment with 1 μM (Figures 6.3B and 6.3C).

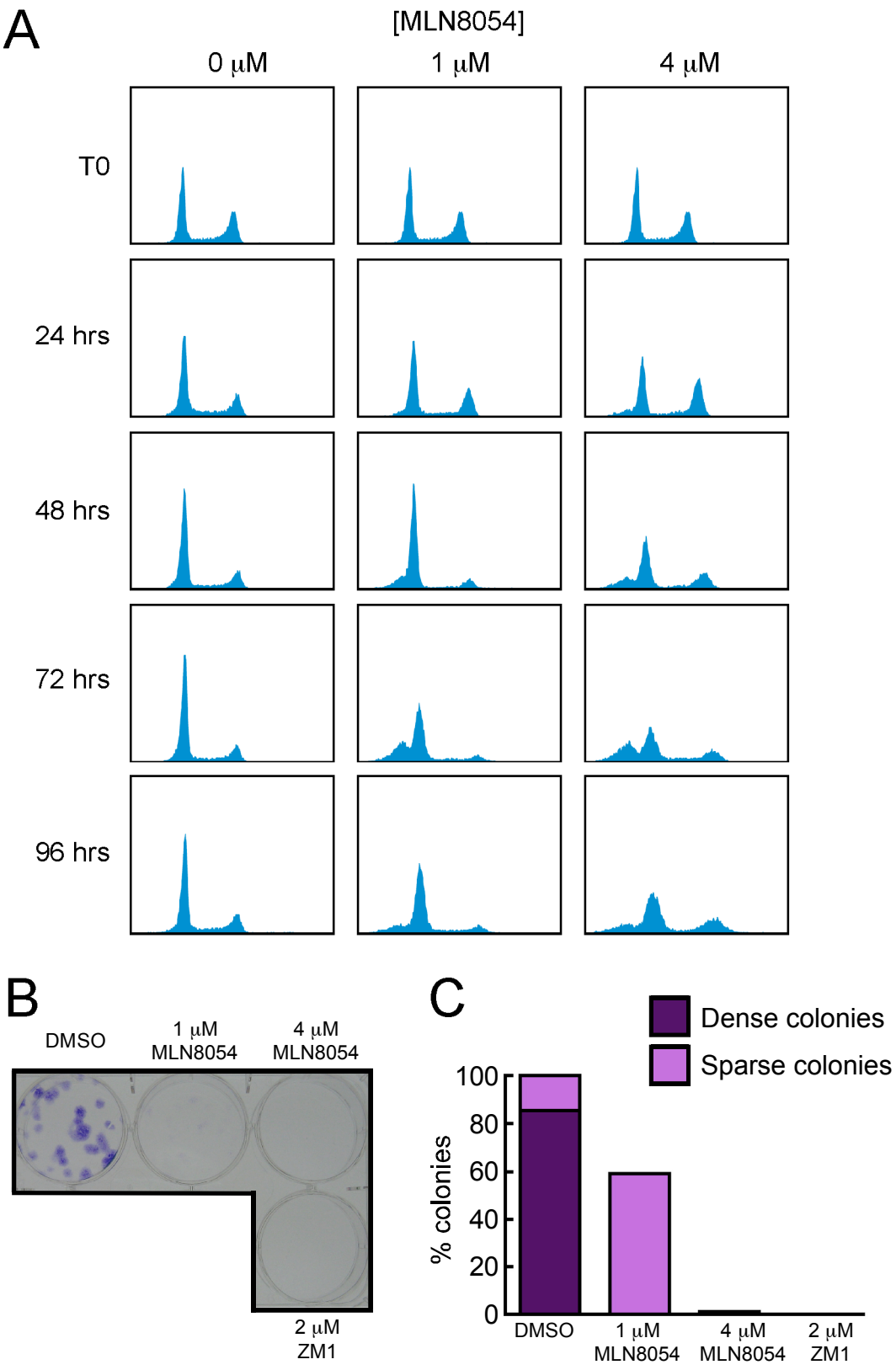

Figure 8.5 Long-term Aurora A inhibition causes RPE cells to accumulate with 2N DNA and the formation of sparsely populated colonies
(A) Flow cytometry PI profiles of RPE cells treated with 0 μM, 1 μM and 4 μM MLN8054. Cells were harvested and processed after every 24 hours for a total of 96 hours. (B) RPE cells were treated with the indicated drugs for 14 days before fixing and staining with crystal violet. (C) Qunatitation of the colony formation assay in (B).

In the equivalent experiment in RPE cells, control cells were shown to form a large number of densely populated colonies during the growth period. In contrast, treating the cells with ZM1 completely prevented the formation of colonies, which is consistent with previously described HeLa data (Figure 6.3C). RPE cells exposed to sustained 1 μM MLN8054, were unable to form colonies that were identical to those of control samples, however surprisingly, smaller and less densely populated colonies could be detected. Importantly however, not only was the density of the colonies reduced after the 1 μM MLN8054 treatment but also the quantity, with a 41% reduction in the number of colonies detected. Interestingly, the sustained treatment of cells with 4 μM MLN8054 prevented the growth of almost all colonies, denoting the higher toxicity of the treatment.

Although RPE cells were rendered unviable after their extended exposure to 4 μM MLN8054, the observation that 1 μM MLN8054 allowed the formation of a number of sparsely populated colonies was in stark contrast the complete loss of HeLa cell viability after the same treatment. Furthermore, the finding that long-term Aurora A inhibition caused RPE cells to accumulate with 2N DNA, while a profound sub-G1 population was produced in HeLa cells, highlighted some important differences in how the transformed and cancer cells responded to the treatment. Supporting these observations, a recently published report described how MLN8054 caused a higher proportion of cell death in transformed cells compared to non-transformed cell lines (Shang et al., 2009).

8.6 The effect of Aurora A inhibition on the localisation of RPE proteins

The data in this chapter so far has outlined some clear differences between the effects of Aurora A inhibition on transformed and non-transformed cell lines. To uncover an explanation for these differences, I examined changes in the localisation of various proteins in response to Aurora A inhibition.

I have previously described that Aurora A localisation in HeLa cells was not dependent on its activity, however its activity was partially required for its stable association to the spindle (Figure 4.7A). A similar result was also seen in fixed and immunostained RPE cells (Figure 8.6A). When RPE cells were not extracted before their fixation, there appeared to be no change to the Aurora A antibody localisation after MLN8054 treatment (data not shown). However, when including the extraction step in the protocol, Aurora A inhibition caused a reduction of Aurora A to be detected on the spindle, although consistent with the HeLa data, its localisation to the centrosome did not appear to be affected.

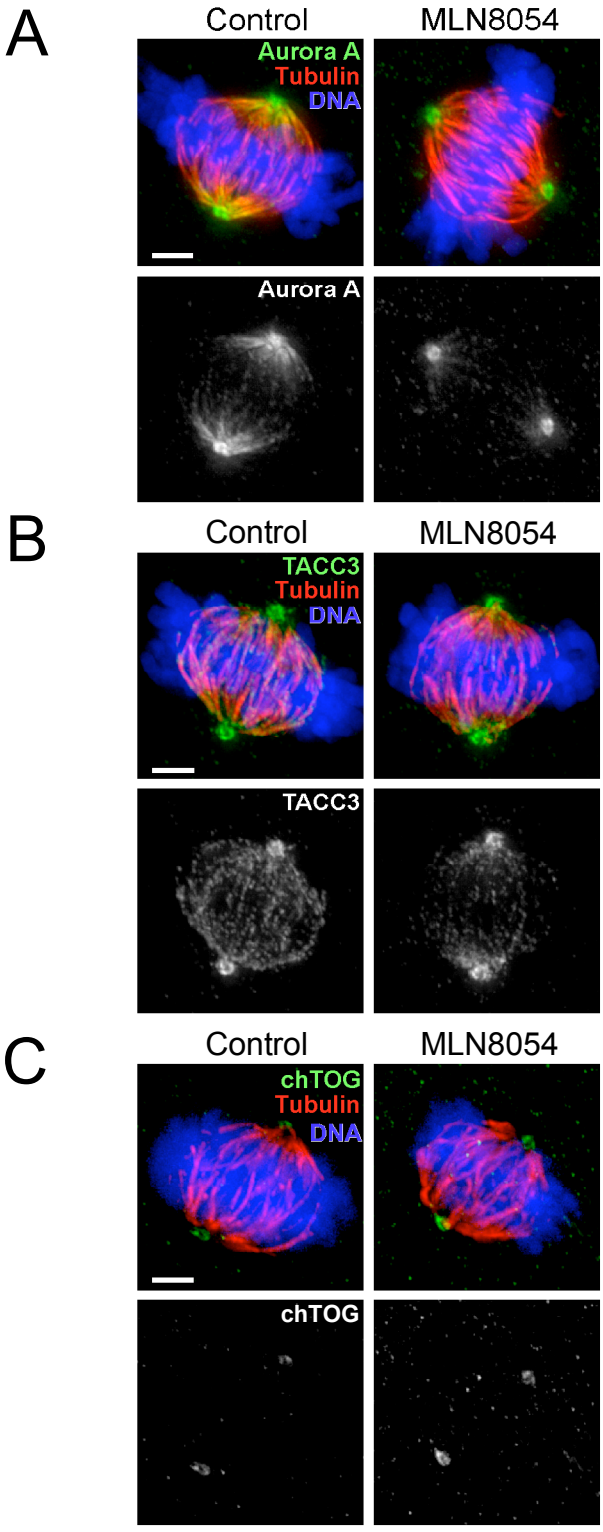

Figure 8.6 Aurora A activity is partially required for the stable association of Aurora A protein to the spindle, however TACC3 and chTOG localisation are not as affected by Aurora A inhibition as previously described in HeLa cells
RPE cells were treated with 0 μM MLN8054 (control) or 1 μM MLN8054 for 2 hours before being fixed and stained with the indicated antibodies. Z-stacked images were taken of the samples to observe the affect of Aurora A inhibition on Aurora A localisation (A), on TACC3 localisation (B), and chTOG localisation (C). Scale bars = 4 μm.

The localisation of TACC has been attributed to Aurora A activity, with a recent study even suggesting that the mislocalisation of TACC3 seen after MLN8054 treatment could be a pharmacodynamic method of measuring Aurora A activity (LeRoy et al., 2007). Furthermore, the localisation of chTOG/XMAP215 to the minus-ends of microtubules has been proposed to be dependent on TACC (Barros et al., 2005, Giet et al., 2002, Kinoshita et al., 2005, LeRoy et al., 2007, Deluca et al., 2006, Peset et al., 2005, Lee et al., 2001, Gergely et al., 2003). Consistent with these findings, I previously showed that TACC3 was completely mislocalised and chTOG levels were reduced after Aurora A inhibition in HeLa cells (Figures 4.7B, 4.7C and 4.7D). Intriguingly however, MLN8054 treatment of RPE cells did not cause the same level of TACC3 mislocalisation as previously seen in HeLa cells (Figure 8.6B). Instead, its localisation was only slightly reduced on spindles after the treatment. This finding is in agreement with the findings of Barros and colleagues (2003), which showed that a D-TACC that could not be phosphorylated by Aurora A, was still localised to the centrosomes of *Drosophila*, but its localisation was reduced on microtubules. Interestingly, the localisation of chTOG to the centrosomes did not seem to be reduced at all, with its localisation instead often appearing to be more intense on some centrosomes after the treatment (Figure 8.6C).

8.7 Summary

Comparing the effects of Aurora A inhibition in non-transformed cells with cancer lines, revealed some interesting differences. One of the most profound of these differences was the ability of RPE cells to more efficiently form bipolar spindles and align their chromosomes in the absence of Aurora A activity compared to cancer cells despite equivalent levels of Aurora A inhibition. The comparative efficiency of these processes in the absence of Aurora A activity meant that non-transformed cells only suffered relatively minimal mitotic delays after the treatment. Furthermore, after extended Aurora A inhibition RPE cells were caused to accumulate with 4N DNA and were able to form sparsely populated colonies, while HeLa cells quickly become aneuploid and died after the same treatment.

The ability of RPE cells to maintain relatively efficient cell division after Aurora A inhibition may be due to a capacity to either sustain the activity of particular cellular pathways in the absence of Aurora A activity or exploit alternatives, where the cancer cells were less able to do so. This ability to withstand the effects of Aurora A inhibition was demonstrated through the continued efficient localisation of chTOG and TACC3 to centrosomes even after MLN8054 treatment, a phenomenon that was not seen in the cancer cell line tested. Interestingly, both chTOG/XMAP215 and TACC3 have been attributed to the normal growth of spindles (Barros et al., 2005, Giet et al., 2002,

Kinoshita et al., 2005, LeRoy et al., 2007, Deluca et al., 2006, Peset et al., 2005, Lee et al., 2001, Gergely et al., 2003). The sustained localisation of chTOG and TACC3 after MLN8054 treatment could possibly therefore be used to explain how RPE cells are able to form spindles of a normal length after the treatment, whereas HeLa cells could not.

The apparent higher sensitivity to Aurora A inhibition of cancer cell lines compared to RPE cells suggests that targeting the kinase activity of Aurora A may be able to effectively target transformed cells while leaving the healthy cells comparitivley less effected, a theory that is supported by a number of reports (Tomita and Mori 2010, Manfredi et al., 2007, Dees et al., 2010). Despite the differences observed between the cancer cell lines and the RPE cells, it is important to highlight that RPE cells were the only non-transformed cells to be tested during this investigation. The differences observed between the two cell types may therefore simply be the result of inter-cell line differences. More work with alternative non-transformed cell lines would need to be undertaken before any concrete conclusions could be made regarding the different effects of Aurora A inhibition in healthy and cancer cells.

Furthermore, the process of removing RPE cells from the retinal sample to growing them in culture involves a major level of disruption to their normal living conditions, causing the development of a degree of continuous adaptation in the cell population (Engelmann and Valtink, 2004). Such adaptations make it possible to effectively culture the cells, however the resulting cell population is also consequently made integraly different from the sample from which they were derived. Thus, although RPE cells have been used during this investigation to represent non-transformed cells, they are not truly synonymous with naturally occurring cells of the human body. A true observation of the role of Aurora A activity within healthy cells would therefore instead, have to be in the form of *in vivo* experiements, which unfortunately were not possibile during this investigation.

Desepite the requirement for *in vivo* studies to make accurate conclusions about the role of Aurora A activity in healthy cells, the differences highlighted between transformed and the RPE cell line demonstrated the danger of assuming commonality between distinct cell lines, and was thus an important part of the investigation.

9 Discussion

9.1 Introduction

The Aurora kinase family has received much attention in recent years, with investigations revealing roles for the kinases throughout mitosis, with links even reported between the expression of the kinases and the development and progression of cancer (Zhou et al., 1998, Keen and Taylor, 2004, Bischoff et al., 1998, Carmena and Earnshaw, 2003). In the case of Aurora A kinase, much of this work comprised of depleting, disabling or mutating endogenous protein, or exogenously expressing mutated forms to determine the role of the Aurora A kinase (Marumoto et al., 2002, Marumoto et al., 2003, Hannak et al., 2001, Mori et al., 2007, Glover et al., 1995, Motegi et al., 2006). These techniques provided important insight into the role of Aurora A kinase, exposing many intriguing qualities of the kinase. Despite the elegant use of the experimental techniques, the requirement to constitutively express mutated forms of the kinase or induce the expression or depletion of proteins, meant that temporal control over the experiments was unachievable. Furthermore, the removal or depletion of the protein either by genetic knockouts or nulls or by RNAi obstructs the ability to distinguish between the structural functions of the kinase and its catalytic roles within the cell. Therefore instead of causing permanent changes to cellular Aurora A or employing methods that are characteristically slow to take effect, a technique that permits the intricate and reversible manipulation of Aurora A activity, would help to develop a more complete understanding of the kinase. Throughout the last decade, small molecule inhibitors have proved to be both efficient anti-cancer therapies and valuable experimental tools, and have made it possible to specifically and potently inhibit Aurora A activity in an efficient and reversible way.

Using the small molecule inhibition of Aurora A activity, I have been able to reveal some interesting features of the kinase, some of which conflict with established perceptions within the literature. Throughout this report I have detailed these findings, showing that Aurora A activity is involved in the formation of a functional spindle in order to efficiently align chromosomes and enable the efficient detection of alignment defects. Therefore when Aurora A activity was inhibited, cells displayed segregation errors and the cell population became eventually unviable. Despite this prominent role for Aurora A activity in mitosis, I showed that cells can divide and activate the spindle assembly checkpoint in its absence, demonstrating that Aurora A activity is not required for cytokinesis and does not play a prominent role in the checkpoint. Aurora A activity was also not required for cytokinesis or the activation of the spindle checkpoint in the non-

transformed cells tested, although surprisingly, non-transformed cell spindles were not as profoundly affected by Aurora A inhibition as cancer cell lines.

9.2 The limitations of the study

The use of small molecules to inhibit Aurora A activity permitted fast, easily controllable, dose-dependent and reversible kinase inhibition, without directly affecting protein levels. However, the specificity of the inhibitors towards Aurora A was an issue (for review see [Taylor and Peters, 2008]). To determine the ability of MLN8054 to specifically target Aurora A activity, Western blotting was used to reveal that MLN8054 was the most specific Aurora A inhibitor out of those available (Figure 3.3). At 1 μM MLN8054 inhibited Aurora A activity to more than 95% in HeLa cells and to around 95% in DLD-1 cells, and importantly didn't also show signs of also inhibiting Aurora B activity (Figures 3.3C and 3.3D). The specificity of MLN8054 for Aurora A activity over the other Aurora family members was further confirmed by the observation that the majority of cells did not suffer cytokinesis defects or exhibit stabilised merotelic attachments following treatment (Figures 5.5, 6.1 and 6.2A) (Hauf et al., 2003, Ditchfield et al., 2003, Giet and Glover, 2001, Yang et al., 2010b).

This data therefore demonstrated that MLN8054 could be used to potently inhibit Aurora A activity, over that of the other family members, however it was also important to determine whether MLN8054 affected any additional kinases. The specificity of the 1 μM MLN8054 treatment over the other members of the kinome was investigated by comparing the cellular effects of the treatment with those of two other Aurora A inhibitors, through the inspection of immunofluorescently-stained cell samples. Encouragingly, similarities between the effects of MLN8054 and the Aurora inhibitor ZM3 to cells indicated that both treatments appeared to be have no obvious off-target action. This deduction is consistent with published *in vitro* data, in which MLN8054 was found to be more than 40-times more selective for Aurora A activity than for that of Aurora B (Manfredi et al., 2007). In the same publication, *in vitro* data was used to show MLN8054 was more potent towards Aurora A activity than a panel of 226 other kinases. A recent publication futher highlighted the importance of testing chemical inhibitors on a large panel of protein kinases such as this to ensure their specificity, which is of particular importance when considering that there are over 500 protein kinases in the human genome (Cohen, 2010). Despite the promising result of the comparative immunofluorescence however, it could not be ruled out that additional off-target kinases were being inhibited by the MLN8054 treatment, and that this was simply not realised by using this technique.

To further tackle this issue, I therefore attempted to create a cell line which expressed a form of Aurora A that was mutated in a way that rendered it resistant to 1 μM MLN8054. This cell line could then be used to identify any off-target effects by treating the cells with MLN8054 and observing any differences that occurred between untreated cells. Any differences between the treated and untreated mutant cell lines would therefore highlight off-target effects of the inhibitor. Despite attempting to introduce a number of different mutations into Aurora A however, none conferred sufficient resistance to MLN8054, and thus could not be used to investigate any off-target effects of the treatment (data not shown). Thus, although immunofluorescence comparison experiments and published *in vivo* data appeared to indicate that MLN8054 was not having off-target effects when used at 1 μM, I cannot completely rule out the occurrence of such effects (Figure 3.4; (Manfredi et al., 2007)). Despite the disappointing inability to produce MLN8054-resistant Aurora A mutants, this difficulty may have important connotations for the development of resistancy in treated patients, and thus the future success of Aurora A inhibitors as anti-cancer treatments.

In addition to being unable to fully investigate off-target MLN8054 effects, a further weakness of the investigation was highlighted in that Aurora A activity may not be sufficiently inhibited by 1 μM MLN8054. Indeed, the application of MLN8054 at 1 μM to HeLa and DLD-1 cells did not produce as markedly pronounced spindle effects as those described in may other reports, despite the apparent potent inhibition indicated in the Western blot data (Figures 3.3, 3.4, 4.4A and 4.4B) (Liu and Ruderman, 2006, Giet and Prigent, 2000, Giet and Prigent, 2001, Roghi et al., 1998, Marumoto et al., 2003, Glover et al., 1995, Hannak et al., 2001, Girdler et al., 2006). Therefore, to test whether the potency of Aurora A inhibition could be increased, I used a HCT116 line which endogenously expressed a mutated drug-resistant form of Aurora B kinase (Girdler et al., 2008). This mutant HCT116 cell line was able to suppress the effects of high concentrations of MLN8054 on Aurora B activity, making it possible to increase the concentration of MLN8054, and therefore to investigate whether more potent Aurora A inhibition was possible (Figures 7.2 and 7.3).

Surprisingly however, treating the mutant HCT116 cells with even 1 μM MLN8054 produced the profound occurrence of monopolar spindles in mitotic cells, although raising the concentration still further to 4 μM caused a further increase in the proportion of cells with shorter spindles (Figure 7.4). These observations were extremely similar to those of cells treated with an Eg5 inhibitor, as well as previous reports of the effects of removing the activity of Aurora A from cells (Figure 4.4A and 4.4B) (Liu and Ruderman, 2006, Giet and Prigent, 2000, Giet and Prigent, 2001, Glover et al., 1995, Hannak et al., 2001, Girdler et al., 2006). Whether the increased monopolarity of the HCT116 cells following MLN8054 treatment represents more potent Aurora A inhibition compared to the other cell lines tested however is up for debate, although Western blots indicated

that the Aurora A inhibition achieved throughout was equivalent in the cell lines tested (Figure 7.2). HCT116 cells may simply have a distinct balance of signalling pathways, causing them to be more dependent on Aurora A activity for bipolar spindle assembly.

Finally, although deductions could be made regarding the differing dependencies of non-transformed and cancer cells on Aurora A activity for the efficient formation of mitotic spindles (Chapter 8), no concrete conclusions could be made due to the use of only one non-transformed cell line. This limitation leaves the study open to the criticism that the differences observed between the two cell types may simply be the result of inter-cell line differences in the effect of Aurora A inhibition. Further work with multiple cell lines would have to be completed before any firm conclusions could be formed regarding the differences between transformed and non-transformed cells in response to Aurora A inhibition.

9.3 Aurora A activity is required for the development of normal mitotic spindle structures in cancer cell lines

Although relatively little was known regarding Aurora A activity at the beginning of this project, depletion and mutagenesis experiments had been used to indicate that Aurora A activity was required for the formation of a bipolar spindle, and without it mitotic cells were shown to predominantly exhibit monopolar spindles (Liu and Ruderman, 2006, Giet and Prigent, 2000, Giet and Prigent, 2001, Roghi et al., 1998, Marumoto et al., 2003, Glover et al., 1995, Hannak et al., 2001, Girdler et al., 2006). By using MLN8054 to potently inhibit Aurora A activity, I was able to demonstrate that although Aurora A activity was indeed required for the normal development of a mitotic spindle, some interesting differences were observed to occur between my data and the published literature.

9.3.1 Aurora A activity is not required for the formation of bipolar spindles in the majority of DLD-1 and HeLa cells

Western blotting of cell samples treated with 1 μM MLN8054 indicated that the treatment caused specific inhibition of Aurora A activity (Figure 3.3). Despite this, the majority of DLD-1 and HeLa cells were seen to form bipolar spindles during mitosis (Figure 3.4), which is in contrast with the finding that the removal of Aurora A activity produced the prominent occurrence of monopolar spindles in mitotic cells (Liu and Ruderman, 2006, Giet and Prigent, 2000, Giet and Prigent, 2001, Roghi et al., 1998, Marumoto et al., 2003, Glover et al., 1995, Hannak et al., 2001, Girdler et al.,

2006). This intriguing discrepancy provoked the closer inspection of the mitotic spindles formed in the absence of Aurora A activity, which revealed some interesting spindle abnormalities.

9.3.2 Aurora A activity is required to define spindle length

Although the majority of HeLa and DLD-1 cells appeared to have bipolar spindles following Aurora A inhibition, the number of long K-fibres was found to be reduced and the spindles were less capable of separating and maintaining centrosome separation (Figures 4.2, 4.3, 4.5 and 5.6). This shortening of inter-pole distance in the absence of Aurora A activity is intriguingly similar to reports describing shorter spindle lengths in cells where Aurora A is prevented from interacting with its activator TPX2 (Bird and Hyman, 2008). Similarly, the depletion of TPX2 in *C. elegans* embryos was also reported to lead to a reduction in spindle lengths, a phenomenon which was linked to causing a reduction in TPX2/Aurora A down spindle microtubules (Greenan et al., 2010). Indeed TPX2 has previously been determined to be required for both Aurora A localisation to the spindle microtubules and for activating and maintaining its Aurora A kinase activity (Eyers et al., 2003, Tsai et al., 2003, Kufer et al., 2002, Kufer et al., 2003, Bayliss et al., 2003, Gruss et al., 2001, Eyers and Maller, 2004). Interestingly, the stable association of Aurora A to the spindle was found to be reduced after treatment with MLN8054 (Figure 4.7A). This may indicate a direct role for the activity of Aurora A in its stable attachment to spindle microtubules. Alternatively the association of MLN8054 to Aurora A may negatively affect the formation of the TPX2/Aurora A complex, which may as a result hinder Aurora A recruitment to spindle microtubules in a TPX2-dependent way. Thus, both the direct inactivation of Aurora A activity in my experiments or the prevention of Aurora A from interacting with TXP2 appears to reduce the length of the mitotic spindle structure, highlighting the importance of Aurora A activity in the regulation of spindle formation.

9.3.3 Aurora A activity regulates the stability of spindle microtubules

The exact role of Aurora A on the spindle is as yet unknown, however Aurora A activity is required for the proper localisation of TACC3 to both the centrosomes and spindle microtubules (Figure 4.7B), a finding that is supported in the literature (Giet et al., 2002, Kinoshita et al., 2005, LeRoy et al., 2007, Peset et al., 2005, Mori et al., 2007). When the *Xenopus* TACC protein Maskin was prevented from localising to the spindle and centrosomes through its depletion in egg extracts, the resulting spindles were reported to be on average 30% smaller than control cells with markedly reduced microtubule nucleation (O'Brien et al., 2005, Kinoshita et al., 2005, Peset et al., 2005). Furthermore, the over-expression of TACC3, D-TACC or Maskin, has been shown to cause the increase in the level and length of spindle microtubules (Gergely et al., 2000b, Peset et al., 2005,

Lee et al., 2001). These findings therefore suggest that through regulating the localisation of TACC3, Aurora A activity may regulate microtubule length, thus providing an explanation as to why reduced K-fibre length and inter-pole distances were observed following MLN8054 treatment (Figures 4.2 and 4.3 and for model see Figure 9.1). The role for the TACC proteins in maintaining microtubule length is not thought to be derived from the increase of microtubule nucleation or centrosomal maturation, but by the stabilisation of the microtubules (reviewed in [Peset and Vernos, 2008]; (Peset et al., 2005, Bellanger and Gonczy, 2003)). Thus Aurora A activity may therefore regulate spindle length by increasing the protection of spindle microtubules from depolymerisation.

This method of microtubule stabilisation may occur through the regulation of the microtubule stabilising family of proteins. Indeed, TACC proteins have been found to interact with chTOG/XMAP215, and aid its localisation to the centrosome and spindle minus-ends, thus

countering the effects of the microtubule destabiliser MCAK (Bellanger and Gonczy, 2003, Srayko et al., 2003, Sato et al., 2004, Conte et al., 2003, Lee et al., 2001, Gergely et al., 2003, Barros et al., 2005). Interestingly, the depletion of chTOG/XMAP215 has been shown to shorten the length of spindles, thus producing effects similar to those seen after Aurora A inhibition (Figures 4.2 and 4.3) (Goshima et al., 2005b). Further supporting the link between Aurora A and the regulation of chTOG/XMAP215, the phosphorylation of TACC3 by Aurora A has been shown to stabilise microtubules by loading chTOG/XMAP215 to the minus-ends of centrosomal spindle fibres (Lee et al., 2001, Gergely et al., 2003, Barros et al., 2005, Kinoshita et al., 2001). Consistent with these findings, I found chTOG to be reduced at centrosomes after MLN8054 treatment in HeLa cells (Figures 4.7C and 4.7D). This level of regulation over microtubule stability may therefore explain why there are less long K-fibres following the inhibition of Aurora A (Figure 4.3 and for model see Figure 9.1)

Therefore Aurora A activity appears to regulate spindle formation through the protection of spindle microtubules from depolymerisation. Aurora A activity has however also been implicated in regulating microtubule stability by directly controlling the activity and localisation the microtubule depolymerisors Kif2a and MCAK. Through the delicate balancing of their action, these two microtubule depolymerisors have been shown to work together, enabling the formation of bipolar spindles (Ganem and Compton, 2004, Zhang et al., 2008). Indeed the depletion of proteins involved in microtubule deploymerisation has previously been reported to increase the length of spindles (Goshima et al., 2005b). Furthermore, yeast have has been shown to selectively depolymerise long microtubules rather than short ones, highlighting the requirement for microtubule depolymerisors to maintain the length of individual spindle fibres (Varga et al., 2006). Therefore, by regulating the intricate balancing of depolymerise activity and localisation to

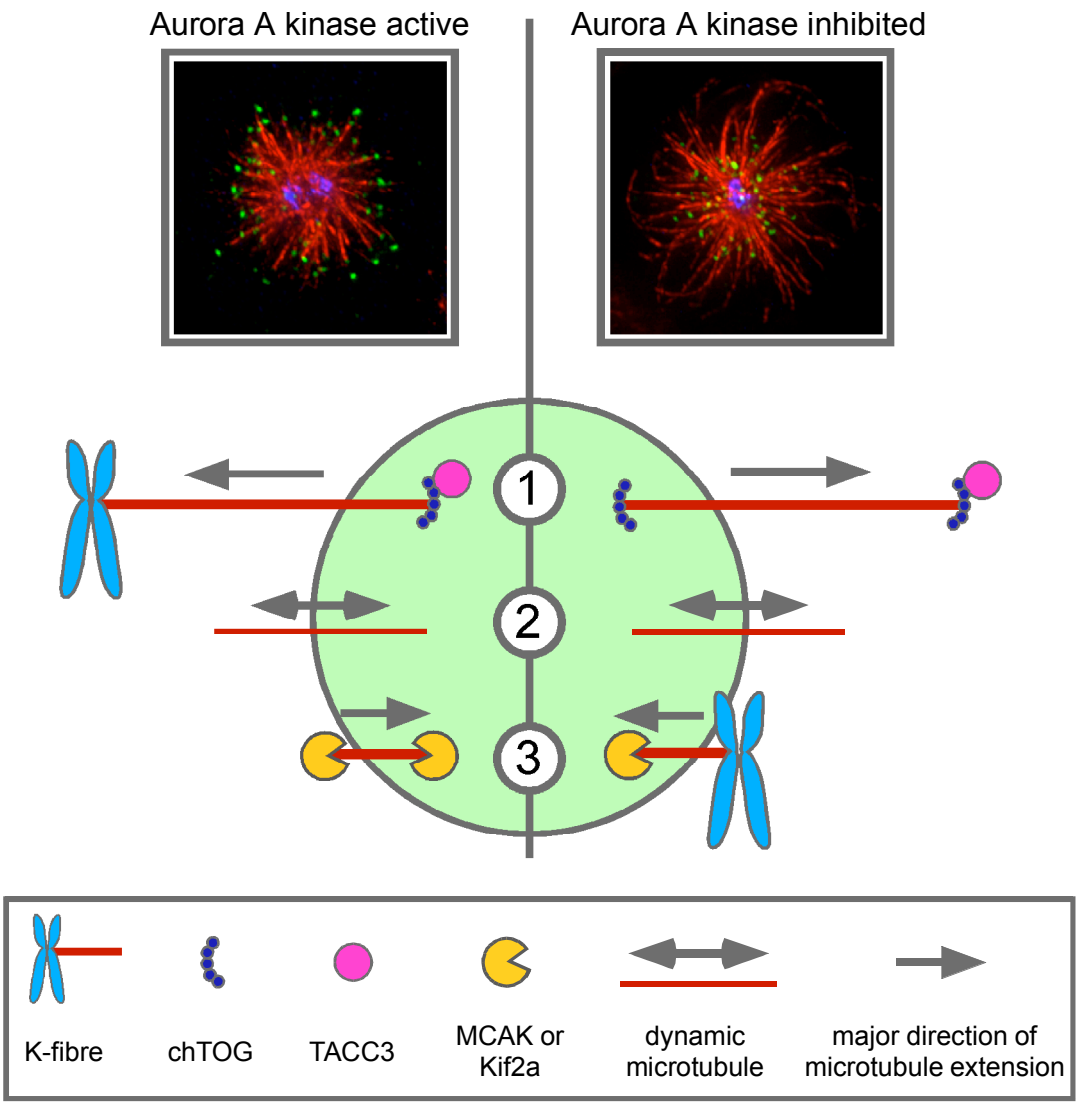

Figure 9.1 Model of changes in microtubule stability following Aurora A inhibition
In control cells and those experiencing Aurora A inhibition three major types of spindle microtubule were observed: 1) long and relatively stable, 2) dynamic, 3) short. Aurora A activity was found to be required to determine that chromosomes were attached to long and relatively stable microtubules, while unattached kinetochores were dynamic or destabilised. Without Aurora A activity, shortened K-fibres were observed, while long and apparently stable unattached microtubules were often also detected.

These changes observed after Aurora A inhibition were predicted to occur as a result of the misregulation of the delicate balancing of polymerisation and depolymerisation events within the cell. When Aurora A kinase activity is inhibited TACC3 is mislocalised from the centrosome (Figure 4.7B), which reduces the localisation and activity of chTOG/XMAP215 at the minus-ends of microtubules (Figure 4.7C) (Kinoshita et al., 2005). As a consequence, centrosomal microtubule growth and protection from MCAK may be reduced, causing the shortening of spindle fibres. Furthermore, due to their mislocalisation from the centrosomal area, TACC3 and chTOG/XMAP215 may become increasingly localised to the plus-ends of microtubules, and in the case of chTOG/XMAP215, also along the lengths of microtubules (Spittle et al., 2000; Lee et al., 2001). This may enable inappropriate microtubule protection and plus-end polymerisation, allowing the growth of abnormally long non-kinetochore microtubules. In addition, the inhibition of Aurora A may cause the over-activation of MCAK at early stages of mitosis (Zhang et al., 2008) and an increase of Kif2a on spindle microtubules (Jang et al., 2009). This misregulation help may also help explain the problems with bipolar spindle formation as well as the shorter microtubule length characteristic of cells depleted of Aurora A activity.

different areas of the spindle, Aurora A activity may control the length of spindle fibres (for model see figure 9.1). Kif2a is also implicated in chromosome congression, a process that was found to be disrupted following Aurora A inhibition (Figure 5.1B) (Jang et al., 2008, Jang et al., 2009). The requirement for Aurora A to regulate Kif2a function in chromosome congression may therefore provide an explanation to the reduced ability of cells to align their chromosomes following Aurora A inhibition (Figure 5.1B). During my investigation however, no difference could be detected in the localisation of the microtubule destabilisers Kif2a and MCAK following Aurora A inhibition (data not shown). I cannot however, rule out the possibility that Aurora A activity does wield some form of control, either over the function or localisation of microtubule depolymerases.

9.3.4 Aurora A activity is required for the proper maturation of the centrosomes

Aurora A activity may also control the formation of the spindle structure via its role in centrosome maturation (Berdnik and Knoblich, 2002, Hannak et al., 2001, Hirota et al., 2003, Terada et al., 2003, Giet et al., 2002, Barros et al., 2005). This relationship with the regulation of spindle formation has been attributed to both the nucleation capacity of the matured centrosomes and the determination of microtubule length by contributing to centrosome size (Greenan et al., 2010, Bird and Hyman, 2008, Berdnik and Knoblich, 2002, Hannak et al., 2001, Hirota et al., 2003, Terada et al., 2003, Giet et al., 2002, Barros et al., 2005). Consistent with the control of centrosome maturation by Aurora A activity, I showed that MLN8054 treatment caused a reduction in the localisation of various centrosomal components in HeLa cells (Figure 4.7), while also causing a slight reduction in centrosomal size (Figures 4.1 and 4.7). Interestingly however, such dramatic mislocalisation of centrosomal components was not seen in non-transformed cells, and neither was there an obvious reduction in centrosome size following Aurora A inhibition (Figure 8.6). This difference in the two cell lines is consistent with their differing abilities to form a bipolar spindles capable of efficiently aligning chromosomes, highlighting the importance of proper centrosome maturation in proper spindle formation (Figures 5.1 and 8.3).

Additionally, the role of Aurora A activity in centrosome maturation may also contribute to the determination of spindle length through the production of astral microtubules. Aurora A activity and its regulation of TACC proteins have been attributed to the efficient nucleation and stabilisation of astral microtubules (Giet et al., 2002, Motegi et al., 2006, Srayko et al., 2003). The importance of their presence is seen in early mitosis where even before the creation of a spindle centrosomes have been shown to move apart, which is proposed to occur through the nucleation of astral microtubules (Waters et al., 1993, Rosenblatt et al., 2004). Astral microtubules have also been found to act as spindle tethers, contributing to the spindle positioning process with in the cell (Thery et al., 2007). The dependency on Aurora A activity for the existence of astral microtubules,

may therefore contribute to the reduction inter-pole distances seen after MLN8054 treatment (Figure 4.2).

Aurora A has also been implicated in centriole separation, with the injection of anti-Aurora A antibodies or the use of MLN8054 appearing to prevent the separation of centriole pairs (Marumoto et al., 2003, Hoar et al., 2007). Despite the reported defects in centriole separation, most cells were capable of organising more than one spindle pole, although some spindle poles were observed to be lacking NuMA or Centrin 3. This may therefore further demonstrate the role of the Aurora A kinase activity in the regulation of centrosome development and function, and may subsequently help to explain the abnormal spindle assembly observed following Aurora A inhibition.

9.3.5 Aurora A activity may regulate microtubule antiparallel sliding

In addition to controlling spindle formation via centrosomal maturation, Aurora A activity may also control spindle length through regulating the antiparallel sliding of its microtubules. Indeed, Aurora A has been shown to interact with and phosphorylate the plus-end directed motor protein Eg5, which has been reported to be required for bipolar spindle formation (Giet et al., 1999, Giet and Prigent, 2000, Koffa et al., 2006, Mayer et al., 1999). The prominent role of Eg5 in bipolar spindle formation is highlighted by the observation that Eg5 inhibition causes a severe monopolar spindle phenotype (Figures 4.4A and 4.4B). Such a dramatic effect to spindle polarity was however not seen in the majority of cells following Aurora A inhibition during this investigation (Figures 3.4, 8.3C and 8.3D), suggesting that other kinases may also be able to activate Eg5. Importantly however, Aurora A inhibition in HCT116 cells did cause a severe monopolar spindle phenotype (Figure 7.4), which could demonstrate the lack of possible alternative Eg5 activating pathways in the cell line. Alternatively, HCT116 cells may be the only cell line where complete Aurora A inhibition was achieved, therefore residual Aurora A activity in the other cell lines may be sufficient to maintain the activity of Eg5. This is however unlikely, as Western blot data indicated that equivalent Aurora A inhibition was achieved by MLN8054 in all of the cell lines tested, as determined by phosphor-Aurora A signal (Figures 3.3, 7.2 and 8.1).

9.3.6 Aurora A activity is required for the proper organisation of the spindle

One of the most notable features of HeLa cell spindles treated with MLN8054 was that they were decidedly less organised than those of control cells (Figures 4.1 and 5.3). During late prometaphase to metaphase, control cells displayed robust and clearly visible K-fibres, which formed bipolar spindles with a canonical structure and neatly aligned chromosomes (Figure 4.1A). In comparison,

MLN8054-treated HeLa cells exhibited poorly defined K-fibres which formed an unfocused mass of microtubules (Figures 4.1B, 4.1D, 5.3C and 5.3D). Furthermore, although the kinetochores within these cells appeared to be attached to spindle microtubules, the chromosomes were often misaligned, forming broader metaphase plates with some chromosomes pulled closely into the poles. These structural abnormalities formed in the absence of Aurora A activity demonstrate that the activity is required not only for spindle length determination, but also for microtubule organisation. These roles for Aurora A activity may also stem from its function in bringing about the proper maturation of centrosomes, a process which promotes both efficient microtubule nucleation and importantly microtubule organisation (Berdnik and Knoblich, 2002, Hannak et al., 2001, Hirota et al., 2003, Terada et al., 2003, Giet et al., 2002, Barros et al., 2005, Greenan et al., 2010).

9.3.7 Aurora A activity regulates the length of non-kinetochore microtubules

In addition to Aurora A inhibition causing the shortening and disorganisation of spindles, abnormally long non-kinetochore microtubules were often observed to protrude from the spindle structure of MLN8054-treated HeLa cells (Figures 4.1D and 5.3D). The disruption of the highly regulated and delicately balanced action of microtubule stabilisers and depolymerisors may go some way in explaining the occurrence of these abnormal fibres. In untreated cells, the balancing of these forces usually enables the formation of exceedingly dynamic yet structurally constant mitotic spindles. Therefore, the deregulation of this process may lead to the stabilisation of microtubules that are normally dynamic, and the reduction in the stability of spindle fibres that are typically stable (for model see Figure 9.1). The shortening of the relatively stable K-fibres following Aurora A inhibition demonstrates that the latter of these two cases is true (Figure 4.3), with the mislocalisation of chTOG also indicating the mislocalisation of microtubule stabilisers (Figures 4.7C and 4.7D). The mislocalisation of microtubule stabilising factors from the minus-ends of microtubules after Aurora A inhibition may produce a more disperse localisation throughout the cell allowing the stabilising factors to abnormally localise or increase their localisation to various positions throughout the cell. Indeed, both chTOG/XMAP215 and TACC proteins have been found to be associated with microtubule plus-ends, a situation which may be heightened after Aurora A activity is prevented from targeting them to the centrosomes (Figure 4.7B) (Lee et al., 2001, Tournebize et al., 2000, Kinoshita et al., 2001). Furthermore, the role of Aurora A activity in regulating MCAK may also contribute to the presence of abnormal long microtubules, as MCAK RNAi has been shown to increase the proportion of astral microtubules (Zhang et al., 2008, Rankin and Wordeman, 2010). Situations such as these may therefore cause the production and

stabilisation of long non-kinetochore microtubules while reducing the length and stability of K-fibres, similar to the situation seen observed after Aurora A inhibition (Figure 9.1).

9.4 Aurora A activity is required for the maintenance of genomic stability

Despite the spindle abnormalities seen after Aurora A inhibition, cells were still capable of dividing, although they soon lost viability, highlighting the importance of the kinase in the maintenance of genomic stability (Figures 6.1, 6.2 and 6.3).

9.4.1 The efficient alignment of chromosomes requires active Aurora A

Although Aurora A inhibition did not create the potent monopolar spindle phenotype as described in the literature in the majority of cell lines tested, Aurora A activity was nonetheless found to have a prominent role in the formation of a normal spindle (Figure 4.1). Despite the clearly abnormal spindles produced by inhibiting Aurora A activity however, the K-fibres within these spindles were shown to attach to the kinetochores and generate a degree of force to the chromosomes (Figures 5.3 and 5.4). This K-fibre attachment was marked by a decrease in Mad2 staining at kinetochores, indicating equivalent if not slightly increased microtubule attachment compared to control cells. This slight increase may be related to the shortened K-fibres of the cells causing their kinetochores to be placed closer towards the microtubule rich centrosomal area, thus making them more available for microtubule attachment. Alternatively, the slight increase in microtubule attachment may simply be the result of the disorganised nature of spindles produced in the absence of Aurora A activity.

Interestingly however, the centromere stretch of chromosomes within cells exposed to MLN8054 treatment was greater than those in cells exposed to Nocodazole, but significantly less than observed in control cells (Figure 5.4C). This reduction in centromere stretch is most likely to be derived from the reduction in length and level of organisation of spindles formed in the absence of Aurora A activity (Figures 5.3D and 5.4D). The ability of K-fibres to form apparently stable attachments to the kinetochores despite the relatively low levels of centromere stretch, suggests that the inter-kinetochore tension applied by the spindle was adequate to prevent microtubule destabilisation by tension sensing pathways (for review see [Santaguida and Musacchio, 2009, Maresca and Salmon, 2010]; (King and Nicklas, 2000, Kapoor et al., 2000, Waters et al., 1998)). It is highly likely that the low levels of force generated by the structurally disorganised spindle following the inhibition of Aurora A, prevented the affected cells from efficiently aligning their chromosomes (Figure 5.1B).

9.4.2 Aurora A activity is required to prevent segregation errors

The inability of cells to efficiently align their chromosomes in the absence of Aurora A activity was found to cause significant delays in mitosis (Figures 6.1, 6.2A, 7.5 and 8.2). Live cell analysis later showed that after prolonged mitotic delay, HeLa cells were observed to suffer from segregation errors and decondense their chromatin without first dividing (Figure 6.2B). In light of these observations, it was unsurprising to learn that long–term Aurora A inhibition caused the development of aneuploidy as well as causing a substantial increase in the sub-G1 peak in flow cytometry PI profiles of HeLa cells (Figure 6.3A(ii)). Colony formation assays also demonstrated that HeLa cells exposed to long-term Aurora A inhibition eventually lost viability, demonstrating the degenerative effect of the segregation errors (Figures 6.3B and 6.3C). The occurrence of such segregation defects and the consequential development of aneuploidy have been attributed to the dependency on Aurora A activity to maintain the activation of the spindle assembly checkpoint (Wysong et al., 2009, Kaestner et al., 2009). This did not however appear to be the case, as cells were arrested in mitosis for significant periods after Aurora A inhibition (Figures 6.1, 7.6 and 8.2C). Therefore cells must instead be able to satisfy the spindle assembly checkpoint despite the presence of incorrectly attached and aligned chromosomes.

This inappropriate satisfaction of the spindle checkpoint despite misaligned chromosomes may be derived from the disorganised nature of the spindles formed in the absence of Aurora A activity (Figures 4.1B and 5.3D). Their abnormal construction and disorganised nature may permit the generation of sufficient tension across kinetochores to satisfy the spindle checkpoint, despite the unaligned state of the chromosomes (Li and Nicklas, 1995, Nicklas et al., 1998, Nicklas et al., 1995, Skoufias et al., 2001, Biggins and Murray, 2001, Stern and Murray, 2001). Alternatively, cells may be able to satisfy the spindle checkpoint despite low levels of centromere stretch, relying only on the levels of microtubule attachment at kinetochores (Figures 5.4B and 5.4C). The concept that a low level of tension does not sustain checkpoint activation has been supported by a range of reports, which range from using laser ablation, to exploiting replication defects to support their argument (Dewar et al., 2004, O'Connell et al., 2008, Rieder et al., 1995). Despite the compelling evidence in support of this theory however, it is difficult to apply it to the situation seen after Aurora A inhibition. This difficulty stems from the observation that spindles appear able to relatively efficiently attach to kinetochores in the absence of Aurora A activity (Figure 5.4B), although the cells still experience extended mitotic delays following the treatment (Figure 8.2C). Indeed, the only divergence from the control cell phenotype appears to be the reduced centromere stretch, suggesting that this may maintain checkpoint activation, thus causing the extended mitotic delays.

Despite the abnormal spindles and chromosome alignment defects, the majority of cells were able to divide following Aurora A inhibition (Figures 6.1 and 6.2). Indeed, even when a cell population was seen to be predominantly monopolar following Aurora A inhibition, unlike with Eg5 inhibition, cells were still able to divide (Figure 7.5B). This unexpected finding was predicted to be due to the cells eventually being capable of forming adequate spindles even in the absence of Aurora A activity. Indeed, the observation of live HeLa cell data revealed the sequential collapsing and reformation of the mitotic spindle after Aurora A inhibition (data not shown). This observation indicates that although bipolar spindle assembly was possible, the weakened spindle structure formed in the absence of Aurora A activity may make it impossible for cells to continually maintain their bipolarity. If the HCT116 cells observed to be mostly monopolar following Aurora A inhibition spent more time with their spindles in the collapsed state, this would explain the prominent occurrence of monopolar spindles and relatively few bipolar spindles observed in the fixed cell samples (Figure 7.4). The ability to form unstable bipolar spindles would therefore also help explain how the cells are able to divide despite spindle defects produced in the absence of Aurora A activity.

9.5 Aurora A activity is not required for regulation of spindle length in non-transformed cells

Cancer cells such as HeLa and HCT116 lines are commonly used in laboratories, due to their ease of use and extensively studied profiles. However, to build a more universal understanding of the role of Aurora A kinase activity, I used a non-transformed RPE cell line that had been immortalised through the expression of hTERT. The requirement to investigate the role of Aurora A in non-transformed cells was further supported by the observation that they responded differently to anti-mitotic drugs compared to transformed cell lines, indicating the same may be true for the effects of inhibiting Aurora A activity (Orth et al., 2008, Brito et al., 2008, Gascoigne and Taylor, 2008).

Indeed, comparing Aurora A inhibition in non-transformed cells and cancer lines revealed some interesting differences. One of the most profound differences observed was the ability of RPE cells to form bipolar spindles which were not significantly reduced in length when compared with control cells (Figures 8.3C and 8.3D). Furthermore, the non-transformed cell line was also more able to align its chromosomes in the absence of Aurora A activity compared to cancer cells (Figures 8.3B and 8.4). The comparative efficiency of these processes in the absence of Aurora A activity meant that non-transformed cells only suffered relatively small mitotic delays after the treatment (Figure 8.2). Furthermore, after extended Aurora A inhibition, RPE cells accumulated

with 2N DNA and were able to form sparsely populated colonies (Figure 8.5). In contrast, HeLa cells quickly became aneuploid and lost viability following the same treatment (Figure 6.3).

9.5.1 Non-transformed RPE cells may be able to maintain signalling pathways down-stream of Aurora A in the absence of Aurora A activity

The ability of RPE cells to maintain bipolar spindle formation after the same level of Aurora A inhibition experienced as the cancer lines may be due to their capacity to either sustain the activity of particular cellular pathways in the absence of Aurora A activity, or exploit alternatives, whereas the cancer cells may be less able to do so. The existence of such a system may be demonstrated by the presence of phospho-TACC3 even in RPE cells treated with 10 μM MLN8054, despite the loss of phospho-Aurora A (Figure 8.1). Interestingly, this situation was not observed in HeLa or DLD-1 cells (Figures 3.3 and 8.1), and may indicate a method by which RPE cells retain the activation of TACC3 even after Aurora A inhibition. Similarly, although Aurora B activity appeared to be inhibited to equivalent levels by 1 μM MLN8054 in both the transformed and non-transformed cell lines, as judged by the reduction of phospho-Aurora B signal, the phospho-histone H3 signal was only reduced at much higher concentrations in the non-transformed cells. The ability of RPE cells to retain the activation of downstream kinases even in the absence of the upstream Aurora kinase activity, could therefore demonstrate the presence of redundant pathways which may not be present in the cancer cell lines tested.

Furthermore, in RPE cells chTOG and TACC3 were efficiently localised to centrosomes, and TACC3 maintained a relatively high degree of spindle localisation after MLN8054 treatment, which is in contrast to HeLa cells which were completely unable to localise TACC3 and showed reduced localisation of chTOG (Figures 4.7 and 8.6). Both chTOG/XMAP215 and TACC3 have been linked with the normal growth of spindles, and their sustained localisation after Aurora A inhibition in RPE cells could therefore explain how RPE cells are able to form spindles of a relatively normal length and organisation after the MLN8054 treatment (Barros et al., 2005, Giet et al., 2002, Kinoshita et al., 2005, Peset et al., 2005, Lee et al., 2001, Gergely et al., 2003). HeLa cells which may not be able to retain the activation of Aurora A-driven signalling pathways may not consequently be able to sustain the localisation of cellular components that are essential for the development of normal spindles, such as TACC3 and chTOG.

Although both chTOG and TACC3 maintained a relatively constant localisation after Aurora A inhibition in RPE cells, the stable localisation of Aurora A to the spindle microtubules appeared to be reduced after the treatment in a similar way to that seen in HeLa cells (Figures 4.7A and 8.6A). This similarity in the effect of MLN8054 on Aurora A localisation, together with the equivalent

loss of the phospho-Aurora A in Western blot samples (Figure 8.1), indicates that Aurora A activity was indeed inhibited to a comparable levels by the MLN8054 in HeLa and RPE cells. Despite the Western blot evidence, it could however be argued that RPE cells are somehow more resistant to the inhibitory effects of MLN8054, thus providing an alternative theory as to how RPE cells were able to more efficiently form functional bipolar spindles than the cancer cells tested.

9.5.2 In the absence of Aurora A activity RPE cells show signs of senescence

In addition to showing a differing ability to form bipolar spindles following Aurora A inhibition, RPE cells exposed to long-term Aurora A inhibition did not develop a sub-G1 population (Figure 8.5A). Instead they accumulated with a 2N DNA content and formed sparsely populated colonies (Figures 8.5B and 8.5C). In contrast, HeLa cells quickly became aneuploid and became unviable following the treatment (Figure 6.3). Consistent with these observations, MLN8054 has recently been shown to cause a higher proportion of cell death in transformed cells compared to non-transformed cell lines (Shang et al., 2009).

The tendency of RPE cells to accumulate with 2N DNA in response to long-term Aurora A inhibition, may indicate that senescence is triggered. Senescence is a state in which cells are no longer permitted to proliferate, and are instead usually held in G0 (for review see [Reddel, 2010]). The induction of senescence is a stress response, and has been found to be triggered by a range of situations including (Chen et al., 2009, Collado et al., 2007, Collado and Serrano, 2010, Mo et al., 2007, Young et al., 2008, Courtois-Cox et al., 2006). Interestingly, Aurora A over-expression has been suggested to extend the life-span of cells by increasing the expression of the human telomerase reverse transcriptase (hTERT) and activating telomerase activity (Yang et al., 2004). It may therefore be a possibility that the inhibition of Aurora A has the reverse effect, and causes the shorting of telomeres, thus providing a mechanism by which senescence may be triggered after Aurora A inhibition. The exact conditions by which senescence is triggered, is however dependent on the cell type and the culture conditions. Factors which cause increased senescence in some cell lines lead to increased apoptosis in others, and it may therefore be possible for MLN8054 treatment to have alternate effects in different cell lines (Ventura et al., 2007, Xue et al., 2007).

Senescent cells are prevented from further proliferation and can be cleared from the body by the immune system, which can result in the regression of tumours (Xue et al., 2007, Braig et al., 2005, Collado and Serrano, 2010). Indeed, senescence is widely regarded as a barrier to tumour formation, as markers for it are often found on pre-malignant tumours, but are absent in their malignant states, and senescence markers in biopsies are linked with the success of chemotherapy treatment (Chen et al., 2005b, Collado and Serrano, 2006, Gray-Schopfer et al., 2006, Roberson et

al., 2005, te Poele et al., 2002). Chemotherapies designed to induce senescence when used may even represent a valuable therapy, either on their own or in combination with additional treatments (Schmitt et al., 2002). Therefore, the observation of possible senescence in the immortalised RPE cells following Aurora A inhibition may indicate a method of targeting the abnormally cycling cells and removing them from the body. Indeed, although RPE cells are a non-transformed cell line, their immortalisation may mimic benign tumour growth. The ability of MLN8054 to induce a senescent response may therefore indicate an important method of anti-cancer therapy.

The differences highlighted between transformed and non-transformed cells, as well as the observed variations in response between the cancer lines, demonstrate the danger of assuming commonality between distinct cell lines. However, the high occurrence of cell death in cancer cell lines in response to MLN8054 treatment is encouraging. Consistent with *in vivo* reports, it suggests that targeting the kinase activity of Aurora A could be a successful anti-cancer therapy (Shang et al., 2009, LeRoy et al., 2007, Manfredi et al., 2007, Kaestner et al., 2009, Dar et al., 2008, Hoar et al., 2007).

9.6 Conclusion and the future targeting of Aurora A

Throughout this thesis I have shown that in HeLa, DLD-1 and HCT116 cells, Aurora A activity is required for the formation of a functional spindle able to efficiently align its chromosomes and the effective detection of alignment defects. As a consequence, when Aurora A is inhibited, segregation errors are produced in the cancer cell line tested causing the cell population to eventually become unviable. Despite this prominent role for Aurora A activity in mitosis, I have shown that cells can divide and activate the spindle assembly checkpoint in its absence, showing that Aurora A activity is not required for cytokinesis and does not play a prominent role in the checkpoint. Interestingly however, the effects of Aurora A inhibition on non-transformed cells were not as profound as those observed in the cancer cell lines, with cells being able to retain their ability to form spindles and align their chromosomes relatively efficiently when compared to cancer cell lines.

Therefore, in addition to highlighting the importance of Aurora A activity in the regulation of spindle formation in many cell lines, this work also demonstrates the existence of important inter-cell line differences, not just between non-transformed cells and cancer lines, but also between all the cell lines tested. This observation has important connotations with regards to making broad generalisations from the characteristics of just one cell type. Furthermore, the observation that cancer cells became aneuploid after Aurora A inhibition, while non-transformed cells were able to sustain relatively efficient chromosome alignment, indicate that inhibiting Aurora A activity may

be an efficient method of specifically targeting cancer cells within patients. Indeed, early success in both laboratory tests and clinical trials indicates that Aurora A activity is a promising anti-cancer target (Shang et al., 2009, LeRoy et al., 2007, Manfredi et al., 2007, Kaestner et al., 2009, Dar et al., 2008, Hoar et al., 2007). Despite these promising results, the success of therapies such as Aurora A inhibition will rely on the proper appreciation of their effects in a patient and tumour-specific basis, so as to enable the intelligent application of the appropriate therapy or combination of treatments. Therefore, clinical approaches to treating cancer in patients will be increasingly reliant on findings such as those detailed in this report in order to determine the most efficient methods of cancer treatment.

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