Mitotic catastrophe: the role of chromosome-binding proteins and DNA damage

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MITOTIC CATASTROPHE: THE ROLE OF CHROMOSOME-BINDING PROTEINS AND DNA DAMAGE

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A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2010

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LIST OF ABBREVIATIONS

3F3/2	tension sensing kinetochore phosphoepitope
ACA	anti centromeric antigen
ActD	actinomycin D
AIM-1	Aurora B kinase
AP-1	activator protein 1
Apaf-1	apoptotic protease activating factor 1
APC/C	anaphase promoting complex/cyclosome
ATM	ataxia-telangiectasia mutated
ATR	ataxia- telangiectasia and Rad3-related
ATRIP	ATR interacting protein
Bad	Bcl-XL/Bcl-2-associated death promoter
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell Lymphoma 2
Bcl-XL	B-cell leukemia XL
BH2	Bcl-2 homology
Bid	BH3-interacting domain death agonist
Bik	pro-apoptotic Bcl-2 family member
Bim	Bcl-2 interacting mediator of cell death
BIR	Baculoviral IAP Repeats
Bok	Bcl-2-related ovarian killer
BRCA1	breast cancer 1

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BSA	bovine serum albumin
Bub	budding uninhibited by benzimidazole
BubR1	Bub Receptor 1
CAD/DFF40	caspase activated DNase/DNA Fragmentation Factor 40
Caly A	Calyculin A
Caspase	cysteine aspartic acid protease
Cdc20	cell division cycle 20homologue
Cdc25A	cell division cycle 25 homologue A
Cdc42	cell division cycle homologue 42
CDK	cyclin-dependent kinase
CENP-A	centromeric protein A
CENP-E	centromeric protein E
CENP-F	centromeric protein F
CENP-I	centromeric protein I
c-FLIP	cellular FLICE-inhibitory protein
CFP	cyan fluorescent protein
Chk2	checkpoint kinase 2
CLASP	cytoplasmic linker-associated protein
CO ₂	carbon dioxide
CPC	chromosome passenger complex
DAPI	4'6-diamidino-2-phenylindole
ddH ₂ O	distilled deionized water
DDR	DNA damage response

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DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	double stranded break
dsDNA	double stranded DNA
ECL	enhanced chemiluminescence
E.coli	Escherichia coli
Eg2	Aurora A kinase
ERK	extracellular signal-related kinase
FANCD2	Fanconi anemia, complementation group D 2
FasL	Fas ligand
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FLIP	Fluorescence Loss In Photobleaching
FRAP	Fluorescence Recovery After Photobleaching
FRET	Fluorescence Resonance Energy Transfer
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate
HeLa	Henrietta Lacks
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis protein

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ICAD/DFF45 45	inhibitor of caspase activated DNase/DNA fragmentation factor
INCENP	inner centromere protein
JNK/SAPK	c-jun N terminal kinase/ Stress activated kinase
KCl	potassium chloride
KH ₂ PO ₄	potassium dihydrogen phosphate
LB	Luria Bertani
Mad2	mitotic arrest deficient
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MCAK	microtubule-destabilizing Kin 1 kinesin
MCC	mitotic checkpoint complex
Mcf-1	Makes caterpillars floppy 1
Mcd1	mediator of DNA damage checkpoint 1
MEK	MAPK or ERK kinase
MKK4	mitogen-activated protein kinase kinase 4
MKK7	mitogen-activated protein kinase kinase 7
MPF	M phase promoting factor
Mps1	mono spindle 1
Mre11	meiotic recombination 11
MRN	Mre11-Rad50-NBS1
MT	microtubule

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NaCl	sodium chloride
Na ₂ HPO ₄	sodium phosphate
NBS1	Nijmegen breakage syndrome 1
Nuf2	Nuclear Filament-containing protein
Ndc80	Nuclear division cycle 80 (yeast)
Ndc80/Hec1	Nuf2-Ndc80 complex/highly expressed in cancer
Noc	nocodazole
NPC	nuclear pore complex
NUP	nucleoporin
NES	nuclear export signal
NLS	nuclear localization signal
NuMA	nuclear mitotic apparatus protein
P53	tumour protein 53
PARP	poly (ADP-ribosyl)polymerase
PBS	phosphate-buffered saline
Pen-strep	penicillin-streptomycin
pH3	phosphorylated histone H3
PI3K	Phosphatidyl inositol 3-kinase-like serine/threonine protein kinases
pJNK	phosphorylated c-jun N terminal kinase
PLK1	polo like kinase 1 (Drosophila)
PLX	polo like kinase (Xenopus)
PP1	protein phosphatase 1

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PP2A	protein phosphatase 2A
Raf	protein kinase in MAPK/ERK pathway
Rad17	radiation absorbed dose 17
Rad50	radiation absorbed dose 50
Ran	ras-related nuclear protein
RanGAP1	Ran GTPase-Activating Protein 1
RanBP2	Ran Binding Protein 2
RCC1	regulator of chromosome condensation-1
RNAi	RNA interference
Rod	rough deal, binding partner of Zw10
ROI	region of interest
RPA	replication protein A
SAF	spindle assembly factor
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ser/thr	serine/threonine
siRNA	small interfering ribonucleic acid
SMC1	structural maintenance of chromosome 1
SPF	S phase promoting factor
SQE	serine-glutamine-glutamate motif
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TNFR	tumour necrosis factor receptor
TopBP1	human DNA topoisomerase II binding protein 1

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TPX2	targeting protein for Xklp2
TRAIL	tumor necrosis (TNF) related apoptosis inducing ligand
UbcH10	Ubiquitin-conjugating Enzyme E2 C
USP44	ubiquitin specific peptidase 44
UV	ultraviolet
UVB	ultraviolet B
VEGF	vascular endothelial growth factor
YIC	YFP-Importin β binding domain (IBB)-CFP fusion
YFP	yellow fluorescent protein
Zw10	zeste-white 10 protein

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ABSTRACT

Mitotic catastrophe is a phenomenon displayed by cells that undergo aberrant mitosis so that cells that fail to repair the errors would be eliminated. Currently, the mechanisms involved in this form of cell death remain poorly understood. Why and how mitotic catastrophe would lead to cell death remains to be resolved and the answer will prove valuable in design of better therapeutic agents that specifically target such cells in mitosis.

We found that actinomycin D induces mitotic catastrophe with severe spindle assembly defects. We have studied the nature of three groups of chromosome binding proteins in mitotic cells treated with actinomycin D. We found that actinomycin D reduced the binding affinity of RCC1 to the mitotic chromosome, which led to a reduction of RanGTP level. In addition, Mad2 was not concentrated at the kinetochores, indicating that the mitotic spindle checkpoint was affected. Furthermore, the localization of survivin was altered in cells. These data suggested that chromosomal binding of the mitotic regulators such as RCC1, Mad2 and survivin is essential for mitotic progression. Mitotic chromosomes not only carry the genetic material needed for the newly synthesized daughter cells, but also serve as docking sites for some of the mitotic regulators. Perturbation of their binding to the mitotic chromosome by actinomycin D could affect their functions in regulating mitotic progression thus leading to severe spindle defects and mitotic catastrophe.

The antibiotic actinomycin D has also been shown to induce chromosomal lesions in lower order organisms as well as in human interphase cells. However, relatively few studies have been conducted to elucidate molecular events in the context of mitotic DNA damage. Here, we show that actinomycin D introduced cellular stress via DNA damage during mitosis. The higher order packing of chromosomes during mitosis might impede efficient DNA repair. γ H2AX serves as a marker for DNA repair and active JNK interacts with γ H2AX in actinomycin D treated mitotic extracts. We believe JNK might be in part, responsible for the phosphorylation of H2AX and thereby, facilitate the propagation of a positive signal for cell death, when repair is not achieved. The mitotic cell activates JNK mediated cell death response that progresses through a caspase cascade downstream of the mitochondria. In the mean time, remaining checkpoint signals might be sufficient to put a restraining hand on entry into anaphase and the cell eventually dies in mitosis.

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1. Introduction

1.1 The cell cycle

The cell cycle refers to the sequence of events that takes place in a cell between divisions and is composed of 4 distinct phases: G_1 phase, S phase, G_2 phase, and M phase (Figure 1). Interphase is a collective term that encompasses the G_1 , S, and G_2 phases. The G_1 (G refers to 'gap' or 'growth') phase have $2N$ genetic material and biosynthetic activities which are halted during the previous M phase resume in preparation for subsequent divisions as well as to provide cellular fuel for growth. Deoxyribonucleic acid (DNA) synthesis commences in the S phase. This is when genetic content is doubled for later distribution into daughter cells. As such, faithful replication is of paramount importance to maintaining genomic stability. A normal cell contains duplicated sets of chromosomes in the form of sister chromatid pairs as S phase goes to completion. The G_2 phase lasts until the cell enters mitosis. Normal cellular metabolism, growth, and differentiation occur during G_2 . The M phase is composed of two coupled processes called mitosis and cytokinesis. In mitosis, the genetic material, in the form of chromosomes, is partitioned between the 2 daughter cells. This process is aptly named karyokinesis, which literally translates to division of the nucleus. The cytoplasm physically divides in cytokinesis that follows, forming the 2 daughter cells.

Chromosome condensation is the earliest visible phenomenon in M phase. Segregation

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of the replicated chromosomes requires the machinery in the form of the mitotic spindle. Assembly of the bipolar mitotic spindle is a process involving microtubules and various microtubules associated proteins and microtubule-dependent motor proteins. The centrosome is the principal microtubule-organizing centre in animal cells and centrosome replication occurs in interphase prior to entry into mitosis.

Five distinct stages – prophase, prometaphase, metaphase, anaphase and telophase constitute the M phase and occur in strict sequential order (Figure 2). The spindle starts to form outside the nucleus during prophase. The interphase microtubule array is converted into shorter and more dynamic microtubules surrounding each centrosome. In animal cells, each of the replicated centrosomes nucleates its own array of microtubules and later, the two sets of microtubules interact to form the mitotic spindle. After nuclear envelope breakdown at prometaphase, the spindle microtubules come in contact with the replicated chromosomes and through a search-and-capture mechanism, which eventually result in the sister chromatids being aligned at the metaphase plate. At anaphase, separation of sister chromatids occur and the spindle elongates, pushing the chromosomes poleward. This action continues during telophase and the chromosomes arriving at the poles are released from the spindle microtubules and the nuclear envelope reforms around them. Cytokinesis begins in anaphase and is completed by the end of telophase.

Figure 1. Cell cycle phases. The cell cycle is composed of 4 distinct phases: G₁ phase, S phase, G₂ phase, and M phase. Interphase is a collective term that encompasses the G₁, S, and G₂ phases. Biosynthetic activities which were suspended during the previous M phase resume in G₁ phase in preparation for subsequent divisions and to provide cellular fuel for growth. DNA synthesis occurs in the S phase. Normal cellular metabolic processes continue during G₂ to prepare the cell for mitosis. In mitosis, chromosomes are divided equally between the 2 daughter cells and this is followed by cytokinesis to form 2 daughter cells. Five distinct stages – prophase, prometaphase, metaphase, anaphase and telophase constitute the M phase (adapted from <http://www.le.ac.uk/ge/genie/vgec/he/cellcycle.html>).

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Figure 2. Mitotic phases. Centrosome duplication occurs outside the nucleus during G₂ phase, giving rise to a pair of centriole, which will later form asters to organize an early mitotic spindle. The chromatin begins to condense during prophase. The nuclear membrane disintegrates during prometaphase and spindle microtubules attach to kinetochores on the sister chromatids which eventually result in the chromosomes being aligned at the metaphase plate. Following which, sister chromatids separate during anaphase. Kinetochore microtubules shorten and the spindle elongates, resulting in the poleward movement of the 2 sets of chromosomes. Chromosomes arriving at the poles are released from spindle microtubules and the nuclear envelopes reforms around them. Cytokinesis completes cell division as 2 daughter cells are formed at the end of the M phase (adapted from http://kvhs.nbed.nb.ca/gallant/biology/mitosis_phases.jpg).

Many a time, a cell might leave the cell cycle. Cells that have temporarily stopped dividing are said to have entered a quiescent state called G_0 phase. Given the proper stimulation though, these cells are fully capable of re-entering the cell cycle. Often G_0 cells are terminally differentiated: they will never reenter the cell cycle but instead will carry out their function in the organism until they die. G_0 represents not simply the absence of signals for mitosis but an active repression of the genes needed for mitosis. Cancer cells cannot enter G_0 and are destined to repeat the cell cycle indefinitely. Molecular events in the cell cycle progress in a sequential and ordered manner. The timing of the cell cycle and the relative lengths of the various stages depends on the specific type of cell and on the local conditions.

1.2 Control of the cycle cycle

The molecular events of the cell cycle are controlled by 2 key regulatory molecules: cyclins and cyclin-dependent kinases (CDKs) (Figure 3). A heterodimer is formed from the regulatory cyclin subunit and the CDK catalytic subunit. Cyclin-bound CDKs act to phosphorylate target proteins important in promoting or preventing advancement to the next stage of the cell cycle. Different cyclin-CDK pairs are selective for specific target proteins. The plethora of cyclins and CDKs constitute the autonomous oscillator that drives the cell cycle. Different cyclin-CDK complexes are activated at different points of the cell cycle and destruction of cyclins must occur before cells can exit from mitosis (Murray, 1989; Murray et al, 1998). Thus, their levels fluctuate in correspondence to the stages of the cell cycle. Cyclins are associated with G1 (cyclin D), S phase (cyclins E and A), and mitosis (cyclins B and A).

Figure 3. Cell cycle regulators – cyclins. Cyclins are produced and degraded in a cyclical fashion in order to drive the cell through different stages of the cell cycle. A cyclin forms a complex with a cyclin-dependent kinase (CDK) which will activate the CDK counterpart. Different cyclins are active in different phases of the cell cycle (adapted from <http://www-rcf.usc.edu/~forsburg/cclecture.html>).

Extracellular pro-mitotic signals activate G₁ cyclin-CDK complexes that promote the expression of transcription factors controlling the expression of S cyclins and enzymes required for DNA replication. G₁-cyclin CDK complexes also promote the ubiquitination of S phase inhibitors, causing them to be degraded by the proteasome. A rising level of S phase promoting factor (SPF), which includes A cyclins bound to Cdk2, enters the nucleus and signals the cell to duplicate its genetic material. S cyclin-CDK complexes phosphorylate pre-replication complexes assembled during G₁. This phosphorylation ensures that DNA is replicated only once. As DNA replication progresses, Cyclin E is destroyed, and the level of mitotic cyclins begin to rise. Mitotic

cyclin-CDK complexes are synthesized but inactivated during S and G₂ phases. The M phase promoting factor (MPF) refers to the complex of mitotic B cyclins with the M phase Cdk1. They help initiate mitosis by activating proteins important in mitotic spindle assembly and chromosome condensation. These events take the cell to metaphase. At this point, the MPF activates the anaphase-promoting complex (APC/C), which promotes separation of paired sister chromatids by degradation of securin and at the same time, targets mitotic cyclins for degradation, allowing cytokinesis to proceed. Synthesis of G₁ cyclins is turned on once again for the next cycle and geminin, which has kept the duplicated DNA from being re-replicated before mitosis is also degraded.

1.3 Cell cycle checkpoints

Checkpoints exist at various stages of the cell cycle. These molecular mechanisms not only provide a surveillance system on progression through the phases. They also prevent damaged or incomplete DNA from being passed on to daughter cells, thus ensuring replication fidelity (Figure 4).

1.3.1 The G₁/S checkpoint

Among the known cell cycle checkpoints, the G₁/S, S, and G₂/M are relatively well-characterized while the spindle assembly checkpoint is less understood. The G₁/S checkpoint halts DNA replication if any DNA damage is sensed. It is believed that DNA lesions of the double-stranded breaks type resulting from ionizing radiation or radiomimetics will cause activation of ataxia-telangiectasia mutated kinase (ATM),

which will then phosphorylate p53 and checkpoint kinase 2 (Chk2), setting in motion a signaling cascade to initiate and maintain the G₁/S arrest (Bartek and Lukas, 2001). Phosphorylation of p53 is necessary in maintenance of G₁/S arrest as p53 loss of function results in dysfunction of G₁/S checkpoint.

1.3.2 The S phase checkpoint

The S phase checkpoint had been shown to be activated in budding yeast by damage that occurred in S phase or by unrepaired damage that escapes the G₁/S checkpoint (Paulovich and Hartwell, 1995). ATM and breast cancer 1 (BRCA1) are required to activate the checkpoint when double-stranded break type of DNA damage occur (Howlett et al, 2002). These repair proteins activate the S phase checkpoint through a kinase signalling cascade that could either proceed through a ATM-Chk2-cell division cycle 25 homologue A (Cdc25A) pathway, which is a checkpoint response or a second pathway that activates the recovery process which depends on structural maintenance of chromosome 1 (SMC1) phosphorylation by ATM in the presence of BRCA1, Fanconi anemia, complementation group D 2 (FANCD2) and Nijmegen breakage syndrome 1 (NBS1) (Kim et al, 2002; Yazdi et al, 2002). When DNA damage is induced by ultraviolet light (UV) or chemicals, ataxia-telangiectasia and Rad2-related (ATR) protein binds to UV-induced lesion (Unsal-Kacmaz et al, 2002) or to replication protein A (RPA)-coated single-stranded DNA (Wang et al, 2003) produced from the repair mechanism, and becomes activated. Activated ATR then phosphorylates Chk 1, which in turn phosphorylates and downregulates Cdc25A, and as a result, inhibits firing of replication origins (Hefferman et al, 2002).

1.3.3 The G₂/M checkpoint

The G₂/M checkpoint prevents cells from entering mitosis in the presence of DNA damage. The ATM-Chk2-Cdc25 signalling pathway and/or the ATR-Chk1-Cdc25 pathway is activated to halt cell cycle progression (Brown and Baltimore, 2003). DNA damage induced by UV irradiation results in activation of ATR-Chk1 signalling, which arrests the cell cycle. However the maintenance of arrest is regulated by ATM-Chk2 signalling (Abraham, 2001). Checkpoint kinases inhibit mitosis entry through downregulation of Cdc25 and upregulation of Wee1, which later together control the activity of Cdc2/Cyclin B (Yarden et al, 2002). Cdc25A phosphatase is the main effector of G₂/M checkpoint. Phosphorylated Cdc25A phosphatase binds to 14-3-3 proteins and is degraded by ubiquitin-proteasome pathway (Furnari et al, 1997; Sanchez et al, 1997).

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Figure 4: Mammalian Cell Cycle Checkpoint Pathways. A general overview of the G1, S, and G2 cell cycle checkpoint pathways is indicated (left, center, and right panels, respectively). In response to DNA damage, ATM and/or ATR trigger the activation of a checkpoint that leads to cell cycle arrest or delay. The checkpoint pathways are characterized by cascades of protein phosphorylation events, which in turn alter the activity, stability, or localization of the modified proteins (adapted from

http://www.rndsystems.com/mini_review_detail_objectname_MR03_DNADamageResponse.aspx).

1.3.4 The spindle checkpoint/ Mitotic checkpoint

The spindle assembly checkpoint also plays an important role in cell cycle regulation. The checkpoint detects defects in spindle structure or the alignment of chromosomes on the spindle and delays the onset of chromosome segregation until the defects are repaired. Mammalian cells that lack functional p53 gene such as HeLa has an increase in ploidy when cultured in nocodazole or colcemid (Cross et al, 1995). This highlights the involvement of p53 in this checkpoint.

The spindle checkpoint monitors the state of the kinetochores in mitotic cells. The spindle checkpoint has been proposed to involve two separate arms mediated by two distinct families of proteins: mitotic arrest defective (Mad 1-3) and budding uninhibited by benzimidazole (Bub 1, 3, and R1) proteins (Lampson and Kapoor, 2005; Li and Benezra, 1996; Logarinho et al, 2004; Millband and Hardwick, 2002). The BubR1 component of the pathway has been suggested to monitor spindle tension (Skoufias et al, 2001; Taylor et al, 2001) whereas the Mad2 pathway monitors microtubule attachment at the kinetochore (Waters et al, 1998). Incomplete kinetochore-microtubule attachment or the lack of tension between microtubules transmits a signal to the cell cycle machinery that delays anaphase onset. The cell cycle progression resumes after all kinetochores are attached to the microtubules and proper tension has been established across all kinetochores. It is a subject for open debate whether these represent two completely independent, parallel pathways or a single pathway that depends on both BubR1 and Mad2 (Shannon et al, 2002).

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The spindle checkpoint proteins Bub1, Bub3, Mad1, Mad2, mono spindle 1 (Mps1) and a Mad3/Bub1-related protein kinase BubR1, only localize to kinetochores during mitosis (Musacchio and Hardwick, 2002; Shah and Cleveland, 2000). The Bub proteins localize to kinetochores in all mitotic cells while the soluble Mad proteins respond only to lack of proper kinetochore attachment to microtubules (Millband and Hardwick, 2002). It has been suggested that the checkpoint proteins are recruited to kinetochores in an orderly fashion. More precisely, the protein kinase Bub1 localizes to the kinetochore very early during mitosis, followed by centromeric protein F (CENP-F), BubR1, centromeric protein E (CENP-E) and finally Mad2 (Johnson et al, 2004).

The most downstream level of the checkpoint pathway involves the inhibition of the Anaphase Promoting Complex/Cyclosome (APC/C), which requires association of cell division cycle homologue 20 (Cdc20) for its activation (Bharadwaj and Yu, 2004). When the checkpoint is satisfied, Cdc20 is freed and is able to interact with APC/C. The APC/C ubiquitinylates its partner Cdc20 with the help of the ubiquitin-conjugating enzyme E2 C (UbcH10). These ubiquitin tags are removed by ubiquitin-specific peptidase 44 (USP44) while the cell is in its holding pattern (Stegmeier et al, 2007). APC/C induces anaphase onset by promoting cleavage of the cohesins, proteins responsible for cohesion of sister chromatids. Securin is targeted for proteasomal degradation via ubiquitinylation by the APC/C. This releases separase, which then causes the dissociation of the proteins of the cohesion complex, resulting in separation of sister chromatids (Vodermaier, 2004; Vodermaier and Peters, 2004). Checkpoint

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proteins target Cdc20 to inhibit its role in inducing anaphase onset (Ciliberto et al, 2005) (Figure 5).

Figure 5. The spindle checkpoint/mitotic checkpoint. A dynamic balance of ubiquitination and de-ubiquitination events regulates anaphase onset. The mitotic spindle must lasso each sister chromatid for correct segregation of a complete genome to daughter cells. Untethered kinetochores continuously signal through checkpoint proteins Mad2 and BubR1 to restrain the anaphase-promoting complex/cyclosome (APC/C) and its activator Cdc20 from dissolving the molecular glue binding the sister chromatids. The APC/C frees itself by ubiquitinating its partner, with the help of the UbcH10 enzyme. These ubiquitin tags are removed by USP44 while the cell is in its 'wait anaphase' stage. When the last kinetochore is attached, the APC/C and Cdc20 would no longer be restrained by the checkpoint proteins, and anaphase ensues (adapted from Nature **446**, 868-869).

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Central to the mitotic spindle checkpoint are the kinetochores. It has been proposed that the 'wait anaphase' signal is generated by kinetochores. This was further proven by classic experiments done by Rieder and colleagues (1995) that a single unattached kinetochore was sufficient to inhibit anaphase and that laser ablation of that kinetochore resulted in rapid onset of anaphase. Mammalian kinetochores recruit additional proteins besides the classical Mad and Bub proteins. Among these proteins are Rod (rough deal), Zw10 (zeste-white 10) and Zwilch, which are required for the recruitment of dynein/dynactin to kinetochores (Chan et al, 2000; Scaerou et al, 2001; Williams et al, 2003; Karess et al, 2005; Famulski et al, 2008). Rod/Zw10 was found to be necessary for the metaphase checkpoint in *Drosophila* (Basto et al, 2000). Recently, it was reported that Rod/Zw10 plays a dual role in checkpoint signaling. It contributes to checkpoint activation through its role in promoting Mad2 accumulation on kinetochores and conversely, to checkpoint inactivation by removing Mad2 via dynein/dyactin recruitment subsequently (Buffin et al, 2005).

As discussed above, multiple players are involved in the functioning and maintenance of the mitotic spindle checkpoint. Two models on how the APC/C is inhibited by the checkpoint have been put forward. The Sequestration Model proposed that Mad2 and BubR1 bind and sequester Cdc20, thus preventing ubiquitination of essential mitotic regulators Cyclin B1 and securin, both of which are needed to keep the cell in mitosis. The unattached kinetochore converts Mad2 into a catalytically active form which binds Cdc20. In the Direct Inhibition Model, the mitotic checkpoint complex (MCC) is made

up of BubR1, Bub3, Mad2 and Cdc20 binds and inhibits APC/C independently of the kinetochore. In this case, however, the 'wait anaphase' signal generated by unattached kinetochores sensitizes APC/C to prolonged inhibition by MCC (Chan and Yen, 2003).

1.4 The kinetochore

In order to ensure correct segregation of genetic material into daughter cells, amphitelic attachments must be formed with each centromere of the sister chromatid pair attached to spindle fibers from opposite spindle poles. Amphitelic attachments are often preceded by monotelic, syntelic or merotelic attachments. During this search-and-capture period, chromosomes oscillate in a series of poleward and anti-poleward movements, mediated by the pulling and pushing forces generated by the microtubules.

The centromere consists of repetitive DNA sequences termed α -satellite DNA, which has little or no homology among species. A specific histone, called centromeric protein A (CENP-A), replaces histone H3 at certain intervals along the chromatin (Blower et al., 2002). Heterochromatin flank the centromeric region (Pidoux and Allshire, 2005). Put together, these repeating units form a three-dimensional array on the chromatin face pointing poleward. This surface forms the recruitment site of centromere proteins and kinetochore proteins. This process is thought to be regulated by epigenetic modifications (Karpen and Allshire, 1997).

Figure 6. Organization of the mammalian kinetochore. The vertebrate kinetochore is a complex structure that specifies the attachments between the chromosomes and microtubules of the spindle. Kinetochore assemble on centromeric heterochromatin and can be divided into inner, middle and outer plates (adapted from Journal of Cell Science 117, 5461-5477 (2004).

Kinetochore have several important functions during mitosis. First, they attach to the plus ends of spindle microtubules. Second, they generate forces for chromosome movement. Third, they prevent premature anaphase onset until all the chromosomes are properly attached to the spindle kinetochore. The kinetochore is dynamic with changing constituent proteins as cells enter mitosis and as it encounters microtubules.

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The vertebrate kinetochore is a trilayered structure consisting of an inner, middle and outer kinetochore (Brinkley and Stubblefield, 1966) (Figure 6). A fibrous corona region extends away from the outer kinetochore. The inner kinetochore forms on highly repetitive DNA sequences and its components include the chromosomal passenger proteins, proteins involved in binding of the kinetochore to DNA and proteins partaking in kinetochore assembly. The middle kinetochore has proteins involved in sensing tension across the kinetochore (tension sensing kinetochore phosphoepitope (3F3/2 antigen)) as a result of microtubule pulling forces. The outer kinetochore is composed of a dynamic network of resident and transient components that are involved in the spindle checkpoint and the attachment of microtubules as well as regulation of their behaviour (Maiato et al, 2004). The outer plate of kinetochores in animal cells has ~20 attachment sites for spindle microtubules, compared to only one in yeast.

Kinetochore proteins can be grouped by whether their kinetochore concentrations remain constant or vary during mitosis and by whether they turnover rapidly or slowly at their binding sites. Proteins that remain nearly constant in level from prophase through late anaphase include the constitutive components of the inner plate and the stable outer kinetochore components, such as the Nuclear Filament-containing protein (Nuf2)- yeast nuclear division cycle 80 (Ndc80) complex/highly expressed in cancer (Ndc80/Hec1) complex (Ciferri et al, 2007). Dynamic components that change in concentration at kinetochores during mitosis include the microtubule motors (CENP-E and dynein) and the spindle checkpoint proteins (Mad1, Mad2, BubR1 and Cdc20). These proteins assemble at high concentrations at kinetochores and are reduced in

concentration following subsequent interactions with spindle microtubules. These reductions in kinetochore protein concentrations are proposed to be important for inactivating the spindle checkpoint and for anaphase onset (Millband and Hardwick, 2002; Shah and Cleveland, 2000).

The checkpoint signal is generated at the kinetochores and restrains anaphase onset before all replicated chromatids are properly attached to the bipolar spindle (Chan and Yen, 2003). The spindle checkpoint is highly conserved throughout evolution and ensures correct segregation of chromosomes during cell division. Failure of the spindle checkpoint can lead to aneuploidy and genomic stability, events that have been linked to tumorigenesis in many types of cancer.

1.5 Chromosomal Passenger Proteins

1.5.1 Components and localization

The chromosomal passenger complex (CPC) comprises of Aurora B kinase, its targeting and activation unit inner centromere protein (INCENP), and two other subunits survivin and Borealin. The chromosomal passenger proteins localize to multiple sites on the mitotic apparatus, including centrosomes, microtubules of the metaphase and central spindle, kinetochores, and midbodies (Fortugno et al, 2002) during distinct mitotic phases and this localization pattern is cell cycle dependent. During early mitosis, CPC members can be found on chromosome arms, which later concentrate at the inner centromere and remain there until the metaphase-to-anaphase transition. The CPC

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migrates to the spindle midzone during anaphase and are finally localized at the midbody during cytokinesis. The distinct localization of CPCs reflect their involvement in diverse mitotic processes, ranging from chromosome condensation and cohesion, correction of microtubule-kinetochore interactions, spindle checkpoint activities and cytokinesis.

Aurora B belongs to the Aurora kinase family of Ser/Thr kinases which mediate distinct functions during mitosis. Aurora B forms the enzymatic core of the CPC. INCENP is required for Aurora B activation and acts as a scaffold to bring the rest of the CPC members together. Aurora B in turn phosphorylates INCENP in a positive feedback loop (Sessa et al, 2005). Survivin and Borealin bind to the N-terminal of INCENP whereas Aurora B binds near the carboxyl terminus (Ainsztein et al, 1998, Bolton et al, 2002, Klein et al, 2006). Survivin and Borealin mediate CPC localization to centromeres and midzone together with the N terminal of INCENP (Klein et al, 2006, Vader et al, 2006a; 2006b).

Survivin is also phosphorylated by Aurora B but the functional significance of this event is unclear. Survivin and Aurora B activities intertwine closely. Aurora B regulates survivin dynamics, which in turn, might be a crucial factor in regulating its activities (Beardmore et al, 2004). It has been suggested that survivin can enhance the activity of Aurora B (Bolton et al, 2002) and a model has been put forth whereby survivin regulates spindle formation through Aurora B. Borealin's role is not well-characterized

but it may regulate Aurora B activity and contribute to interaction between INCENP and survivin (Vader et al, 2006, Jelluma et al, 2006).

CPC proteins display high interdependency in both localization and function. If one member is targeted with small interfering ribonucleic acids (siRNAs) or dominant negative mutants, other members are mislocalized and the CPC cannot perform its function as a collective whole. This is further supported by a recent crystal structure of the CPC comprising of INCENP, Survivin and Borealin in a 1:1:1 stoichiometry forming a three-helix bundle, which shows complex molecular interactions (Jeyaprkash et al, 2007).

1.5.2 CPC functions during mitosis

Mitotic chromosomes are condensed entities. Condensation confers rigidity to chromosomes, a property which is necessary for chromosome segregation during anaphase. Aurora B kinase recruits and phosphorylates condensin subunits (Lipp et al, 2007). Aurora B also phosphorylates histone H3, an event believed to be involved in chromosome condensation although exact mechanisms remain unclear (Corces et al, 2004).

Incorrect attachments or unattached chromosomes activate the checkpoint and it is of paramount importance that these errors are corrected to allow formation of proper, bipolar attachments (Figure 7). It has been proposed that the chromosomal passenger complex is a regulator of kinetochore attachment (Adams et al, 2001) and is important

for bipolar spindle formation in a pathway involving Aurora B-dependent phosphorylation of the microtubule-destabilizing Kin 1 kinesin (MCAK)(Gassmann et al, 2004; Sampath et al, 2004). The Aurora B kinase was reported to promote kinetochore microtubules turnover to reduce chromosome segregation errors (Cimini et al, 2006) . Aurora B phosphorylates Ndc80/Hec1, which is pivotal in the establishment of stable kinetochore microtubule attachments at the outer kinetochore (McClelland et al, 2003; DeLuca et al, 2002; 2005; Lin et al, 2006). Ndc80/Hec1 phosphorylation weakens its interaction with microtubules thereby releasing improper attachments.

Figure 7. Chromosomes must be captured in amphetelic manner i.e. sister kinetochores are attached to kinetochore microtubules from opposite poles of the mitotic spindle. Errors in chromosome orientation (syntelic, monotelic and merotelic attachments) result in segregation errors if uncorrected.

It was recently showed that a number of chromosomal passenger proteins play an important role in the spindle checkpoint. CPCs regulate the spindle checkpoint indirectly through destabilization of incorrect microtubule-kinetochore attachments (Kallio et al, 2003). It was reported that the chromosomal passengers are required for the targeting of checkpoint proteins BubR1 and Mad2 to kinetochores (Carvalho et al, 2003; Ditchfield et al, 2003; Lens et al, 2003). Apart from that, stable targeting of CENP-E, dynein and MCAK to centromeres requires Aurora B function and presence of microtubule (Murata-Hori and Wang, 2002). Survivin was also reported to be involved in stable checkpoint activation and a sustained checkpoint signal in response to lack of tension across sister chromatids (Carvalho et al, 2003).

1.6 The Ran signaling system in spindle assembly

The small GTPase Ran (Ras-related nuclear protein) is a 25 kilodalton protein which is abundant in the cell. Ran exists in GDP- and GTP-bound forms regulated by nucleotide exchange and GTP hydrolysis. Regulator of Chromosome Condensation 1 (RCC1) is the only known guanine nucleotide exchange factor for Ran. Upon association with chromatin, RCC1 catalyzes production of RanGTP (Li et al, 2004). The low, intrinsic GTPase activity of Ran is enhanced by a GTPase-activating protein (RanGAP) and Ran-binding protein (RanBP1), both of which are localized in the cytoplasm during interphase. During mitosis, a significant fraction of RanGAP1 is associated with kinetochores (Jomon Joseph, 2002). The asymmetrical distribution of regulators of the Ran system explains the presence of a gradient of Ran's nucleotide-bound forms across the nuclear envelope, with a higher concentration of RanGTP in the nucleus (Figure 8).

The Ran GTPase cycle at the animal nuclear pore

Figure 8. The RanGTPase cycle at the animal nuclear pore. Ran exists in GDP- and GTP-bound forms regulated by nucleotide exchange and GTP hydrolysis. RCC1 is the nucleotide exchange factor for Ran and converts RanGDP to RanGTP. RCC1 is associated with the chromatin in the nucleus. Two other proteins, RanGAP and RanBP, which are responsible for GTP hydrolysis, localize onto the filaments of the nuclear pore complex (adapted from http://www.biosci.ohio-state.edu/pcmb/osu_pcmb/images/content/people/meier_Fig1Lrg.jpg).

Ran is well-characterized for its role in mediating nucleocytoplasmic transport, which is highly selective with regard to the cargo and also directionality of transport. Passive diffusion is restricted to small, water-soluble particles smaller than 40-50 kilodalton whereas molecules above the diffusion limit require active transport. Molecular trafficking is mediated through the 125 megadalton structures called nuclear pore complexes (NPCs), which are comprised of at least 50 different nucleoporins (NUPs).

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Passage of proteins above the nuclear size exclusion limit through the NPC is regulated by the import and export receptors. Ran functions in concert with a myriad of nuclear import and export receptors, termed importins and exportins. These specialized molecules regulate nucleocytoplasmic passage of proteins bearing nuclear localization signal (NLS) and/or nuclear export signal (NES). Import cargoes harbouring an NLS are complexed by the importin α/β heterodimer in the cytoplasm and translocated through the NPC. In the nucleus, binding of RanGTP to the import receptor-cargo complex promotes cargo release. The importin-RanGTP complex is then recycled to the cytoplasm. RanGTP is displaced from the complex and subsequently hydrolysed by RanGAP. In the case of nuclear export, RanGTP promotes the formation of export complexes consisting of an export substrate carrying an NES, the exportin and RanGTP itself. After translocation to the cytoplasm, hydrolysis of RanGTP occurs, releasing RanGDP and free exportin (Gorlich, 1997).

Apart from its prominent role in mediating nucleocytoplasmic trafficking, Ran has captured the attention of the scientific community in recent years through a variety of emerging roles in mitosis such as spindle assembly (Kalab et al, 1999; Quimby and Dasso, 2003; Wilde and Zheng, 1999), nuclear envelope formation (Zhang and Clarke, 2000) and cell cycle control (Dasso, 2002).

Spindle assembly is an event which is tightly coupled to the Ran cycle. The collective activities of multiple proteins are involved in generating localized microtubule nucleation, dynamics and organization (Karsenti and Vernos, 2001). Breakthrough

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studies came from several groups, which demonstrated the control that the small GTPase Ran exerts on microtubules during mitosis (Dasso, 2001; Heald and Weis, 2000; Kahana and Cleveland, 2001; Karsenti and Vernos, 2001). Interestingly, Ran adopts a mechanism akin to that used in nuclear transport to regulate spindle assembly during mitosis. After nuclear envelope breakdown, the importins bind to and sequester several regulatory proteins in the mitotic cytosol, including targeting protein for Xklp2 (TPX2) and nuclear mitotic apparatus protein (NuMA), which are essential for spindle microtubule assembly and spindle pole formation. Locally-high RanGTP concentration generated by chromatin-bound RCC1 coordinates the nucleation, dynamics and organization of microtubules during spindle assembly by binding to the importins, thereby releasing TPX2 and NuMA in the vicinity of chromosomes to facilitate bipolar spindle formation (Kalab et al, 2002; Nachury et al, 2001)

In *Xenopus* egg extracts, microtubule dynamics and spindle assembly are dependent upon the concentration of RanGTP (Dasso, 2002). Note: *Xenopus* oocytes lack centrosomes. RanGTP was found to stimulate the interaction between TPX2 and the *Xenopus* Aurora A kinase Eg2. This interaction promotes phosphorylation and kinase activity of Eg2 in a microtubule-dependent fashion (Tsai et al, 2003). These findings have led to the hypothesis that chromatin-associated RCC1 protein creates an increased local concentration of RanGTP in the vicinity of chromosomes, helping to direct the formation of bipolar spindles. This is also consistent with the proposed mechanism that efficient chromosome capture requires a bias in the search-and-capture process in which dynamically unstable microtubules search space to capture chromosomes. It was

additionally shown that RanGTP introduces a spatial bias into microtubule dynamics and thus improves the efficiency of said mechanism for spindle assembly (Wollman et al, 2005).

1.7 Chromosome-binding proteins play important roles in mediating mitotic progression

During interphase, chromatin is associated with a number of proteins that possess DNA-binding domains and thus have a specific or general affinity to DNA. DNA-binding proteins include transcription factors which modulate the process of transcription, nucleases which cleave DNA, and histone proteins which are involved in DNA packaging into nucleosomes. Chromatin is reorganized into condensed chromosomes during mitosis. Intuitively, it is expected that a different set of proteins would interact with the chromosomes after undergoing such enormous change in architecture. Proteins such as condensin and cohesin play important roles in driving chromosome condensation and sister chromatid cohesion, respectively. Kinetochore constituents such as those involved in checkpoint signaling (Mad and Bub proteins) and those comprising the mammalian chromosomal passenger complex are dynamic entities that coordinate numerous events driving mitotic progression. Their dynamic behaviour and unique localization on the mitotic apparatus are two criteria central to their respective functions, which will form a major part of our study.

1.8 Current understanding of mechanisms behind mitotic catastrophe

1.8.1 Mitotic catastrophe from a historical point of view

The term 'mitotic catastrophe' was coined in 1986 to describe the lethal phenotype in yeast, which involves premature entry into mitosis and failure to complete it effectively. The lethal phenotype was created by combining an activated *cdc2/Cdk1* allele with a recessive temperature-sensitive *wee1* mutation (Russell and Nurse, 1986a, 1986b; Okada and Mak, 2004). Members of the Wee1 kinase family control the timing of cell cycle events by inhibiting phosphorylation of Cdk1 and 2.

1.8.2 Mitotic catastrophe as a consequence of cell cycle errors

Mitotic catastrophe is a form of cell death that results from aberrant mitosis. It has been described that mitotic catastrophe is death that occurs during mitosis, resulting from a combination of deficient cell cycle checkpoints and cellular damage (Castedo et al, 2004a; 2004b; Castedo and Kroemer, 2004). The molecular pathways which culminate in this phenotype remain obscure to date although it has been reported that mitotic catastrophe is linked to the abnormal activation of cyclin B/Cdk1 (Castedo et al, 2004).

Tumor cells are usually associated with compromised cell cycle checkpoints (Roninson et al, 2001). Failure to arrest cell cycle progression in the event of DNA damage or mitotic disruption will trigger improper chromosome segregation. This may lead to activation of a cell death machinery and cellular demise during mitosis (Dash and El-Deiry, 2001; Castedo et al, 2004). The induction of mitotic catastrophe is an important aspect of anti-cancer therapies using DNA-damaging agents. Moreover, it has become

apparent in recent years that chemotherapy and radiation induce antiproliferative responses, cell death through mitotic catastrophe being one of them. This is a refinement of our understanding in the therapeutic effects of anticancer agents which, on most part, have been accounted for by apoptosis (Roninson, 2002). Most importantly, the suppression of mitotic catastrophe can favor asymmetric division and the generation of aneuploid cells.

1.8.3 DNA damage response (DDR) during interphase and the players involved

DNA double-stranded breaks (DSBs) is one of the deadliest type of DNA lesion. Considerable progress has been made in understanding DDR during interphase (Bartek and Lukas, 2007). All three phosphoinositide 3-kinase related kinases (PIKKs) ATM, ATR and DNA-dependent protein kinase (DNA-PK) are central in mediating the DNA damage checkpoint network, which is comprised of DNA damage sensors, signal transducers and various effector pathways (Shiloh, 2003; Bakkenist and Kastan, 2004; Bartek et al, 2004; Lukas et al, 2004). The Mre11-Rad50-Nbs1 (MRN) complex functions as a sensor that recognizes DNA lesions and in turn, recruits ATM (Petrini and Stracker, 2003; You et al, 2005). ATM is activated and mediates checkpoint events *via* two pathways – a chromatin modification pathway that functions throughout interphase and a DSB resection pathway that leads to ATR activation and is restricted to S and G₂ phases (Jazayeri et al, 2006). In the first pathway, ATM phosphorylates histone H2AX which then serves to recruit a mediator of DNA damage checkpoint 1 (Mdc1) and later facilitate formation of protein complexes at DNA lesions that include

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MRN, 53BP1, BRCA1 and ATM itself (Stucki et al, 2005). Active ATM then targets many substrates, including the effector kinase Chk2, to propagate the signal throughout the nucleus. In the DSB resection scenario, ssDNA become coated with RPA and serves as the key intermediate for ATR-dependent signaling and homologous recombination (Wang et al, 2003). ATR- ATR interacting protein (ATRIP) complex and human DNA topoisomerase II binding protein 1 (TopBP1) recruitment are events required for ATR activation, which then targets substrates, including the effector kinase Chk1 (Kumagai et al, 2006). Collectively, the ATM-Chk2 and ATR-Chk1 pathways mediate the checkpoint responses and facilitate DNA repair.

DNA damage checkpoints permit the cell to repair the damage before entering mitosis. Checkpoint recovery involves degradation of the checkpoint mediator Claspin and mitosis-inhibiting kinase Wee1 via ubiquitin/proteasome-mediated mechanisms. Polo like kinase (PLK)-mediated phosphorylation of Claspin and Wee1 is associated with checkpoint recovery (Mailand et al, 2006; Mamely et al, 2006; Peschiaroli et al, 2006).

Some cells are able to divide following a sustained checkpoint-imposed cell cycle arrest despite the presence of persistent DNA damage (Toczyski et al, 1997). They are said to have undergone checkpoint adaptation, a phenomenon related to, but conceptually distinct from checkpoint recovery. The mitotic re-entry of such adapted cells facing incomplete DNA replication or unrepaired DNA breakage is brought about by resumed activity of PLX/PLK1 and inhibition of Chk1 kinases, through a poorly understood mechanism that involves the adaptor/mediator protein Claspin and possibly other

factors (Yoo et al, 2004; Syljuasen et al, 2006). It remains to be seen whether the only mission of adaptation in vertebrates is to eliminate such defective cells with irreparable damage through mitotic catastrophe. While the mechanisms behind DNA damage-induced G₂ checkpoint are relatively well characterized, little is known about checkpoints triggered by mitotic DNA damage. Recently, mitotic DNA damage was reported to inhibit the kinase activity of Plk1 and block mitotic exit (Jang et al, 2007; Smits et al, 2000).

1.9 Signaling events leading to mitotic catastrophe

1.9.1 Involvement of MAP kinases in cellular proliferation

Mitogen-activated protein (MAP) kinases play an important in signal transduction networks that lead to cellular responses such as proliferation, differentiation, transformation, development and apoptosis. Generally, MAP kinase cascades comprise of three important enzymes that are activated in sequence, namely a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and MAPK. Three main groups of MAPKs have been identified in mammalian cells – classical MAPK (ERK), c-Jun NH₂ terminal kinase/ stress-activated protein kinase (JNK/SAPK) and p38 (Figure 9). Extracellular signal related kinase (ERK) is among the best-characterized MAPK and the Raf-MEK-ERK is the most well-characterized pathway which has been shown to be responsible for G₁/S progression. The p38 MAPK family is activated by cellular stress including UV irradiation, heat shock, osmotic stress, and certain cytokines. p38 activity is involved in mitotic arrest in somatic cell cycles at the spindle assembly checkpoint

and also in cell division cycle homologue 42 (Cdc42)-induced cell cycle arrest at G₁/S (Molnar et al, 1997; Takenaka et al, 1998).

1.9.2 c-Jun NH₂-terminal kinase (JNK)

The c-Jun NH₂-terminal kinases (JNKs) are an evolutionarily conserved subgroup of MAP kinases (Weston and Davis, 2002). The JNK pathway is activated primarily by cytokines and exposure to environmental stress (Ip and Davis, 1998). Specific stimuli trigger the activation of MAPKKKs, which then phosphorylate and activate the MAPKK isoforms MKK4 and MKK7, which in turn phosphorylate and activate JNK. ERKs can activate JNK kinases when the stimulus is an endothelial cell growth factor such as vascular endothelial growth factor (VEGF). The significance of ERK/JNK cross activation is during G₁ to S phase transition. JNK signaling has been implicated in the decision between cellular survival and apoptosis. It has been reported that UV-induced apoptosis in fibroblasts requires JNK for cytochrome c release from the mitochondria.

A major target of the JNK signaling pathway is the activator protein-1 (AP-1) transcription factor, which is activated, in part, by the phosphorylation of c-Jun and related molecules. A new JNK substrate has been identified, that is phosphorylated in apoptotic cells: H2AX, a histone H2A variant. The site of phosphorylation on H2AX corresponds to a non-canonical site for MAPK phosphorylation. H2A functions as a core component of the histone octamer that forms the nucleosome. In response to DNA

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damage, phosphorylated H2AX accumulates at the site of double strand breaks, where it is thought to restructure chromatin and assist in the recruitment of DNA repair and signaling factors. A recent study by Lu et al (2006) showed that JNK-mediated H2AX phosphorylation may be essential for DNA fragmentation in UV-stimulated cells (Sluss and Davis, 2006; Ip and Davis, 1998). *In vitro* studies indicate that H2AX phosphorylation is required for caspase-activated DNase (CAD)-mediated nucleosomal fragmentation of the chromosomal DNA. JNK-mediated phosphorylation of H2AX may therefore contribute to apoptosis.

Figure 9. MAP kinase cascades comprise of 3 important enzymes that are activated in sequence, namely a MAPKKK, a MAPKK and MAPK. Main mammalian MAPKs are classical MAPK (ERK), c-jun NH2 terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38.

1.9.3 Cell death pathways *via* the apoptotic machinery

Apoptosis is defined by distinct morphological and biochemical alterations mediated by a family of cysteine aspartate specific proteases (caspases) which are produced as inactive precursors called pro-caspases and which require proteolytic processing to be converted to their respective activated forms following an apoptotic stimulus. Caspases can be divided into three functional groups: initiator caspases (caspase-2, -9, -8, -10), effector caspases (caspase-3, -6, -7) and caspases involved in cytokine maturation (caspase-1, -4, -5, -11, -12, -13, -14). It is broadly acknowledged that two pathways can separately lead to caspase activation. The extrinsic pathway involves activation of transmembrane death receptors (Fas, TNFR, and TRAIL receptor) upon binding with the respective ligands (FasL, TNF and TRAIL) and the subsequent activation of membrane-proximal caspases (caspase-8, -10). Activated caspases then act to cleave and activate downstream effector caspases (caspase-3, -7). The extrinsic pathway subjected to regulation by c-FLIP and the inhibitor of apoptosis proteins (IAPs).

The intrinsic pathway involves disruption of mitochondrial membrane and release of mitochondrial proteins, a significant example being cytochrome c. Released cytochrome c forms a complex called the apoptosome, together with two other cytosolic proteins, apoptotic protease activating factor-1 (Apaf-1) and pro-caspase 9. The apoptosome in turn activates caspase 9 and initiates the caspase cascade. The intrinsic pathway is primarily regulated at the level of cytochrome c release, mediated by the B-cell lymphoma 2 (Bcl-2) family proteins. Mammalian Bcl-2 proteins can be subdivided into pro-apoptotic and anti-apoptotic family members. Pro-apoptotic proteins include Bcl-2-

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associated X protein (Bax), bcl-Xs, Bcl-2 antagonist/killer (Bak), Bcl-2-related ovarian killer (Bok), which contain 2 or 3 Bcl-2 homology (BH) domains and Bcl-XL/Bcl-2-associated death promoter (Bad), Bik, BH3-interacting domain death agonist (Bid) and Bcl-2 interacting mediator of cell death (Bim), which contains only the BH3 region. Anti-apoptotic Bcl-2 family members include Bcl-2, Bcl-XL, and Mcl-1, which contain regions of extensive amino acid similarity to Bcl-2 (BH1-BH4). Over expression of pro-apoptotic proteins promotes cytochrome c release from the mitochondria in response to apoptotic stimuli. On the other hand, upregulation of anti-apoptotic factors inhibit cytochrome c release under similar circumstances (Figure 10).

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Figure 10. Comparison among apoptosis-inducing pathways in nematodes, mammals and fruitflies (adapted from Nature Reviews Molecular Cell Biology 5, 897-907 (November 2004))

There is considerable cross talk between the extrinsic and intrinsic pathways. For example, caspase-8 can proteolytically activate Bid, which can then facilitate the release of cytochrome *c* and amplifies the apoptotic signal following death receptor activation. Most of the anticancer agents either directly induce DNA damage or indirectly induce

secondary stress-responsive signaling pathways to trigger apoptosis by activation of the intrinsic apoptotic pathway, and some can simultaneously activate the extrinsic receptor pathway.

1.9.4 Actinomycin D

Figure 11. Molecular structure of the antibiotic actinomycin D

The antibiotic actinomycin D was used in the project to induce mitotic catastrophe in HeLa cells. The molecule consists of a phenoxazone ring system, to which two cyclic pentapeptides are attached. Structural analysis revealed that actinomycin D intercalates to the DNA at GC steps *via* the aromatic ring system (Chen et al, 1996; Chin et al., 2003; Khan and Lindell, 1980; Liu et al, 1991).

Aims and objectives

We aim to define the cellular and molecular mechanisms of mitotic catastrophe in mammalian cells and to determine the roles of RanGTP signalling pathway, chromosome passenger proteins, and mitotic spindle checkpoint in mitotic catastrophe.

2. Materials and Methods

2.1 Plasmids/constructs

Constructs for RCC1-green fluorescent protein (GFP) and YIC biosensor were kindly provided by Dr. Li Hoi Yeung. Plasmids for Mad2-GFP, Aurora B-GFP and Survivin-GFP were gifts from Dr. Yixian Zheng (HHMI and Carnegie Institution of Washington).

2.2 Antibodies

Antibodies to Actin, Ran, RanBP1, RCC1, PARP, Cyclin B1, phospho Cdc2 and Cdc2 p34 HRP were purchased from Santa Cruz (Beverly, MA, USA). Antibodies to phospho-Histone H3 were from Calbiochem® (San Diego, CA, USA), MAD2 were from Covance (Denver, Pennsylvania), Tubulin-fluorescein isothiocyanate (FITC) were from Sigma (Missouri, USA), ACA were from Antibodies Incorporated (Davis, CA, USA), AIM-1/Aurora B and BubR1 antibodies were from BD Transduction Laboratories (Palo Alto, CA, USA) and Novus Biologicals (Littleton, CO, USA). Survivin antibodies were from R&D Systems (Minneapolis, MN). Cdc20 antibodies were from MBL International (Japan). Gamma H2AX, H2AX, Cleaved ICAD/DFF40 antibodies and JNK PhosphoPlus Kit were from Cell Signaling (Danvers, MA). Secondary antibodies conjugated with appropriate fluorophores were purchased from Invitrogen (Carlsbad, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Dakocytomation (Glostrup, Denmark).

2.3 Cell culture, transfection and drug treatments

Materials:

HeLa cells

DMEM GlutaMax medium (Gibco, Invitrogen, USA)

Fetal Bovine Serum (Hyclone)

Penicillin/Streptomycin (Gibco, Invitrogen, USA)

Lipofectamine® Reagent (Invitrogen, Carlsbad, CA, USA)

Nocodazole (10mg/ml in DMSO) (Sigma, St. Louis, MO, USA)

Actinomycin D (10mg/ml in DMSO) (Sigma, St. Louis, MO, USA)

Methods:

HeLa cells were maintained in DMEM GlutaMax medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1 unit/ml Penicillin/Streptomycin (Pen-Strep) at 37°C in a humidified atmosphere with 5% carbon dioxide.

Transfection was done using Lipofectamine® Reagent according to manufacturer's protocol. Briefly, cells were seeded one day before transfection so that they will reach 80% confluency at the time of transfection.

For drug treatment, HeLa cells were treated with nocodazole for 6 hours and drug washout was done by incubating the cells in DMEM for 30 minutes with 3 changes of medium. For actinomycin D treatment, cells were supplemented with actinomycin D 2

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hours into nocodazole treatment and following nocodazole washout, cells were allowed to recover in the presence of added actinomycin D.

2.4 Time-lapse microscopy

Materials:

HeLa cells

DMEM Glutamax supplemented with 10%(v/v) FBS and 1%(v/v) Pen-Strep

L-15 medium supplemented with 10%(v/v) FBS and 1%(v/v) Pen-Strep

Nocodazole (10mg/ml)

Actinomycin D (10mg/ml)

Methods:

HeLa cells were treated as described in the previous section. Imaging was performed on an Axiovert 200M inverted fluorescence compound microscope (Carl Zeiss, Germany) using a 10x objective (Carl Zeiss, Germany). The culture was maintained at 37°C with 5% CO₂ for the duration of the time-lapse acquisition. Images were recorded at 5-minute intervals and analyzed using the Axiovision software (Carl Zeiss, Germany).

2.5 Site-directed Mutagenesis

Materials:

pEGFP-N3 RCC1

Stratagene Quikchange Site-Directed Mutagenesis Kit

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List of primers for mutations using **pEGFP-N3 RCC1**

S2A forward: 5' ctgcagtcgacatggcacccaagcgcatagc 3'

S2A reverse: 5' gctatgcgcttgggtgcatgctgactgcag 3'

S2P forward: 5' ctgcagtcgacatgccacccaagcgcatagc 3'

S2P reverse: 5' gctatgcgcttgggtgcatgctgactgcag 3'

P3Q forward: 5' ctgcagtcgacatgtcacagaagcgcatagctaaaag 3'

P3Q reverse: 5' ctttagctatgcgttctgtgacatgctgactgcag 3'

ASPK forward: 5' ctgcagtcgacatggcttcacccaagcgcatagc 3'

ASPK reverse: 5' gctatgcgcttgggtgaagccatgctgactgctg 3'

K4R forward: 5' gtcgacatgtcacccaggcgcatagctaaaag 3'

K4R reverse: 5' ctttagctatgcgcctgggtgacatgctgac 3'

K4Q forward: 5' gtcgacatgtcacccagcgcatagctaaaag 3'

K4Q reverse: 5' ctttagctatgcgcctgggtgacatgctgac 3'

Methods:

In vitro site-directed mutagenesis was carried out using Stratagene's QuikChange® Site-Directed Mutagenesis Kit. The basic procedure uses a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The primers, which are complementary to opposite strands of the vector, are extended during thermal cycling by *PfuTurbo* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid with staggered nicks. Following thermal cycling, the product is treated with *DpnI* endonuclease, which is specific for methylated and hemimethylated DNA (parental DNA template isolated from almost all *E.coli* strains is dam methylated and is a target for *DpnI* digestion). The nicked vector DNA is then transformed into DH5α competent cells where the nicked ends will be repaired by the host enzymes.

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Primers for use in this reaction are designed to contain desired mutation in the middle of the sequence with ~10-15 correct bases on both sides. Primers anneal to same sequence on opposite strands of the parental plasmid, and have melting temperatures (T_m) of $\geq 78^\circ\text{C}$.

To estimate the T_m of primers, the following formula was used:

$$T_m = 81.5 + 0.41(\% \text{ GC}) - 675/N - \% \text{ mismatch}$$

For calculating T_m :

N is the primer length in bases

Values for % GC and % mismatch are whole numbers

To estimate T_m for primers intended for introduction of insertions, the following formula was used:

$$T_m = 81.5 + 0.41 (\% \text{ GC}) - 675/N$$

where N does not include the bases which are being inserted.

The reaction was prepared as indicated below:

5 μl of 10x reaction buffer

X μl (50ng) of dsDNA template

X μl (125ng) of oligonucleotide primer #1

X μl (125ng) of oligonucleotide primer #2

1 μl of dNTP mix

ddH₂O to a final volume of 50 μl

Finally, 1 μl of *PfuTurbo* DNA polymerase (2.5U/ μl) was added to the reaction.

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Cycling parameters for QuikChange Site-Directed Mutagenesis Method

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	12* 16** 18***	95°C	30 seconds
		55°C	1 minute
		68°C	1 minute/kb of plasmid length

Legend:

- * Point mutations
- ** Single amino acid changes
- *** Multiple amino acid deletions or insertions

Following thermal cycling, the reaction mix was placed on ice for 2 minutes to cool it to $\leq 37^{\circ}\text{C}$. Subsequently 1 μl of *DpnI* restriction enzyme (10U/ μl) was added directly to each reaction and mixed gently by pipetting. Reactions were spun down and incubated at 37°C for 1 hour to digest the parental supercoiled dsDNA.

Transformation into DH5 α competent cells was done as follows: Competent cells were thawed on ice. 1 μl of the *DpnI*-treated DNA from each sample reaction was added to aliquots of the competent cells and the reactions were gently mixed and incubated on ice for 30 minutes. Heat shock was done at 42°C for 1 minute and the reactions were returned to ice for 2 minutes. 1 ml of Luria-Bertani (LB) broth without antibiotics was added to the reactions and incubated with shaking at 225rpm for 1 hour at 37°C . The cells were spreaded on LB-kanamycin plates containing 50 $\mu\text{g/ml}$ kanamycin.

2.6 Chromosome spread

Materials:

HeLa cells

0.56%(w/v) KCl

Methanol:glacial acetic acid (3:1)

Glass slide

Cytospin machine (Thermo Shandon, Pittsburgh, PA)

Methods:

HeLa cells were treated as mentioned and the cell suspension was collected via trypsinization. The pellet was collected via centrifugation, resuspended in remaining media and swelled using pre-warmed 0.56% potassium chloride (KCl). Cells were again spun down and resuspended in remaining liquid before the fixative (ice-cold methanol:glacial acetic acid (3:1)) was added. Cells in fixative can be stored at 4 °C for 16 hours. The resuspension step was repeated with ~100-200µl of fixative (depending on cell number) before dropping 30µl of cell suspension onto a slide pre-cleaned with fixative.

2.7 Immunofluorescence

Materials:

HeLa cells

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4% (w/v) paraformaldehyde

0.5% (v/v) Triton X-100 in PBS

Tris-buffered saline (TBS) with 0.05% (v/v) Tween 20

Prolong Gold Anti-Fade Mounting medium with DAPI (Invitrogen, Carlsbad, CA, USA)

Methods:

Cells were grown on coverslips and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes at room temperature. For cold treatment, cells were incubated for 15 minutes at 4°C before fixation. Primary antibodies in 4% bovine serum albumin in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST) were incubated for 1 hour at room temperature or overnight at 4°C. Cells were washed three times and incubated with appropriate secondary antibodies for 1 hour at room temperature. Slides were mounted in Prolong Gold Anti-Fade medium with DAPI. Images were collected and analyzed on an Axiovert 200M inverted fluorescence compound microscope (Carl Zeiss, Germany).

2.8 Fluorescence Recovery after Photobleaching (FRAP)

Materials:

HeLa cells

Cover slips

Depression glass slide

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Zeiss 510 LSM confocal microscope with laser unit (Carl Zeiss, Germany)

Methods:

Cells were plated on a coverslip and mounted onto a glass slide with a depression containing culture medium. FRAP was performed on a Zeiss 510 LSM confocal microscope (Carl Zeiss, Germany) using the 488nm laser line of an argon laser. For FRAP experiments, five single scans were acquired, followed by a single bleach pulse of 200-500ms at maximum laser power for 80 iterations in defined regions without scanning. Single section images were then collected at 1s intervals. For imaging, the laser power was attenuated to 1% of the bleach intensity. FRAP recovery curves were generated from background-subtracted images. The total cellular fluorescence was determined for each image and compared with the initial total fluorescence to determine the amount of signal lost during the bleach pulse and during imaging. In FRAP experiments, typically ~10% of total fluorescence was lost during the bleach pulse and less than 5% of fluorescence was lost during the entire imaging process. The fluorescence signal measured in a region of interest normalized to the change in total fluorescence was determined as

$$I_{rel} = \frac{T_0 I_t}{T_t I_0}$$

where T_0 is the total cellular intensity during prebleach, T_t the total cellular intensity at timepoint t , I_0 the average intensity in the region of interest (ROI) during prebleach, and I_t the average intensity in the ROI at timepoint t . Double normalization through

application of this expression accounts for the small loss in total intensity caused by the bleach itself and yields a more accurate estimate of the immobile fraction (Misteli et al, 2003; Phair and Misteli, 2000)

2.9 Fluorescence Loss In Photobleaching (FLIP)

Materials:

HeLa cells

Cover slips

Depression glass slide

Zeiss 510 LSM confocal microscope with laser unit (Carl Zeiss, Germany)

Methods:

Cells were plated on a coverslip and mounted onto a glass slide with a depression containing culture medium. FLIP were performed on a Zeiss 510 LSM confocal microscope (Carl Zeiss, Germany) using the 488nm laser line of an argon laser. For FLIP experiments, five single scans were acquired, followed by repeated photobleaching using a single bleach pulse at intervals of 1s for 200 iterations in defined regions. Single section images were then collected at 1s intervals. For imaging, the laser power was attenuated to 1% of the bleach intensity. The relative fluorescence intensity in a region of interest was determined by normalizing fluorescence intensity in the region to the total fluorescence in the same region during prebleach.

2.10 Fluorescence Resonance Energy Transfer (FRET)

Materials:

HeLa cells

Cover slips

Depression glass slide

Zeiss 510 LSM confocal microscope with laser unit (Carl Zeiss, Germany)

Methods:

FRET detection was performed on a Zeiss 510 LSM confocal microscope (Carl Zeiss, Germany) via the Acceptor Photobleaching method of FRET detection. Fluorescence of donor cyan fluorescent protein (CFP) and acceptor yellow fluorescent protein (YFP) in the YIC probe both before and after photobleaching of the YFP fluorophore were recorded. CFP was excited by 458nm light and the emission was collected through an HQ 470-500nm bandpass filter. YFP was excited by 514nm light and the emission was collect through an HQ 525-575nm bandpass filter. Photobleaching of YFP was performed by repeatedly scanning a region of the specimen with the 514nm laser line set at maximum intensity to photobleach the original acceptor fluorescence. The fluorescence emission from the donor and the acceptor are collected sequentially using the multitrack function of the Zeiss LSM 510 software. Average fluorescence intensities of the donor are measured before and after bleaching. FRET efficiency was calculated as

$$E_F \% = (I_6 - I_5) \times 100 / I_6$$

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where I_5 and I_6 represent the CFP fluorescence intensity of the fifth and sixth images immediately before and after photobleaching of YFP (Karpova et al, 2003). The change in donor fluorescence was assessed in at least 10 cells for each experimental set. Mean FRET efficiencies were then reported $\pm$ standard error.

2.11 Western Blotting

Materials:

HeLa cells

Phosphate-Buffered Saline (PBS) (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, pH 7.4)

M-PER Mammalian Lysis Buffer (Pierce, Rockford, IL, USA)

1x Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA)

2x Laemli Buffer (Biorad, Hercules, CA, USA)

10-15% Polyacrylamide Gel

Gel Electrophoresis Buffer (196mM glycine / 0.1% SDS / 50mM Tris-HCl pH 8.3)

Transfer Buffer (25 mM Tris, 192 mM glycine, 10% methanol)

Tris-Buffered Saline (TBS) with 1%(v/v) Tween 20 (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4)

0.45 μ m Nitrocellulose Membrane (Biorad, Hercules, CA, USA)

Enhanced Chemiluminescence (ECL) reagent (GE Healthcare UK Limited, Buckinghamshire, England)

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Methods:

Mitotic cells were collected via shake-off and lysed in M-PER Mammalian Lysis Buffer and samples were boiled for 5 minutes before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to nitrocellulose membrane. HRP-conjugated secondary antibodies were from Dakocytomation (Glostrup, Denmark). Detection was via chemiluminescence using ECL reagent.

2.12 Immunoprecipitation

Materials:

HeLa cells

M-PER Mammalian Lysis Buffer (Pierce, Rockford, IL, USA)

1x Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA)

Protein G - Sepharose® 4B (Invitrogen, Carlsbad, CA, USA)

Wash Buffer (1x TBS with 250 mM NaCl)

2x Laemli Buffer (Biorad, Hercules, CA, USA)

Methods:

HeLa cells were lysed with M-PER Mammalian Lysis Buffer. Lysates were clarified by centrifugation at 13200 rpm for 10 minutes at 4°C. Clarified lysates were incubated with primary antibody at 4°C overnight. Protein G agarose was then added to the lysates and the mixture was incubated for an additional 2 hours. After that, immunoprecipitants

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were washed five times with wash buffer and proteins were recovered by boiling the beads in sample buffer and analyzed by Western blotting.

3. Results

3.1 Alteration of dynamic behaviour of chromosome-binding proteins during mitotic catastrophe

3.1.1 Actinomycin D induces mitotic catastrophe in HeLa cells

One of the characteristics that set cancer cells apart from normal cells is their ability to proliferate in an uncontrolled manner. As such, cancer cells often exhibit an increased sensitivity to DNA-damaging agents, to which they respond by activating cell death machinery.

This forms the basis behind the therapeutic effects of many anti-tumour agents. It has been proposed that the cell has many options when challenged and growth arrest in mitosis is one of them. Mitotic catastrophe is a form of cell death that results from abnormal mitosis. To date, this molecular event remains a poorly understood area in science. Aberrance in mitosis can lead to cell death in the form of the necrotic or apoptotic pathway. The deciding factor is complex, which may depend on the nature of the inducer and the status of cell cycle checkpoints. Re-entry of tumour cells into the cell cycle after prolonged growth arrest can also be causal.

We have tested a panel of cytotoxic drugs to induce mitotic catastrophe. Surprisingly, we found that actinomycin D was able to cause cell death in mitosis, as opposed to its

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effect on interphase cells that seemed predominantly apoptotic after prolonged treatment. To investigate the effect of the actinomycin D on mitotic cells, we arrested HeLa cells stably expressing histone H2B-GFP in mitosis by incubating them in 600nM nocadazole for 2 hours before actinomycin D was added. The cells were released from nocadazole arrest by thorough washing with normal medium and recovery was followed using time-lapse microscopy in the absence or presence of actinomycin D (Figure 12A). We found that treatment of actinomycin D caused significant cell death during mitosis or cytokinesis (~70%). In contrast, there was only about 12% of cell death during mitosis or cytokinesis in control experiments. H2B-GFP serves as a marker to visualize chromatin. Several abnormalities were observed more frequently in actinomycin D treated cells. Some cells reverted to interphase with multinucleated phenotype while others appear to die in G1 phase after cell division has occurred, however, the segregation of chromosomes was clearly asymmetric. We also observed cases in which actinomycin D treated cells undergo division before proper metaphase alignment had been achieved, resulting in mis-segregation and finally death (Figure 12B, 12C).

Figure 12. Actinomycin D induces mitotic catastrophe in HeLa cells. (A) Schematic diagram showing the nocodazole arrest and release experiment. Cells were incubated with 600nM nocodazole for 2 hours and 400nM actinomycin D was added for an additional 4 hours before nocodazole washout was done by washing with complete medium. Recovery was allowed in the

presence or absence of 400nM actinomycin D. (B) Time lapse experiment was carried out using a H2B-GFP stable cell line. Representative cells displaying different phenotypes in control and actinomycin D treated samples are shown in phase contrast, GFP and merged views. Scale bar indicates 20µm. (C) Quantification of various phenotypes observed. An average of 100 mitotic cells was quantified in 3 independent time-lapse experiments.

3.1.2 Actinomycin D affects spindle assembly during mitosis

We hypothesized that disrupted spindle assembly might plausibly account for the cell death in mitotic progression. Cells were fixed and stained for Histone H3 phosphorylated at Ser10 and tubulin after nocodazole washout at the indicated time points as shown in Figure 13A. Phosphorylation of histone H3 occurs exclusively in mitosis (Hans and Dimitrov, 2001) and therefore it serves as a mitosis marker. The samples were co-stained with anti-tubulin antibodies to visualize the spindle structure that formed.

We found that most of the cells treated with actinomycin D remained at prometaphase whilst those in control samples completed cell division within 2 hours after nocodazole washout (Figure 13B). This observation is consistent with time lapse experiments performed earlier and are suggestive of an activated and prolonged spindle checkpoint. Actinomycin D treatment caused approximately two fold more of cells exhibiting severe spindle defects as compared with control one hour after nocodazole washout, which formed normal bipolar spindles with properly aligned chromosomes at the metaphase plate (Figure 13C). Furthermore, most of the kinetochores were not attached by microtubules in cells treated with actinomycin D. This is probably due to a decrease

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in kinetochore fibres emanating from the spindle poles that were able to capture chromosomes successfully. This data suggested that actinomycin D inhibited spindle assembly during mitosis (Figure 13D). Moreover, defects in chromosome congression were also rampant in actinomycin D treated cells as shown in cases in which a bipolar spindle has been assembled but misaligned chromosomes were still evident. To further investigate the nature of chromosomal aberrations in these samples, we adopted the chromosome spreading method to visualize individual chromosomes. Chromosome aberrations such as fragmentation could be observed in actinomycin D treated samples (Figure 14).

It has been well documented that actinomycin D inhibits transcription by preventing the binding of transcriptional factors to the chromosomes thus leading to apoptosis (Braverman et al, 1977). Structural analysis revealed that actinomycin D intercalates to the DNA at GC steps (Chen et al, 1996; Chin et al., 2003; Khan and Lindell, 1980; Liu et al, 1991). We believed that actinomycin D could intercalate to the DNA of the mitotic chromosomes and therefore some of the essential chromosomal binding proteins for spindle assembly are knocked off from the mitotic chromosomes. To test this hypothesis, the nature of several chromosomal binding proteins, which are essential for spindle assembly and mitotic progression, were analyzed.

Figure 13. Actinomycin D affects spindle assembly during mitosis. (A) Time line showing experimental procedure and drug treatment used. (B) Immunofluorescence images of representative cells stained for microtubules (green), phosphorylated histone H3 (red) and DNA (blue) at different time points indicated after drug treatment. (C) Spindle morphologies in control and actinomycin D treated cells. Spindles are shown in green and DNA is shown in blue. The percentage of normal, bipolar spindles and abnormal spindles were calculated. (D) Microtubule attachments to kinetochores appear to be disrupted in actinomycin D treated cells.

Microtubules are shown in green and the centromere marker ACA in red. Insets show magnified regions boxed in white. DAPI staining is not shown. Bar, 10µm

Figure 14. Chromosome aberrations caused by actinomycin D treatment. Chromosome spread showing control chromosomes as condensed sister chromatids while actinomycin D treated chromosomes show aberrations such as fragmentations and breaks. Individual chromosomes (boxed in white) are magnified as shown in right panel.

3.1.3 Actinomycin D disrupts RCC1 Mobility and RanGTP production

While extensive research has firmly established the importance of the cell cycle machinery in spindle assembly and chromosome segregation, recent studies have revealed that a signaling pathway mediated by RanGTPase plays an equally important role in mitosis (Li et al, 2003). In *Xenopus* egg extracts, microtubule dynamics and spindle assembly are dependent upon the concentration of RanGTP (Dasso, 2002). This finding has led to the hypothesis that chromatin-associated RCC1 protein creates an increased local concentration of RanGTP in the vicinity of chromosomes, helping to direct the formation of bipolar spindles.

Endogenous RCC1 is associated with the chromosome throughout all stages of mitosis and during interphase (Hood and Clarke, 2007). The amino terminus of RCC1 binds to DNA while the rest of the protein associates with chromatin *via* an interaction with histones H2A and H2B. Binding of these histones stimulates a modest increase in RCC1's GEF activity (Nemergut et al, 2001). It is believed that the binary complex of RCC1-Ran has better affinity for the core histones on the chromatin than RCC1 or Ran alone (Li et al, 2003). The stable binding of the binary complex could allow coupling of nucleotide exchange to the chromatin. Upon successful nucleotide exchange, the two proteins would have reduced binding affinity thus enabling them to dissociate from chromatin, driving RanGTP production on the chromatin surface (Figure 15).

Figure 15. A model depicting interaction of RCC1, Ran and reaction intermediates with the chromatin. RCC1, RanGDP and RanGTP interact with the chromatin reversibly due to low affinity binding whereas the binary complex of RCC1-Ran associates with chromatin stably (adapted from J Cell Biol (2003) 160: 635-44).

It is possible that actinomycin D interferes with the binding of the amino terminus of RCC1 to mitotic chromosomes by intercalating to the DNA. Consequently, RanGTP production may be suppressed and thus spindle assembly would be affected. To study the effect of actinomycin D on the nature of RCC1, we examined the kinetic behaviour of RCC1-green fluorescent protein (GFP) fusion protein in HeLa cells. The RCC1-GFP construct was transfected into HeLa cells and we found that RCC1-GFP was concentrated on the mitotic chromosomes in control cells whereas it was found in both mitotic cytosol and mitotic chromosomes in actinomycin D treated cells (Figure 16A, 16B). This suggested that the binding affinity of RCC1-GFP to mitotic chromosomes was reduced in the presence of actinomycin D. To verify this idea, we examined the dissociation kinetics of RCC1-GFP using Fluorescence Loss in Photobleaching (FLIP). In a FLIP experiment, a fluorescent cell is repeatedly photobleached within a small

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region while sequential images of the whole cell are continuously collected. FLIP is useful in assessing whether RCC1-GFP undergoes interactions with chromatin that impedes its motion. Upon actinomycin D treatment, an increase in the rate of fluorescence loss was observed as compared to control, indicating that actinomycin D reduced the binding affinity of RCC1-GFP to the chromosomes in HeLa cells (Figure 16E, 16F).

Figure 16. Actinomycin D disrupts RCC1 mobility and RanGTP production. (A) Representative images of HeLa cells transfected with RCC1-GFP for control and actinomycin D treated cells. (B) Intensity profiles of live cells expressing RCC1-GFP for control and actinomycin D treated cells. (C) Fluorescence intensity (A.U.) of whole cell expressing RCC1-GFP for both actinomycin D treated cells or untreated cells were measured. Intensities were comparable between actinomycin D treated cells and control cells. (D) Western blotting shows expression

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levels of Ran, RCC1 and importin β in mitotic cells to remain constant both in control and in actinomycin D treated sets. (E) A spot marked by a white box in the mitotic cytosol was repeatedly photobleached in HeLa cells expressing RCC1-GFP. Representative FLIP images are shown. Scale bar indicates 10 μ m. (F) Fluorescence intensity of the RCC1-GFP was quantified after each bleach pulse and plotted as relative intensity versus time. (G) RanGTP production is affected under drug treatment. Histogram shows FRET efficiency calculated for mitotic cells in the absence and presence of actinomycin D. Error bars indicate standard deviation.

A recent model proposed a bimodal mechanism for RCC1 interaction with nucleosomes (Chen et al, 2007; Hao et al, 2008). This model proposes that the amino terminus tail anchors RCC1 protein to chromatin when it is not associated with Ran. This low affinity binding state is converted to one of higher affinity when Ran binds during the nucleotide exchange cycle. As such, the amino terminal region and its modified state are important for chromatin binding. We have created several RCC1 mutants with amino acid substitutions in the N terminal region that reportedly blocked methylation required for association with chromatin (Figure 17A) (Chen et al, 2007). Wild type RCC1, S2A and S2P retained N terminal methylation, while P3Q, K4Q and ASPK are unmethylated, presumably because putative methylation sites that serve as a recognition motif for N terminal methylation have been mutated. Expression levels of various mutants are comparable in HeLa cells and intensity profiles show generally more diffuse localization as observed in live cells expressing these constructs (Figure 17B, 17C). Fluorescence recovery after photobleaching (FRAP) and FLIP experiments suggest that abolished N terminal methylation will lead to decreased binding to chromatin as seen in mutants P3Q, K4Q and ASPK (Figure 17D). We noticed this observation is similar with our earlier results obtained after actinomycin D treatment

and this might hint at a possible way through which actinomycin D disrupts RCC1 binding, which is by blocking N terminal tail association.

The application of FRAP allows investigation of the diffusion and mobility of fluorescently labeled proteins. In a FRAP experiment, the fluorescently tagged protein is photobleached at a defined region of interest (ROI) and recovery is followed over time at low laser intensity. The working principle for FLIP has been elaborated in section 3.1.1. FLIP is closely related to and complements FRAP data. FLIP follows the path of the bleached fluorophore while FRAP measures the recovery of fluorescence in the bleached region.

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Figure 17. Expression profiles and kinetic behaviour of RCC1 N terminal methylation mutants in HeLa cells. (A) Schematic diagram showing RCC1 domain structure consisting of a short sequence at the N-terminus, followed by seven RCC1 repeats. Various mutations are introduced at the N terminus as shown. (B) RCC1 N terminal mutants show comparable levels of expression in HeLa cells. (C) Intensity profiles of wild type RCC1 and RCC1-ASPK. N-terminal methylation-deficient RCC1 mutant ASPK has lower binding affinity with chromosomes. RCC1-ASPK has an alanine residue inserted at position 2 and lacks N-terminal methylation purported to be important for association with chromosomes. RCC1-ASPK was expressed as a fusion with GFP at the C-terminus as N-terminus fusion reported blocked binding and methylation. (D) FRAP and FLIP profiles of various mutants in mitotic HeLa cells. N = 5 cells each with 200 time points.

We next investigated whether RanGTP production was affected in actinomycin D treated cells. We used YIC as a biosensor to detect the presence of RanGTP in the mitotic cells using the fluorescence energy resonance transfer (FRET) technique. The YIC probe composed of the importin β -binding domain of importin α (IBB) fused to YFP and CFP (Kalab et al, 2002) was expressed in HeLa cells. The probe is held in an extended conformation by binding of importin β in the absence of RanGTP, inhibiting occurrence of FRET. In the presence of RanGTP, the importin is released. Following which, the donor-acceptor pair can interact and give rise to FRET (Figure 18). FRET was performed by the acceptor photobleaching technique in live cells. In acceptor photobleaching FRET, energy transfer between the donor and acceptor is reduced or eliminated when the acceptor is irreversibly bleached, resulting in an increase in donor fluorescence as an indicator of physical interaction between the test proteins (Kalab et al, 2002; Karpova et al, 2003). Upon photobleaching, an increase in donor CFP fluorescence of approximately 8.7% was observed in control cells, indicating RanGTP

production. Upon drug treatment, we saw an approximately 60% reduction in FRET efficiency (Figure 16G).

Figure 18. The YIC probe which consists of the importin β -binding domain of importin β (IBB) fused to YFP and CFP (Kalab et al, 2002) was expressed in HeLa cells. The YIC probe was designed as a means to monitor the release of importin β cargoes, which is regulated by RanGTP. FRET is observed when the probe is released from importin β in the presence of RanGTP. Therefore, the probe serves as a convenient method to gauge RanGTP levels in the cell.

Collectively, these results thus indicate that RanGTP production is obliterated in HeLa cells treated with actinomycin D, presumably via its effect in the interruption of RCC1 from binding to the mitotic chromosomes. This, in turn, could result in continual sequestration of spindle assembly factors, which explains the deformed spindles and prolonged arrest.

3.1.4 Spindle Checkpoint Regulators/Kinetochores components are altered upon actinomycin D treatment

The spindle assembly checkpoint has been proposed to be bipartite, with one arm monitoring kinetochore occupancy with microtubules (Waters et al, 1998) and the other

spindle tension exerted on kinetochores (Taylor et al, 2001). We have observed an extended prometaphase in actinomycin D treated cells and this suggested that the spindle assembly checkpoint might be prolonged. Activation of spindle assembly checkpoint signaling is initiated by recruitment of a stably bound Mad1/Mad2 complex to unattached kinetochore early in prometaphase. Spindle checkpoint regulators accumulate on unattached kinetochores in close correlation with checkpoint activation. As few as one unattached kinetochore is sufficient for checkpoint activation.

It has been reported that an active spindle checkpoint is a prerequisite for cells to undergo mitotic cell death (Nitta et al, 2004). On the other hand, premature checkpoint inactivation before correct microtubule attachments to kinetochores have been achieved causes cells to exit mitosis with multinuclei or similarly undergo cell death. We reasoned that actinomycin D could interfere with the spindle checkpoint by preventing checkpoint proteins from binding to the unattached kinetochores. To test this idea, we performed double immunostaining against the centromere marker (ACA) and checkpoint protein Mad2 or BubR1. Kinetochores assembled on chromatin in nocodazole-arrested HeLa cells can be visualized as discrete foci by immunostaining with antibodies against ACA. Addition of actinomycin D did not alter ACA staining in any detectable manner, suggesting that the underlying structure of the kinetochore remains intact.

In accordance to its role in checkpoint signaling, Mad2 and BubR1 are depleted from kinetochores after microtubule capture and alignment. The overall Mad2 or BubR1

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levels at prometaphase and metaphase kinetochores were determined by normalizing their immunofluorescence intensities against ACA immunofluorescence intensity. The fluorescence signal for an average of 30 kinetochores for each sample was quantified using the Axiovision software. After a nocodazole block, we found that Mad2 was predominantly localized to the kinetochores in control cells, indicating that Mad2 is retained at kinetochores due to the lack of microtubule attachment at the kinetochores. In contrast, there was a significant reduction of Mad2 detected on kinetochores in mitotic cells treated with actinomycin D (Figure 19A, B and E). We considered two possible explanations for the lack of Mad2 accumulation at kinetochores. First, stable kinetochore-microtubule attachments could be formed in actinomycin D treated mitotic cells. Second, the drug affects Mad2 protein localization such that it has little bearing to kinetochore-microtubule attachment status. Double immunofluorescence against centromeric ACA and microtubule showed misalignment of DNA coupled with defective microtubule attachments (Figure 13D). To test if this effect was attributable to destabilization of kinetochore-microtubule attachments, we assayed for the presence of cold-stable microtubules, as kinetochore microtubules are preferentially stabilized at 4°C. In control cells, the spindle remains intact after cold treatment, with microtubule fibres clearly attached to kinetochores stained with ACA antisera. In actinomycin D treated cells, some kinetochores were observed to have no microtubules attached (Figure 20). We quantified this discrepancy and overall, actinomycin D treated cells had 29.8 ± 17.9 while control had 8.1 ± 6.3 unattached kinetochores (student's t test, $p < 0.05$).

Figure 19. The spindle checkpoint signal is sustained by the BubR1-mediated but not the Mad2-mediated pathway. Immunofluorescence images of representative cells stained for Mad2 (A) or BubR1 (C) (green), centromere marker ACA (red) and DNA (blue) in control and cells after drug treatment. (B) and (D) Selected kinetochores were magnified to show Mad2/BubR1 levels on kinetochores. (E) An average of 20 cells was selected for quantification purposes and

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fluorescence intensity of Mad2 and BubR1, respectively, was normalized against ACA intensity and presented as a ratio. Error bars indicate standard deviation. Bar, 10 μ m. (F) Western Blotting shows expression levels of Mad2 and BubR1 to remain constant in both control and actinomycin D treated mitotic lysates.

Together, this data demonstrated that upon actinomycin D treatment, mitotic cells have impaired ability to maintain stable kinetochore-microtubule interactions. Under such circumstances, a large fraction of the checkpoint protein Mad2 which is normally recruited to kinetochores was mislocalized, which could be the prerequisite for gross mis-segregation of chromosomes and premature exit from M phase with a final fate of mitotic cell death (Figure 12B and C). To further dissect the mechanism which lies behind this phenomenon, we also examined levels of BubR1, a known regulator responsible for recruiting Mad2 in early prometaphase. Quantification was done in the same manner as in the case for Mad2. BubR1 levels seemed to be unaffected after actinomycin D treatment (Figure 19C, D and E). As such, BubR1 mediated checkpoint signaling might be important in maintaining the checkpoint signal leading to mitotic catastrophe.

Figure 20. Actinomycin D destabilizes microtubule-kinetochore interactions. The presence of cold stable microtubules was checked for in control and actinomycin D treated mitotic cells. Kinetochore microtubules are stabilized at 4°C compared to interphase microtubules arrays. Representative immunofluorescence images stained for ACA (red), microtubules (green) and DNA (blue) are shown. Bar, 10µm.

3.1.5 Alteration in Mad2 dynamics in actinomycin D treated HeLa cells

Spindle assembly checkpoint components such as products of the MAD (mitotic arrest defective) genes are recruited to kinetochores in prometaphase, where they monitor the attachment of spindle microtubules to the kinetochores of sister chromatids. We probed Mad2 dynamics using FLIP. Mad2-GFP was transfected into HeLa cells and its dynamics was studied in the presence of actinomycin D. Mad2 is a dynamic protein with a turnover rate of 25s at the kinetochores (Howell et al, 2000; Howell et al, 2004). Photobleaching on Mad2-GFP bound kinetochores eliminates the stable pool and a transient component. It had been reported that ~50% of Mad2 is stably associated with unattached kinetochores, whereas a remaining 50% of Mad2 exchanges rapidly (Chan et al, 2005; Nezi et al, 2006; Poddar et al, 2005). Upon drug treatment, Mad2 dynamics showed only a slight change according to FLIP experiments (Figure 21).

Figure 21. Actinomycin D has little effect on Mad2 mobility. Fluorescence intensity of Mad2-GFP was quantified after each bleach pulse and plotted as relative intensity versus time. Bar, 10µm.

3.1.6 Dynamics and localization of the chromosomal passenger protein survivin is altered upon actinomycin D treatment during prometaphase

Previous reports have shown that a number of chromosomal passenger proteins play an important role in the spindle checkpoint, both in terms of recruitment of various checkpoint proteins such as Mad2 and BubR1, and also for correction of attachment errors.

It had been previously reported that the two proteins Aurora B and survivin show distinct kinetics during mitosis (Delacour-Larose et al, 2004; Howell et al, 2004). Survivin-GFP, in contrast to Aurora B-GFP, was shown to be highly mobile at prometaphase. Survivin, unlike Aurora B, is said to be weakly associated with the centromeric chromatin at prometaphase and this association is dependent on the presence of Aurora B.

Together with INCENP and Borealin, survivin and Aurora B form the chromosome passenger complex in human. We examined the localization of survivin and Aurora B in cells treated with actinomycin D. We performed double immunostaining against the centromere marker ACA with either survivin or Aurora B during mitosis. We observed an increased number of kinetochores positive for ACA with diffused survivin staining in actinomycin D treated cells. However, control cells show strong, focused survivin staining at kinetochores (Figure 22A and B). On the other hand, Aurora B staining did not show striking differences upon drug treatment (Figure 22C and D). This was

reflected in the quantification done in similar manner as for Mad2 and BubR1. Survivin showed a ~40% reduction in intensity in actinomycin D treated samples as compared to control whereas Aurora B showed no obvious changes in both sets (Figure 22E). Thus, actinomycin D treatment disrupted survivin, but not Aurora B localization, which could lead to chromosome missegregation and aberrant mitosis.

We studied the dynamic behaviour of survivin-GFP and Aurora B-GFP at prometaphase stage in HeLa cells by FLIP and confirmed these earlier observations. Upon actinomycin D treatment, Aurora B-GFP did not show any changes in mobility (Figure 24). Aurora B-GFP recovery kinetics is indicative of a stable association at the kinetochores. In contrast, survivin-GFP proved to be a highly mobile protein and its dynamics was further enhanced by addition of actinomycin D. (Figure 23). Taken together, actinomycin D prevents survivin from binding to kinetochores, which could affect mitotic progression.

Figure 22. Actinomycin D disrupts survivin localization in mitotic cells. Immunofluorescence images of representative cells stained for survivin (A) or Aurora B (C) (green), centromere marker ACA (red) and DNA (blue) in control and cells after drug treatment. (B) and (D) Selected kinetochores were magnified to show survivin/Aurora B levels on kinetochores. (E)

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An average of 30 cells was selected for quantification purposes and fluorescence intensity of survivin and Aurora B, respectively, was normalized against ACA intensity and presented as a ratio. Error bars indicate standard deviation. Bar, 10 μ m. (F) Western Blotting shows expression levels of survivin and Aurora B to remain constant in both control and actinomycin D treated mitotic lysates.

Figure 23. Actinomycin D disrupts survivin mobility. (A) A spot marked by a white box in the mitotic cytosol was repeatedly photobleached in HeLa cells expressing survivin-GFP. Representative FLIP images are shown. (B) Fluorescence intensity of survivin-GFP was quantified after each bleach pulse and plotted as relative intensity versus time. Bar, 10 μ m.

Figure 24. Actinomycin D has no effect on Aurora B mobility. (A) A spot marked by a white box in the mitotic cytosol was repeatedly photobleached in HeLa cells expressing Aurora B-GFP. Representative FLIP images are shown. (B) Fluorescence intensity of Aurora B-GFP was quantified after each bleach pulse and plotted as relative intensity versus time. Bar, 10µm.

3.1.7 Mitotic regulators are still intact upon actinomycin D treatment

HeLa cell lysates were prepared as described and processed for SDS-PAGE, followed by western blotting analysis. Indeed, the regulators in the Ran signaling pathway, chromosomal passenger proteins and checkpoint proteins appeared to be expressed at comparable levels as control (Figure 16D, 19F, 22F). This gives further evidence that our observations were not due to reduction in protein levels but rather, they reflect a change in the protein's subcellular localization.

3.2 JNK is a possible candidate to mediate H2AX phosphorylation and downstream intrinsic pathways leading to mitotic catastrophe

The second part of this thesis is focused on the mechanisms that preceded and culminated in mitotic cell death.

3.2.1 Highly compacted mitotic DNA can be damaged in the presence of actinomycin D

In 3.1, we examined mitotic cells for acquired DNA aberrations. Control mitotic cells generated from a 6h nocodazole block displayed little spontaneous aberrations. But mitotic figures prepared from actinomycin D treated cells showed obvious DNA lesions at the chromosomal level such as fragmentations and dicentric chromosomes (Figure 14). Previously, it has been reported that actinomycin D indirectly induces DNA double-strand breaks in interphase chromatin, when a stalled topoisomerase I collides with a replication fork (Mischo et al, 2005). We showed in this report that this antitumour agent has the ability to induce chromosomal lesions during mitosis, when the chromosomes are rigid and compact.

3.2.2 DNA damage response is activated and is irreversible

Figure 25. Induction of γ H2AX foci in actinomycin D treated cells. (A) Immunofluorescence images of representative cells stained for phosphorylated histone H2AX (γ H2AX) (green) and DNA (blue) at different time points indicated after drug treatment. (B) The percentages of γ H2AX positive mitotic cells with and without actinomycin D treatment were calculated.

Figure 26. γ H2AX localizes on chromosomes. Chromosome spread of mitotic cells after the indicated treatments were processed for immunofluorescence staining. Immunofluorescence images of representative cells stained for phosphorylated histone H2AX (γ H2AX) (red) and DNA (blue) are shown.

Figure 27. Laser-damaged mitotic cells show similar formation of γ H2AX foci on DNA. Immunofluorescence images of representative cells stained for phosphorylated histone H2AX (γ H2AX) (red), microtubules (green) and DNA (blue) are shown.

Figure 28. γ H2AX levels were upregulated in mitotic cells induced to undergo mitotic catastrophe. (A) Time line showing experimental design and sample collection. Nocodazole was added to arrest cells in mitosis. Actinomycin D or buffer was added 2 hours later and samples were collected over 2 hour intervals via mitotic shake-off. (B) γ H2AX accumulated in actinomycin D treated cells over time, but not in control.

Figure 29. γ H2AX accumulated in mitotic cells even when the death stimulus was removed. (A) Time line showing experimental design and sample collection. Nocodazole was added to arrest cells in mitosis. Actinomycin D or buffer was added at indicated time and removed after 2 hours of incubation. Cells were maintained in mitosis with the continual supply of nocodazole. Samples were collected over 2 hour intervals via mitotic shake-off. (B) γ H2AX accumulated in actinomycin D treated cells over time, but not in control.

The histone H2A variant, H2AX is a well-known marker of DSBs (Forand et al, 2004; Rogakou et al, 1998). H2AX has an extended carboxyl terminal that is characterized by a unique Ser-Gln-Glu (SQE) motif. This sequence in the C terminus of H2AX is a target for post-translational modification during DNA damage, during which serine 139 is phosphorylated, giving rise to the γ H2AX moiety (Rogakou et al, 1998). γ H2AX accumulates around sites of DNA lesions and is thought to recruit downstream repair proteins. γ H2AX formed distinct foci on the chromosomes in mitotic cells induced to undergo mitotic catastrophe (Figure 25A). Cells with three brightly stained foci were scored as γ H2AX positive. Practically all actinomycin D treated mitotic cells (100%) were decorated with this moiety, compared to approximately 13% in control mitotics (Figure 25B). Chromosome spread followed by staining for γ H2AX showed this moiety to be localized on chromosomes (Figure 26). The western blot findings corroborated with immunofluorescence data that γ H2AX levels were dramatically upregulated in actinomycin D treated mitotic lysate (Figure 28A, 28B). Additionally, we have performed similar staining against γ H2AX in laser-damaged mitotic cells whereby chromosomes were cut using high intensity laser. γ H2AX was observed to form on DNA on which the damage was inflicted upon (Figure 27).

γ H2AX facilitates post-replicative DNA repair by recruiting chromatin remodeling complexes at DSB lesions, which have been suggested to decondense chromatin and allowing access for other repair factors (Furata et al, 2003). The importance of γ H2AX in DNA repair has been implied not only by γ H2AX's role as the player which kickstarts the DNA repair signaling cascade, but dephosphorylation of γ H2AX is

required to switch off the checkpoint signal (Chowdhury et al, 2005; Nazarov et al, 2003). We were interested to know if removal of the death stimuli would furnish a chance for mitotic cells to repair their DNA. Mitotic chromosomes are highly condensed entities and are functionally and structurally different from interphase chromatin. It is therefore intriguing to think of how repair factors might get about performing their function in a compact environment. Cells were first arrested in mitosis before addition of actinomycin D, which was withdrawn after 2 hours of treatment. Fresh growth medium with nocodazole was provided subsequently to keep the cells in mitosis. γ H2AX profiles were similar to those obtained earlier (Figure 29A, 29B). This data therefore suggest that actinomycin D was sufficiently intercalated into DNA to exert a lethal effect, which could not be overridden by DNA repair factors, which might be partly due to the compact organization of mitotic chromosomes. This also implied that the repair pathway was not efficient when the cells were maintained in mitosis.

3.2.3 JNK pathway is activated and targets H2AX *in vivo*

The mitogen-activated protein kinases (MAPKs) are a family of proteins mediating distinct signaling cascades that are targets for a myriad of extracellular stimuli. It has been reported in the literature that c-Jun N terminal kinase (JNK) or stress-activated protein kinase (SAPK) is activated in response to a variety of stress factors. JNK activation is mediated by dual phosphorylation. It was previously reported that duration of JNK phosphorylation decides whether the outcome is cell cycle regulation or apoptosis (Chen et al, 1996).

Figure 30. c-jun N terminal kinase (JNK) is activated and targets H2AX *in vivo*. (A) Activation of c-Jun N-terminal kinase (JNK) in mitosis was observed in actinomycin D treated mitotic cells while control mitotic cells did not demonstrate prominent activation of JNK. (B) Phosphorylated JNK co-immunoprecipitated γ H2AX in actinomycin D treated mitotic lysate. (C) Phosphorylated JNK localizes on chromosomes and spindle microtubules. Immunofluorescence images of representative cells stained for phosphorylated JNK (red),

microtubules (green) and DNA (blue) are shown. Images were captured with same exposure times.

JNK was phosphorylated in actinomycin D treated mitotic cells but not in control cells (Figure 30A). We also observed that activation of JNK coincides with phosphorylation of histone H2AX and we proceeded to check if the two events are related. Although members of the phosphatidyl inositol 3-kinase-like serine/threonine protein kinase (PI3K) family, including ATM, ATR and DNA-PK were reported to be involved in mammalian DNA damage response, there is no direct data showing that these kinases phosphorylates H2AX *in vivo* (Bakkenist and Kastan, 2003; Burma et al, 2001; Lu et al, 2006; Park et. al, 2003; Ward and Chen, 2001; Ward et al, 2004). Furthermore, formation of γ H2AX was observed in cell lines deficient in one or three of the PI3Ks (Fernandez Capetillo et al, 2004; Koike et al, 2007; Stiff et al, 2004). JNK was previously proposed and proven to be the kinase responsible for phosphorylating the histone variant H2AX to form γ H2AX in an array of *in vitro* and *in vivo* assays (Lu et al, 2006). We did a pulldown using pJNK antibody and indeed, γ H2AX was precipitated together with pJNK in actinomycin D treated mitotic lysate (Figure 30B). This indicated physically interaction between pJNK and γ H2AX and that JNK is a potential candidate as a kinase for histone H2AX in the context of actinomycin D triggered mitotic catastrophe.

The induction of γ H2AX foci in actinomycin D treated cells demonstrated that DNA damage could occur in highly condensed DNA. It is a possibility that the signal for DNA damage is amplified by γ H2AX foci, which were created by the activity of

phosphorylated JNK. If so, JNK must localize to chromosomes for it to be of close proximity with its target substrate. JNK is associated with centrosomes throughout the cell cycle and is largely found in the cytoplasm relative to the nucleus (MacCorkle-Chosnek et al, 2001). We co-stained for phosphorylated JNK and spindle fibers after drug treatment. In actinomycin D treated cells, we observed phosphorylated JNK to be localized on chromosomes as well as on the spindle apparatus, but not in control cells (Figure 30C). This is reminiscent of the formation of γ H2AX foci on damaged DNA.

3.2.4 Caspase activation and a possible mitochondrial-linked cell death

SAPKs, as their name implies, is related primarily to the relay of stress signals in apoptotic pathways. However, in a non-stress setting, SAPKs, in particular the JNK pathway, have been suggested to contribute to proliferative responses, as suggested by genetic and biochemical evidences. Current data indicate that signaling through the JNK pathway is important in the activation of the mitochondria-dependent apoptotic pathway (intrinsic pathway) but dispensable for apoptosis induced by activation of the death receptors (extrinsic pathway).

We asked whether mitotic catastrophe displays the most prominent biochemical apoptotic hallmark – caspase activation. Caspases execute the cell's decision to undergo apoptosis. Caspases are activated proteolytically and upstream activated caspases cleave their downstream counterparts. Our data showed mitotic catastrophe to be dependent upon caspase activation. Mitotic cells treated with the DNA-damaging agent

exhibited cleaved patterns of all the effector caspases. Moreover, caspase 9, a constituent that makes up the apoptosome together with Apaf-1 and cytochrome c to cleave caspase 3 downstream of the mitochondria in response to apoptotic signals is also activated (Figure 31). Assembly of the apoptosome leads to processing of procaspase 9 to yield the large and small subunits of caspase 9. Our data hints at a possible involvement of the intrinsic pathway *via* release of mitochondrial proteins. Control mitotic cells arrested with nocodazole did not show activation of these apoptotic parameters, ruling out the possibility that mitotically arrested cells display activation of these apoptotic variables per se.

Figure 31. Caspase activation and a possible mitochondrial-linked cell death. Activation of the intrinsic pathway and downstream effector caspases were detected upon continuous treatment with nocodazole and actinomycin D in mitotic cells.

3.2.5 DFF45 (Inhibitor of DFF40) is cleaved

DNA fragmentation factor (DFF) is a heterodimeric protein composed of 45 kilodaltons (DFF45) and 40 kilodaltons (DFF40) subunits, a protein that mediates regulated DNA fragmentation and chromatin condensation in response to apoptotic signals (Widlak et

al, 2000). DFF45 is a specific molecular chaperone and an inhibitor for the nuclease activity of DFF40. Previous studies have shown that upon cleavage of DFF45 by caspase-3, the nuclease activity of DFF40 is relieved of inhibition (Liu et al, 1999). The cleaved DFF45 fragments dissociate from DFF40, allowing DFF40 to oligomerize to form a large functional complex that cleaves DNA by introducing double strand breaks (Gu et al, 1999; Inohara et al, 1999).

DFF40 in UV treated cells cleaved DNA strongly when H2AX was phosphorylated. Without H2AX phosphorylation, caspase-3/CAD (DFF40) cannot induce DNA fragmentation (Lu et al, 2006). Cleaved DFF45 accumulated in actinomycin D treated mitotic cells over time but not in control (Figure 31). Thus we hypothesize that the function of γ H2AX might not be restricted to DNA damage repair but could play a part in regulation of apoptosis.

3.2.6 Phosphorylation of histone H3 is inhibited upon DNA damage

Figure 32. Phosphorylation of histone H3 is inhibited upon DNA damage. (A) Histone H3 is dephosphorylated in actinomycin D treated mitotic cells. (B) Co-treatment with the PP1/PP2A inhibitor calyculin A (Caly A) 1 hour before harvesting mitotic cells did not restore histone H3 phosphorylation.

Phosphorylation of histone H3 at Ser10 usually begins before prophase and is required for chromosome condensation. This modification is added by chromosome passenger protein Aurora B kinase (Goto et al, 2002; Sugiyama et al, 2002). As expected, the level of phosphorylated histone H3 increased in control mitotic cells treated with nocodazole with increasing time but remained at basal level in cells exposed to actinomycin D (Figure 32A). This suggests 2 possibilities, either Aurora B was inactive or conversely, the histone H3 phosphatase PP1 is activated (Novak et al, 2003). We eliminated the second possibility as treatment with PP1/PP2A inhibitor calyculin A did not restore histone H3 phosphorylation (Figure 32B).

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Our FRAP and FLIP data showed Aurora B dynamics were largely unchanged upon exposure to actinomycin D during mitosis (Figure 24). Aurora B kinase levels remain almost constant throughout the period of time the assay was done. This ruled out the possibility that the decrease in histone H3 phosphorylation was a consequence of downregulation of its kinase, Aurora B. Previous reports have shown that poly (ADP-ribosyl)ation of Aurora B by poly (ADP-ribosyl) polymerase (PARP-1) inhibits its kinase activity. Upon DNA damage, active PARP modifies Aurora B kinase and inhibits its activity, subsequently quenching histone H3 phosphorylation and halting mitotic progression (Monaco et al, 2005). Poly (ADP-ribosyl)ation of various nuclear proteins was shown to be a necessary event for the apoptotic pathway in different cell lines, followed by caspase-3 mediated cleavage of PARP (Simbulan-Rosenthal et al, 1998). This inactivation of PARP has been proposed to prevent depletion of NAD (a PARP substrate) and ATP, which are thought to be required for later events in apoptosis (Boulares et al, 1999).

3.2.7 Dephosphorylation of Cdc2 and separase degradation indicate an attempt towards mitosis exit

Figure 33. Cdc2 is dephosphorylated and Separase is degraded upon continuous treatment with nocodazole and actinomycin D.

We observed that upon actinomycin D treatment, mitotic cells lost their Cdc2/Cdk1 phosphorylation signature that was needed to form the active mitosis promoting factor (MPF). Separase was noted to undergo cleavage, possibly via autocatalytic cleavage (Figure 33). However from our mitotic figures, sister chromatid pairs did not appear to have undergone separation (Figure 14). Hence, arrested cells did not appear to have experienced anaphase or mitotic slippage prior to mitotic catastrophe.

Separase initiates sister chromatid separation by cleaving the Scc1 subunit of cohesin and thereby resolving the glue that holds sister chromatids together. Activation of separase requires degradation of its inhibitor securin and removal of inhibitory phosphates (Holland et al, 2006). In human cells, separase activation at anaphase onset not only coincides with securin degradation and cohesin cleavage but also with cleavage of separase itself. It was shown that securin inhibits separase by blocking access of

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substrates to the active site of separase and securin degradation induces separase to undergo autocatalytic cleavage at one of three potential sites. However, separase cleavage is not essential for its activation (Waizenegger et al, 2002). A recent publication showed an additional mechanism keeps separase inactive in *Xenopus* egg extracts displaying high Cdc2/Cdk1 activity. Inhibitory phosphorylation at a specific site functions independently of securin binding and complete activation of separase is proposed to require both securin removal and separase dephosphorylation (Huang et al, 2005; Stemmann et al, 2001).

3.2.8 The mitotic checkpoint complex is disrupted

Figure 34. Disruption of interactions within mitotic checkpoint complex (MCC) among Cdc20, BubR1 and Mad2.

This led us to check whether DNA damage would affect the spindle checkpoint components as it has been shown that an active spindle checkpoint is required for mitotic cell death induced by DNA damage (Nitta et al, 2004). We have shown that the HORMA-domain containing spindle checkpoint protein Mad2 (Aravind and Koonin, 1998) lost its kinetochore localization in mitotic cells when the nocodazole block was lifted and recovery was allowed in the presence of actinomycin D treatment (Figure 19A, B and E).

Mad2, together with BubR1 are crucial members which constitute the spindle checkpoint complex, and activation of which serves to keep the anaphase promoting complex and its activator, Cdc20 in check. The mitotic checkpoint ensures accurate

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chromosome segregation during mitosis. Mutations in genes encoding essential checkpoint proteins such as BUB and MAD lead to chromosomal instability and promote cancer development (Kops et al., 2005). BUB and MAD are negative regulators of the Anaphase Promoting Complex/Cyclosome (APC/C) during mitosis to prevent cells with unaligned chromosomes from entering anaphase prematurely.

Currently, two models explain the mechanism by which the APC/C is inhibited by the checkpoint (Chan and Yen, 2003; Sudakin et al, 2001). In the Sequestration Model, Mad2 and BubR1 are postulated to bind and sequester Cdc20, an APC/C activator so that substrates whose destruction results in mitotic exit no longer ubiquitinated. Herein, Mad2 is purportedly converted to a form which binds Cdc20 by the unattached kinetochore. The Direct Inhibition Model, on the other hand, holds that the Mitotic Checkpoint Complex (MCC) consisting of BubR1, Bub3, Mad2 and Cdc20 binds and inhibits APC/C independently of the kinetochore. However, the “wait for anaphase” signal generated by unattached kinetochores might play a role in sensitizing the APC/C to prolonged inhibition by the MCC through a kinase cascade involving components such as BubR1, Bub1 and Mps1.

We have performed immunoprecipitations of endogenous components of the mitotic checkpoint complex. Briefly, mitotic cells were collected after the relevant treatments and subjected to immunoprecipitation by various antibodies against Mad2, BubR1 and Cdc20, respectively. Cdc20 association with BubR1 appeared to be reduced in actinomycin D treated mitotic cells. Reciprocal immunoprecipitations with a BubR1

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antibody showed consistent results. It was noted that the BubR1 antibody did not pick up Mad2 as an interaction partner of BubR1 (Figure 34). This data lends support to the hypothesis that there exists diffusible complexes of the mitotic checkpoint that is not dependent on kinetochore localization and this, in conjunction with our previous immunofluorescence data, could imply that the diffusible and kinetochore checkpoint complexes might complement each other's activity (Fang et al, 2002). Meanwhile, interactions between freely diffusible Cdc20 and Mad2 did not demonstrate any major changes (Figure 34). All in all, these data further substantiate a weakened but still functional checkpoint signal and cooperative interaction between the two arms of the spindle checkpoint to maintain mitotic arrest preceding catastrophic cell death.

4. Discussion and Conclusion

Chemotherapeutic agents target cancer cells, which divide more robustly than normal cells. Cancer cells have defective cell cycle checkpoints, which allow for certain deregulated events during the cell cycle to go unchecked. Actinomycin D was the first antibiotic to display anticancer properties and have been used for treatment of a wide range of tumours, including retinoblastoma, Kaposi's sarcoma ecetera. Actinomycin D is an example of a cell cycle specific chemotherapeutic agent. It has been known to cause severe damage to genetic material. In interphase cells where transcription occurs to form messenger RNAs, which would be translated into protein molecules later on. Actinomycin D actively inhibits the machinery that powers this event, namely RNA Polymerase. This agent intercalates in between DNA base pairs and forces the Polymerase to fall off, thereby halting transcription (Cooper & Braverman, 1977). Apart from actively inhibiting cellular activities, actinomycin D also causes physical lesions, for example double-stranded breaks in interphase chromatin. Topoisomerase 1, which resolves kinks in DNA, is stalled in its tracks upon encountering intercalated drug molecules and hence, single-stranded nicks in the DNA are not repaired (Mischio et al., 2005). When a replication fork approaches the region, these single-stranded breaks are

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converted into double-stranded lesions, which have been shown to be the most lethal of DNA lesions.

Interestingly however, actinomycin D effects on mitotic cells have not been well-documented. Since its discovery and isolation in 1940, relatively few studies have been aimed at understanding its mechanism of action during mitosis. One particular study published four decades ago reported that actinomycin D treatment induced formation of sticky chromatids, especially at the distal segments, which seemed to have difficulties separating in anaphase; while separated proximal segments were stretched. When treated cells were grown in drug-free medium for several days, high frequencies of endoduplicated mitotic figures were found. In addition to that, chromosome and chromatid breaks and other aberrations were commonly observed (Pathak et al, 1975). Another group observed that mitosis was delayed in the presence of actinomycin D in *Physarum sp.* (Mittermayer et al, 1964; Nair et al, 1991). These few, but insightful studies constituted the first evidence that actinomycin D led cells to undergo mitotic catastrophe, a term that was coined much later on in 1986 to describe the observation that yeast cells with an activated *cdc2/cdk1* allele and a mutation in the *wee1* kinase entered mitosis prematurely but failed to complete it (Russell and Nurse, 1986).

The classification of different forms of cell death has been fine-tuned in recent years. Arguments against the existence of a single active form of cell death suggest that there are many different forms of cell death like terminal differentiation, micronucleation, mitotic catastrophe, or multinucleation, that are regulated by different pathways. Mitotic catastrophe can result from a combination of deficient mitotic checkpoints in tumour

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cells, anti-microtubular drugs and premature mitosis (Castedo et al, 2004). It has been proposed that the irreversibly damaged cell has a broad repertoire of 'final options' that allow it to react to certain stressors, according to the multiposition switch model of cell death (Abend, 2003). This idea is consistent with the concept that a decrease in apoptosis is compensated by increase in the fraction of cells that undergo permanent growth arrest with phenotypic features such as that of mitotic catastrophe.

In this report, we investigated mechanisms that preceded and culminated in cell death in a model of mitotic catastrophe induced by actinomycin D in HeLa cells. We have shown that the drug dosage and duration used caused cells to undergo mitotic arrest followed by cell death. In the first part of this report, we have studied contributions of various mitotic regulators in the event of prolonged arrest as a consequence of disrupted spindle assembly followed by mitotic catastrophe. We highlight the importance of the localization of these regulators onto the mitotic chromosomes during mitosis.

RanGTP has been reported independently in many sources to play a role in providing a directional bias in guiding microtubules to kinetochore regions (Carazo-Salas et al, 2001; Dasso, 2002; Gruss et al, 2002; Li et al, 2004; Torosantucci et al, 2008). A model for RCC1 function, the sole guanine nucleotide exchange factor for Ran, describes a coupled mechanism between interaction with chromosomes and production of RanGTP (Li et al, 2003). RCC1 binds to chromatin *via* its amino terminal region, which harbours a nuclear localization signal (Moore et al, 2002; Nemergut et al, 2000). It is possible that administration of a general DNA intercalator might inhibit the binding of RCC1 to chromatin thus impeding its function. Under normal circumstances, chromosome-bound

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RCC1 generates locally high RanGTP concentration. RanGTP binds to the importins and by which, releases spindle assembly factors (SAFs) such as TPX2 and NuMA from importins to facilitate bipolar spindle formation (Kalab et al, 2002; Nachury et al, 2001). With actinomycin D, formation of RanGTP is compromised. SAFs are inhibited by importin sequestration and cannot take part in organizing microtubules to form a functional mitotic spindle (Figure 35).

Figure 35. A model explaining the mechanism how disruption of the mitotic RanGTP gradient would lead to defects in bipolar spindle formation.

This obviously has reverberating consequences to the dividing cell. For one, the spindle assembly checkpoint is dis-satisfied and the cell is trapped in limbo until all chromosomes can be captured and tethered to kinetochore spindle fibers, of which the accuracy may be questionable. This later transmits into aneuploid cells, should they succeed in escaping elimination. The prolonged arrest could be due to activation of the spindle checkpoint regulated by the BubR1 arm. The spindle checkpoint kinase BubR1

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was detected at kinetochores during prometaphase while Mad2 levels at kinetochores appeared to be significantly reduced in Actinomycin D treated cells.

Mad2 is a highly conserved protein from yeast to vertebrates. This is consistent with its pivotal role in mitotic checkpoint signaling. The HORMA domain (for Hop1p, Rev7p and MAD2) was found to be conserved and homology has been established in yeast proteins involved in DNA repair. HORMA-domain-containing proteins share a common feature, that is, they are able to associate directly with chromatin (Aravind & Koonin, 1998). In sharp contrast, protein-protein interaction seems to be responsible for BubR1 recruitment to the checkpoint signaling complex. Recruitment of checkpoint proteins has to occur in a timely and orderly fashion and through RNAi studies, the protein kinase Bub1 is localized to the kinetochores prior to other components, which follows in the sequence CENP-F, BubR1, CENP-E and Mad2 (Johnson et al, 2004). However, the structural details of how the various components interact with one another have yet to be elucidated and more effort is called for in order to gain a better grasp of the big picture.

Many proteins that function during mitosis relocate to the mitotic apparatus after nuclear envelope breakdown. The chromosomal passenger proteins show unique localization patterns throughout different phases of mitosis (Fortugno et al, 2002). They regulate multiple and diverse aspects of mitosis. Aurora B provides the enzymatic core whilst survivin dictates localization of the chromosomal passenger complex. Deletion mutants of survivin lacking the Baculovirus IAP Repeat (BIR) domain failed to localize properly to the inner centromere. The CPC has been shown to play a role in regulation

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of the spindle checkpoint, indirectly through correction of attachment errors and directly by stably targeting of checkpoint protein BubR1 and Mad2 to kinetochores. Extrapolating from this point, the CPC, in particular survivin, has a role in prevention of mitotic catastrophe. Hence, a decrease in survivin targeting to the kinetochores will impact all these processes and drive the cell towards cell death.

In a way, RCC1, Mad2 and survivin can be considered chromosome-binding proteins and it is logical that intercalation of actinomycin D into DNA disrupts their binding and in turn, affect their biological activity. We have also shown that our observations were not due to the reduction in protein expression level after drug treatment.

Proteins are dynamic entities. The cell assembles its paraphernalia prior to entering mitosis and the proteins move in an orchestrated fashion to fulfill their respective designated roles. This underscores the importance of the ability of certain proteins to relocate to specific locations in the cell or to act in a timely fashion for execution of vital processes needed for activation of downstream effectors. We studied the kinetic behaviour of these groups of proteins using photobleaching techniques. Among the four proteins, Aurora B dynamics seemed to be unchanged after actinomycin D treatment in prometaphase cells thereby implying a tight association with the kinetochores. In contrast though, RCC1, Mad2 and survivin kinetics were accelerated, albeit to varying extents. RCC1 catalyzes the production of RanGTP which has multiple functions in the mitotic cell while Mad2 and the chromosomal passenger proteins survivin and Aurora B are well-documented in the literature for their roles in checkpoint signaling, chromosome segregation and correction of attachment errors. Thus, our results

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demonstrate that protein mobility has far reaching consequences, especially in cases where catalysis is the outcome. Changes in protein mobility accompany changes in conformation during the trans-activation of enzymes, which in turn strongly correlate with the microscopic rates of substrate turnover (Hoofnagle et al, 2001; Eisenmesser et al, 2002).

In the second part of this thesis, we explored whether DNA breakage caused by actinomycin D is reparable and we are interested in the downstream events incurred after detection of DNA lesions. Understanding how DNA damage can lead to cell death will provide valuable information in the design of therapeutic agents. Here, we propose that γ H2AX may serve as an apoptotic signal in addition to its role in the recruitment of DNA repair factors (Ismail and Hendzel, 2008).

Two conceptual models linking DNA repair, cell cycle arrest and cell death responses to DNA damage have been proposed. The concept of integrative surveillance revolves around the idea that cells die when the damage is irreparable and the signal that controls the choice between cell cycle arrest and cell death comes from a signaling network that continually monitors DNA integrity. Although there is no direct biochemical evidence as to how the said network distinguishes between 'reparable' and 'irreparable' damage, this intuitive model remain a plausible explanation for the cell's decision to die in the event of DNA damage. The second model proposed that death signaling pathways are integrated with intrinsic delay mechanisms. DNA repair and cell death are activated simultaneously by the DNA damage signaling network. Several factors may account for the delay onset of cell death, i.e. a requirement for new protein synthesis, built-in

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requirements for negative feedback loops to be overcome and for positive feedforward loops to raise death signals above a threshold that can repress existing survival signals. Based on this model, the continuous and repeated activation of death signaling pathways, resulting from the continuous presence of DNA lesions, is necessary to either stimulate the positive feedforward or inhibit the negative feedback loops, and thus death is a delayed response that correlates with the persistence of DNA damage.

γ H2AX foci labeled damaged sites on DNA. It is widely accepted that γ H2AX foci recruit DSB repair proteins and dissolve when repair is completed. Actinomycin D treated mitotic cells exhibited chromosomal abnormalities, which accumulated over time. In a mitotic cell, the chromosomes are packed tightly into a highly compacted structure. Hence, DNA repair factors may have a tougher time to access damaged sites. Homologous recombinational repair or even DNA end-joining cannot occur efficiently. γ H2AX levels increased and persisted, regardless of whether the damaging stimuli was maintained or removed from cells, indicating inability to repair the damage and to switch off the positive signal for downstream death signaling. The continuous propagation of this signal may lead to activation of downstream caspase-related cascades.

When cytotoxic drugs were first generated, they were believed to mainly target cancer cells because of their rapid proliferation rate. Today, we understand this view is simplistic and inadequate to explain the complex cellular processes that drive tumorigenesis. Cytotoxic agents owe their efficacy not only to the fact that they cause

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cellular injury directly, but also because of the stress response being set into motion as a secondary effect of the insult afflicted upon genetic material or cytoskeleton.

SAPKs are related primarily to the relay of stress signals in apoptotic pathways. Signaling through the JNK pathway is important in the activation of the mitochondria-dependent apoptotic pathway (intrinsic pathway) but dispensable for apoptosis induced by activation of the death receptors (extrinsic pathway). We have investigated the effect of MAPKs on the cellular decision to undergo catastrophic cell death. MAPKs exert broad range of effects on cellular activities. Among them, SAPK/ JNK is activated by stress imposed on cells. The exact effects of JNK on cellular events have not been fully elucidated although JNK1 is mainly associated with the induction of apoptosis while JNK2 seems to have an effect on anaphase progression. JNK activity was induced more rapidly in mitosis compared to that during interphase, and activity peaked at 4 hours and assumed low level activation after at 8 hours. Mitotic cells seem more 'potentiated' than cells in other phases of the cell cycle, i.e. they have higher sensitivity towards stress stimuli.

SEK1 (MKK4), the upstream activator of JNK, has been implicated as a prostrate cancer metastasis suppressor gene (Yoshida et al, 1999). This, among other evidence, has provided support for an emerging view that active SAPKs can suppress cancer progression to advanced stages. A novel JNK substrate in apoptotic cells, histone variant H2AX has been previously linked to caspase-activated DNase (CAD)-mediated fragmentation of chromosomal DNA. JNK phosphorylates H2AX at a non-canonical

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site for MAPKs. We show that active JNK co-immunoprecipitates with γ H2AX in mitotic extracts treated with actinomycin D. It is tempting to speculate that JNK might be in part, responsible for the phosphorylation of H2AX and thereby, facilitate the propagation of a positive signal for cell death, when repair is not achieved.

Mitosis is a valuable target for anticancer drug design. Hence, the cellular determinants that specify drug sensitivity is of great interest to researchers as well as the pharmaceutical industry. The mitotic checkpoint is likely to be a major contributor, as it delays mitotic exit so that cells have extra time to correct incorrect microtubule-kinetochore attachments. Mitotic cells die without completing mitosis after undergoing a delay at prometaphase. As the mitotic checkpoint activity is not infinite, cells cannot remain arrested indefinitely, as seen in cases where cells are treated with anti-mitotic drugs. When checkpoint activity is lost, mitosis exit occurs and cells become aneuploid, of which many would eventually die while those that survived would be selected. As such, the mechanisms and events that ultimately dictate that the cell would die in mitosis are important for us to understand drug sensitivity of cancer cells.

In our model of cell death, cells try to exit mitosis by attempts to switch off the mitotic spindle checkpoint. This is evidenced by the degradation of the checkpoint protein BubR1 and depletion of Mad2 from the kinetochores. However, we hypothesized that a functional checkpoint signal carried by both kinetochore-dependent and -independent complexes exist, complying with the notion that the spindle checkpoint is a prerequisite to drive the cells toward mitotic catastrophe. Another study conducted using taxol to challenge the cells concluded that mitotic slippage, as contrary to mitotic arrest,

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was responsible for apoptotic cell death induced by nanomolar concentrations of the drug (Chen et. al, 2003). Events such as dephosphorylation of histone H3 and Cdc2/Cdk1 as well as degradation of anaphase inhibitor separase point toward an attempt to exit mitosis. Histone H3 has been postulated to adopt a more closed configuration when it is phosphorylated on the N terminal tail as the negative charge on the phosphate group neutralizes the positive charge on the N terminal lysines. Dephosphorylation of histone H3 brings about global decondensation of chromosomes, which, is assumed to be a more practical setting for cells to repair damaged DNA. On the other hand, Cdc2/Cdk1 was activated for prolonged time during the mitotic arrest before it underwent dephosphorylation, an event which may prime the cells for apoptosis.

Hereby, we propose a model of mitotic catastrophe in HeLa cells induced by actinomycin D whereby persistent DNA damage invoked stress responses mediated by the SAPK/JNK. The signal relayed by γ H2AX amplifies the initial insult and drives the cell to activate downstream apoptotic pathways by the intrinsic mechanism. The cell attempts to exit mitosis as a default setting in the event of DNA damage, as uncondensed, relaxed chromatin is more accessible for repair factors. However, the remaining checkpoint signal carried by kinetochore-dependent and -independent mechanisms appear to be sufficient to put a restraining hand on entry into anaphase and the cell eventually dies in mitosis.

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PUBLICATIONS AND PAPERS

Perturbation of the chromosomal binding of RCC1, Mad2 and survivin causes spindle assembly defects and mitotic catastrophe

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Apoptotic histone modification inhibits nuclear transport by regulating RCC1

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DNA damage during mitosis invokes a JNK-mediated stress response pathway that leads to cell death

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Perturbation of the Chromosomal Binding of RCC1, Mad2 and Survivin Causes Spindle Assembly Defects and Mitotic Catastrophe

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ABSTRACT

Mitotic catastrophe is a form of cell death that results from aberrant mitosis. Currently, the mechanisms involved in this form of cell death remain poorly understood. We found that actinomycin D induces mitotic catastrophe with severe spindle assembly defects. We have studied the nature of three groups of chromosome binding proteins in mitotic cells treated with actinomycin D. We found that actinomycin D reduced the binding affinity of RCC1 to the mitotic chromosome, which led to a reduction of RanGTP level. In addition, Mad2 was not concentrated at the kinetochores, indicating that the mitotic spindle checkpoint was affected. Furthermore, the localization of survivin was altered in cells. These data suggested that chromosomal binding of the mitotic regulators such as RCC1, Mad2 and survivin is essential for mitotic progression. Mitotic chromosomes not only carry the genetic material needed for the newly synthesized daughter cells, but also serve as docking sites for some of the mitotic regulators. Perturbation of their binding to the mitotic chromosome by actinomycin D could affect their functions in regulating mitotic progression thus leading to severe spindle defects and mitotic catastrophe. *J. Cell. Biochem.* 105: 835–846, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: SPINDLE ASSEMBLY; RCC1; SURVIVIN; MAD2; MITOTIC CATASTROPHE; ACTINOMYCIN D

Mitotic catastrophe is a form of cell death that results from aberrant mitosis. It has been described in the literature that mitotic catastrophe is death that occurs during mitosis, resulting from a combination of deficient cell cycle checkpoints and cellular damage [Castedo et al., 2004]. The definitions of mitotic catastrophe encompass a variety of phenotypes including those presenting with cellular demise in mitosis or following G1 exit after an aberrant mitosis. Generally, such cells may present with multi- or micro-nucleated phenotype after errors in the checkpoint sensing mechanism or die with hallmarks of apoptosis in cases that cell cycle checkpoints have been bypassed. Very little is known about the mechanism(s) that underlies these responses. Nevertheless, it is agreed upon that cell cycle checkpoints come into play upon sensing of genotoxic insult and hold the cell in arrest until eventual activation of cell death pathways. After mitotic arrest, a cell could exit mitosis to enter G1 followed by reentering the cell cycle, senescence, or apoptosis. Alternatively, a cell might die during a mitotic arrest [Weaver and Cleveland, 2005].

Recently, it has been demonstrated that Polo-like kinase 1 (Plk1) activity is inhibited through dephosphorylation of Plk1 when DNA is damaged in mitosis [Jang et al., 2007]. In addition, a chromosome passenger protein, survivin, is important in protecting the cells from entering mitotic catastrophe [Carvalho et al., 2003; Lens et al., 2003]. These studies suggested that mitotic regulators could play important roles in regulating mitotic catastrophe.

The mitotic chromosome is a highly condensed entity and many of the mitotic regulators are found on it. While cohesins hold sister chromatids together by encircling two DNA duplexes within its coiled-coil arms, condensin I and II regulate mitotic chromosome condensation through provision of a centrally located protein scaffold [Losada and Hirano, 2005; Belmont, 2006]. In addition, chromokinesins, HKIF4A and Kid, bind to chromosome arms. Depletion of HKIF4A has been shown to result in mitotic defects such as chromosome misalignment and spindle abnormalities [Mazumdar et al., 2004; Zhu et al., 2005].

Abbreviations used: GDP, guanosine diphosphate; GTP, guanosine triphosphate; GEF, guanine nucleotide exchange factor; DMEM, Dulbecco's Modified Eagles's Medium; DAPI, 4,6-diamidino-2-phenylindole.

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Chromatin-bound RCC1 generates a mitotic RanGTP gradient to regulate spindle formation. Ran is a small GTPase, which exists in GDP- and GTP-bound forms regulated by guanine nucleotide exchange factor for Ran, RCC1 and RanGTPase activating protein, RanGAP1, respectively. During mitosis, a number of spindle assembly factors are sequestered by importin β binding. RanGTP generated by chromatin-bound RCC1 releases the spindle assembly factors from the inhibitory binding of importin β near the chromosomes [Trieselmann and Wilde, 2002; Tsai et al., 2003; Gruss and Vernos, 2004]. Recently, it has been shown that dynamic interaction of RCC1 with the mitotic chromosome is essential to generate RanGTP gradient in mitosis. RCC1 interacts with histone H2A/H2B of the nucleosomes. This specific interaction enhances RCC1 GEF activity by 20% [Nemergut et al., 2001]. In addition, a model has been proposed in which the binary complex of RCC1-Ran has better affinity for the core histones on the chromatin than RCC1 or Ran alone. The stable binding of the binary complex could allow coupling of nucleotide exchange. Upon successful nucleotide exchange, the two proteins would have reduced binding affinity thus enabling them to dissociate from chromatin, driving RanGTP production on the chromatin surface [Li et al., 2003, 2007]. Post-translational modification of RCC1 could regulate its binding to the chromatin, which in turn affects the RanGTP production. For instance, phosphorylation of RCC1 at serine 11 enhances the binding affinity of RCC1 to the chromatin by preventing the inhibitory binding of importin β [Li and Zheng, 2004; Li et al., 2007]. N-terminal α -methylation of RCC1 is required for stable chromatin association in order to generate RanGTP to sustain the formation of spindle during mitosis [Chen et al., 2007].

Kinetochore constituents such as those involved in checkpoint signaling (Mad and Bub proteins) and those comprising the mammalian chromosomal passenger complex are dynamic entities that coordinate numerous events driving mitotic progression. Their dynamic behavior and unique localization on the mitotic apparatus are two criteria central to their respective functions [Maiato et al., 2004].

Checkpoints constitute a surveillance mechanism to ensure the fidelity of chromosome segregation [Shah and Cleveland, 2000; Musacchio and Hardwick, 2002; Maiato et al., 2004]. Checkpoint proteins localize to the outer plate of the kinetochore, a large assembly of >60 proteins, where they monitor microtubule attachments to kinetochores and mediate checkpoint signaling. The checkpoint signal is generated at the kinetochore and as few as one unattached kinetochore is sufficient to initiate checkpoint activation [Chan and Yen, 2003; Maiato et al., 2004]. The spindle checkpoint proteins Bub1, Bub3, Mad1, Mad2 and BubR1 only localize to kinetochores during mitosis [Shah and Cleveland, 2000; Musacchio and Hardwick, 2002]. The chromosomal passenger complex (CPC) in humans comprise of four members, that is, the aurora B serine/threonine kinase, and three non-enzymatic subunits survivin, the inner centromere protein (INCENP) and Borealin/Dasra-B. The CPC proteins show distinct localization to the mitotic apparatus: they move towards the inner centromeric chromatin from chromosome arms during prometaphase, relocate to the central spindle at the metaphase-anaphase transition, and finally concentrate at the midbody during telophase/cytokinesis [Fortugno

et al., 2002]. This localization is correlated to their role in orchestrating various processes during mitosis, such as chromosome alignment, histone modification, and cytokinesis. Central to these functions is the enzymatic activity of aurora B kinase, which in turn relies on proper and timely localization of this complex throughout cell division [Carvalho et al., 2003].

In this report, we found that actinomycin D induces mitotic catastrophe with severe spindle defects. Actinomycin D is an antibiotic produced by *Streptomyces antibioticus*. This molecule consists of a phenoxazone ring system to which two cyclic pentapeptides are attached. The aromatic ring system is well apt to intercalate into DNA, preferably at GC steps [Hou et al., 2002]. This mode of action interferes with the action of the transcriptase and replicase complexes and also prevents chromosome-binding proteins from interacting with chromatin. Interestingly, we found that chromosomal binding of mitotic regulators such as RCC1, Mad2 and survivin is essential for mitotic progression. Perturbation of these interactions by actinomycin D would lead to failure of mitosis and thus induce mitotic catastrophe.

MATERIALS AND METHODS

CELL CULTURE, TRANSFECTION AND DRUG TREATMENTS

HeLa cells were maintained in DMEM GlutaMax medium (Gibco, Invitrogen) containing 10% fetal calf serum (Hyclone), and 1% Penicillin/Streptomycin at 37°C in a humidified atmosphere with 5% carbon dioxide. Transfection was done using Lipofectamine[®] Reagent (Invitrogen) according to manufacturer's protocol. 1 μ g of plasmid DNA was used per transfection reaction. For drug treatment, HeLa cells were treated with 600 nM nocodazole (Sigma, MO) for 6 h and drug washout was done by incubating the cells in DMEM for 30 min with three changes of medium. For actinomycin D (Sigma) treatment, cells were supplemented with 400 nM actinomycin D 2 h into nocodazole treatment and following nocodazole washout, cells were allowed to recover in the presence of 400 nM actinomycin D.

IMMUNOFLOURESCENCE STAINING AND TIME LAPSE MICROSCOPY

For immunofluorescence staining, cells were collected and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Primary antibodies in 4% bovine serum albumin in Tris-buffered saline (TBS) with 0.05% Tween 20 were incubated for 1 h at room temperature or overnight at 4°C. Cells were washed three times and incubated with appropriate secondary antibodies for 1 h at room temperature. Slides were mounted in Prolong Gold Anti-Fade medium with DAPI (Invitrogen). Images were collected and analyzed on an Axiovert 200 M inverted fluorescence microscope (Carl Zeiss Inc., Germany). For live cell imaging, an Axiovert 200 M inverted fluorescence microscope (Carl Zeiss Inc.) equipped with a 10 $\times$ phase contrast objective (Carl Zeiss Inc.) was used. Cells were maintained at 37°C with 5% CO₂ for the duration of the time-lapse acquisition. Images were recorded at 5 min intervals and analyzed using the Axiovision software (Carl Zeiss Inc.). For imaging of live cells expressing RCC1-GFP, images were obtained using same exposure

times for each channel, offsetting any effect the difference in expression levels might have on the outcome.

FLUORESCENCE LOSS IN PHOTOBLEACHING

Cells were plated on a coverslip and mounted onto a glass slide with a depression containing culture medium. FLIP were performed on a Zeiss 510 LSM confocal microscope (Carl Zeiss Inc.) using the 488 nm laser line of an argon laser. For FLIP experiments, five single scans were acquired, followed by repeated photobleaching using a single bleach pulse at intervals of 1 s for 200 iterations in defined regions. Single section images were then collected at 1 s intervals. For imaging, the laser power was attenuated to 1% of the bleach intensity. The relative fluorescence intensity in a region of interest was determined by normalizing fluorescence intensity in the region to the total fluorescence in the same region during prebleach.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

FRET detection was performed on a Zeiss 510 LSM confocal microscope (Carl Zeiss Inc.) via the Acceptor Photobleaching method of FRET detection. Fluorescence of donor cyan fluorescent protein (CFP) and acceptor yellow fluorescent protein (YFP) in the YIC probe both before and after photobleaching of the YFP fluorophore were recorded. CFP was excited by 458 nm light and the emission was collected through an HQ 470–500 nm bandpass filter. YFP was excited by 514 nm light and the emission was collected through an HQ 525–575 nm bandpass filter. Photobleaching of YFP was performed by repeatedly scanning a region of the specimen with the 514 nm laser line set at maximum intensity to photobleach the original acceptor fluorescence. The fluorescence emission from the donor and the acceptor are collected sequentially using the multitrack function of the Zeiss LSM 510 software. Average fluorescence intensities of the donor are measured before and after bleaching. FRET efficiency was calculated as

$$E_F (\%) = (I_6 - I_5) \times \frac{100}{I_6}$$

where I_5 and I_6 represent the CFP fluorescence intensity of the fifth and sixth images immediately before and after photobleaching of YFP (Karpova et al., 2003). The change in donor fluorescence was assessed in at least 10 cells for each experimental set. Mean FRET efficiencies were then reported $\pm$ standard error.

RESULTS

ACTINOMYCIN D INDUCES MITOTIC CATASTROPHE IN HeLa CELLS

We have tested a panel of cytotoxic drugs to induce mitotic catastrophe. Surprisingly, we found that actinomycin D was able to cause cell death in mitosis, as opposed to its effect on interphase cells that seemed predominantly apoptotic after prolonged treatment. To investigate the effect of the actinomycin D on mitotic cells, we arrested HeLa cells stably expressing histone H2B-GFP in mitosis by incubating them in 600 nM nocadazole for 2 h before actinomycin D was added. The cells were released from nocadazole arrest by thorough washing with normal medium and recovery was followed using time-lapse microscopy in the absence or presence of actinomycin D (Fig. 1A). We found that treatment of actinomycin

D caused significant cell death during mitosis or cytokinesis ($\sim 70\%$). In contrast, there was only about 12% of cell death during mitosis or cytokinesis in control experiments. H2B-GFP serves as a marker to visualize chromatin. Several anomalies were observed more frequently in actinomycin D treated cells. Some cells reverted to interphase with multinucleated phenotype while others appear to die in G1 phase after cell division has occurred, however, the segregation of chromosomes was clearly asymmetric. We also observed cases in which actinomycin D treated cells undergo division before proper metaphase alignment had been achieved, resulting in mis-segregation and finally death (Fig. 1B,C).

ACTINOMYCIN D AFFECTS SPINDLE ASSEMBLY DURING MITOSIS

It is prudent to speculate that disrupted spindle assembly might plausibly account for the cell death in mitotic progression. Cells were fixed and stained with histone H3 phosphorylated at Ser10 and tubulin after nocadazole washout at the indicated time points as shown in Figure 2A. Phosphorylation of histone H3 occurs exclusively as an event in mitosis. Hence, pH3 serves as a mitosis marker. The cells were co-stained with anti- α tubulin antibodies to visualize the spindle.

We found that most of the cells treated with actinomycin D remained at prometaphase whilst majority of the control cells went on to complete cell division within 2 h after nocadazole washout (Fig. 2B). This observation is consistent with time lapse experiments performed earlier. Approximately, actinomycin D treatment caused two folds more of cells exhibiting severe spindle assembly defects as compared with control one hour after nocadazole washout (Fig. 2C). Furthermore, most of the kinetochores were not attached to microtubules in cells treated with actinomycin D. This is probably due to a decrease in kinetochore fibers emanating from the spindle poles that were able to capture chromosomes successfully. These data suggested that actinomycin D inhibited spindle assembly during mitosis (Fig. 3).

It has been well documented that actinomycin D inhibits transcription by preventing transcription factors from binding to the chromosomes thus leads to apoptosis. Structural analysis also revealed that actinomycin D intercalates to the DNA through the GC steps [Hou et al., 2002]. We believed that actinomycin D could intercalate to the DNA of mitotic chromosomes and thereby knocking off some of the essential chromosomal binding proteins for spindle assembly. To test this hypothesis, the nature of several chromosomal binding proteins, which are essential for spindle assembly and mitotic progression, were analyzed.

ACTINOMYCIN D DISRUPTS RCC1 MOBILITY AND RanGTP PRODUCTION

Recent studies have revealed that a signaling pathway mediated by RanGTPase plays an important role in mitosis [Li et al., 2003]. In *Xenopus* egg extracts, microtubule dynamics and spindle assembly are dependent upon the concentration of RanGTP [Dasso, 2002]. It is believed that chromatin-associated RCC1 protein creates an increased local concentration of RanGTP in the vicinity of chromosomes, helping to direct the formation of bipolar spindles.

RCC1 is associated with the chromosome throughout the cell cycle [Li et al., 2003]. The N-terminus of RCC1 binds to DNA and the

Fig. 1. Actinomycin D induces mitotic catastrophe in HeLa cells. A: Schematic diagram showing the nocodazole arrest and release experiment. Cells were incubated with 600 nM nocodazole for 2 h and 400 nM actinomycin D was added for an additional 4 h before nocodazole washout was done by washing with complete medium. Recovery was allowed in the presence or absence of 400 nM actinomycin D. B: Time lapse experiment was carried out using a H2B-GFP stable cell line. Representative cells displaying different phenotypes in control and actinomycin D treated samples are shown in phase contrast, GFP and merged views. Scale bar indicates 20 μ m. C: Quantification of various phenotypes observed. An average of 100 mitotic cells were quantified in three independent time-lapse experiments.

rest of the protein associates with chromatin via an interaction with histones H2A and H2B. Binding of the histones stimulates a modest increase in RCC1's activity as Ran's GEF [Nemergut et al., 2001]. Recently, it has been suggested that the binary complex of RCC1-

Ran has better affinity for the core histones on the chromatin than RCC1 or Ran alone [Li et al., 2003]. The stable binding of the binary complex could allow coupling of nucleotide exchange to the chromatin. Upon successful nucleotide exchange, the two proteins

Fig. 2. Actinomycin D affects spindle assembly during mitosis. A: Schematic diagram showing the nocodazole arrest and release experiment. B: Immunofluorescence images of representative cells stained for microtubules (green), phosphorylated histone H3 (red) and DNA (blue) at different time points indicated after drug treatment. C: Spindle morphologies in control and actinomycin D treated cells. Spindles are shown in green and DNA is shown in blue. The percentages of normal bipolar spindles (upper row) and abnormal spindles (bottom row) were calculated. Scale bar indicates 10 μ m.

would have reduced binding affinity thus enabling them to dissociate from chromatin, driving RanGTP production on the chromatin surface.

It is possible that actinomycin D interferes the binding of the N-terminus of RCC1 to the mitotic chromosome by intercalating to DNA. Consequently, RanGTP production could be suppressed and thus spindle assembly could be also affected. To study the effect of

actinomycin D on the nature of RCC1, we examined the kinetic behavior of RCC1-green fluorescent protein (GFP) fusion protein in HeLa cells. The RCC1-GFP construct was transfected into HeLa cells. Statistically, the average total intensity are comparable, suggesting the expression levels of RCC1-GFP in cells treated with or without actinomycin D are similar (Fig. 4C). Interestingly, however, we found that RCC1-GFP was concentrated on the mitotic chromo-

Fig. 3. Microtubule attachments to kinetochores appear to be disrupted in actinomycin D treated cells. Microtubules are shown in green and the centromere marker ACA in red. Insets show magnified regions boxed in white. DAPI staining is not shown. Scale bar indicates 10 μ m.

somes in control cells whereas it was found in both mitotic cytosol and on the mitotic chromosomes in actinomycin D treated cells (Fig. 4A,B). This suggested that the binding affinity of RCC1-GFP to mitotic chromosomes was reduced in the presence of actinomycin D. To verify this idea, we examined dissociation kinetic of RCC1-GFP by Fluorescence Loss in Photobleaching (FLIP). In a FLIP experiment, a fluorescent cell is repeatedly photobleached within a small region while sequential images of the whole cell are continuously collected. Upon actinomycin D treatment, an increase in the rate of fluorescence loss was observed as compared with control, indicating that actinomycin D increased the dissociation kinetic of RCC1-GFP from the mitotic chromosomes (Fig. 4E,F). Additionally, immunoblots for Ran, RCC1 and importin β in mitotic cells showed that their protein levels remained constant for both control and actinomycin D treated cells (Fig. 4D).

We next investigated whether RanGTP production was affected in actinomycin D treated cells. We used YIC as biosensor to detect the presence of RanGTP in the mitotic cells. The YIC biosensor is held in an extended conformation by binding of importin β in the absence of RanGTP, inhibiting occurrence of FRET. In the presence of RanGTP, the importin is released. Following which, donor-acceptor pair can interact and give rise to FRET. Cells were transfected with YIC followed by fluorescence resonance energy transfer (FRET) experiments. FRET experiments were performed by the acceptor photobleaching technique. In acceptor photobleaching FRET, energy transfer between the donor and acceptor is reduced or eliminated when the acceptor is irreversibly bleached, resulting in an increase in donor fluorescence as an indicator of physical interaction between the test proteins [Kalab et al., 2002; Karpova et al., 2003]. Upon drug treatment, we found that there was a

significant reduction in FRET efficiency as compared to control (Fig. 4G).

Collectively, these results indicated that RanGTP production is obliterated in HeLa cells treated with actinomycin D, presumably via its effect in the interruption of RCC1 binding to mitotic chromosomes. This, in turn, could result in continual sequestration of spindle assembly factors, which explains the deformed spindles and prolonged arrest.

ACTINOMYCIN D DISPLACED Mad2 FROM THE KINETOCHORES

The spindle assembly checkpoint has been proposed to be bipartite, with one arm monitoring kinetochore occupancy with microtubules [Waters et al., 1998] and the other spindle tension exerted on kinetochores [Taylor et al., 2001]. Extended prometaphase in actinomycin D treated cells suggested that mitotic delay could be due to activation of the spindle assembly checkpoint. Activation of spindle assembly checkpoint signaling is initiated by recruitment of a stably bound Mad1/Mad2 complex to unattached kinetochore early in prometaphase [Chung and Chen, 2002; De Antoni et al., 2005]. Spindle checkpoint regulators accumulate on unattached kinetochores in close correlation with checkpoint activation. As few as one unattached kinetochore is sufficient for checkpoint activation [Rieder et al., 1995; Rieder et al., 1997].

It has been reported that premature spindle checkpoint inactivation causes cells to exit mitosis with multi-nuclei or mitotic cell death [Castedo et al., 2004]. We reasoned that actinomycin D could interfere with the spindle checkpoint by preventing checkpoint proteins from binding to the unattached kinetochores and thus initiate mitotic cell death as shown in Figure 1B,C. To test this idea, we performed double immunostaining against the centromere marker ACA and checkpoint protein Mad2 or BubR1. Kinetochores assembled on chromatin in nocodazole-arrested HeLa cells can be visualized as discrete foci by immunostaining with antibodies against centromeric proteins (ACA). Addition of actinomycin D did not alter ACA staining in any detectable manner, suggesting that the underlying structure of the kinetochore remains intact.

In accordance to its role in checkpoint signaling, Mad2 and BubR1 are depleted from kinetochores after microtubule capture and alignment. The overall Mad2 or BubR1 level at prometaphase kinetochores was determined by normalizing their immunofluorescence intensities against ACA immunofluorescence intensity. We found that Mad2 was predominantly localized to the kinetochores in control cells, indicating that Mad2 is retained at kinetochores due to the lack of microtubule attachment at the kinetochores. In contrast, there was a significant reduction of Mad2 levels in cells treated with actinomycin D. (Fig. 5A,B,E). To further dissect the mechanism which lies behind this phenomenon, we also examined levels of BubR1, a known regulator responsible for recruiting Mad2 in early prometaphase. BubR1 levels seemed to be unaffected after actinomycin D treatment (Fig. 5C–E). Expression levels for both proteins were confirmed by immunoblotting (Fig. 5F). We believe that kinetochores devoid of Mad2 could indicate that attachments can be established in the presence of actinomycin D. We have previously shown that microtubule attachments to kinetochores were more often found to be defective in the presence of actinomycin D (Fig. 3). This led us to the hypothesis that

Fig. 4. Actinomycin D disrupts RCC1 mobility and RanGTP production. A: Representative images of HeLa cells transfected with RCC1-GFP for control and actinomycin D treated cells. B: Intensity profiles of live cells expressing RCC1-GFP for control and actinomycin D treated cells. C: Fluorescence intensity (AU) of whole cell expressing RCC1-GFP for both actinomycin D treated cells or untreated cells were measured. Intensities were comparable between actinomycin D treated cells and control cells. D: Western blotting shows expression levels of Ran, RCC1 and importin β in mitotic cells to remain constant both in control and in actinomycin D treated sets. E: A spot marked by a white box in the mitotic cytosol was repeatedly photobleached in HeLa cells expressing RCC1-GFP. Representative FLIP images are shown. F: Fluorescence intensity of the RCC1-GFP was quantified after each bleach pulse and plotted as relative intensity versus time. RanGTP production is affected under drug treatment. G: Histogram shows FRET efficiency calculated for mitotic cells in the absence and presence of actinomycin D. Error bars indicate standard deviation. Scale bar indicates 10 μ m.

Fig. 5. The spindle checkpoint signal is sustained by the BubR1-mediated but not the Mad2-mediated pathway. Immunofluorescence images of representative cells stained for Mad2 (A) or BubR1 (C) (green), centromere marker ACA (red) and DNA (blue) in control and cells after drug treatment. B,D: Selected kinetochores were magnified to show Mad2/BubR1 levels on kinetochores. E: An average of 20 cells was selected for quantification purposes and fluorescence intensity of Mad2 and BubR1, respectively, was normalized against ACA intensity and presented as a ratio. Error bars indicate standard deviation. F: Western blotting shows expression levels of Mad2 and BubR1 in mitotic cells to remain constant both in control and in actinomycin D treated sets. Scale bar indicates 10 μ m.

Fig. 6. Actinomycin D disrupts survivin localization in mitotic cells. Immunofluorescence images of representative cells stained for survivin (A) or aurora B (C) (green), centromere marker ACA (red) and DNA (blue) in control and cells after drug treatment. B,D: Selected kinetochores were magnified to show survivin/aurora B levels on kinetochores. E: An average of 30 cells was selected for quantification purposes and fluorescence intensity of survivin and aurora B, respectively, was normalized against ACA intensity and presented as a ratio. Error bars indicate standard deviation. F: Western blotting shows expression levels of survivin and aurora B in mitotic cells to remain constant both in control and in actinomycin D treated sets. Scale bar indicates 10 μ m.

kinetochores are unable to recruit comparable levels of Mad2, which could signify a weak checkpoint signal that is easily bypassed, resulting in premature exit from mitosis or initiation of mitotic cell death (Fig. 1B,C).

LOCALIZATION OF SURVIVIN WAS ALTERED UPON ACTINOMYCIN D TREATMENT

Previous reports have shown that a number of chromosomal passenger proteins play important roles in the spindle checkpoint, both in terms of recruitment of various checkpoint proteins such as Mad2 and BubR1, and also for correction of attachment errors [Vagnarelli and Earnshaw, 2004].

Together with INCENP and Borealis, survivin and aurora B form the chromosome passenger complex in human. We examined the localization of survivin and aurora B in cells treated with actinomycin D. We performed double immunostaining against the centromere marker ACA with either survivin or aurora B during mitosis. We observed an increased number of kinetochores positive for ACA with diffused survivin staining in actinomycin D treated cells. However, there was a strong focused survivin staining at the kinetochore in control cells (Fig. 6A,B,E). On the other hand, Aurora B staining did not show striking differences upon drug treatment (Fig. 6C–E) whereas both survivin and aurora B levels did not change after drug treatment (Fig. 6F). Thus, actinomycin D treatment disrupted survivin localization but not aurora B, which could lead to chromosome mis-segregation and aberrant mitosis.

DISCUSSION

Mitotic catastrophe can result from a combination of deficient mitotic checkpoints in tumor cells, anti-microtubular drugs and premature mitosis. It has been proposed that the irreversibly damaged cell has a broad repertoire of “final options” that allow it to react to certain stressors, according to the multiposition switch model of cell death [Abend, 2003]. This idea is consistent with the concept that a decrease in apoptosis is compensated for by increase in the fraction of cells that undergo permanent growth arrest with phenotypic features such as that of mitotic catastrophe [Roninson et al., 2001].

In interphase cells, actinomycin D causes cell death by repressing gene transcription through its intercalating action into DNA. In mitotic cells, however, gene expression is minimal mainly due to the highly compressed state of chromatin in forming the mitotic chromosomes. Thus, the concerns of actinomycin D usage in interphase cells do not greatly limit our model in this respect.

In this report, we have studied contributions of various mitotic regulators in the event of prolonged arrest as a consequence of disrupted spindle assembly followed by mitotic catastrophe. RanGTP has been reported independently in many sources to play a role in providing a directional bias in guiding microtubules to kinetochore regions [Gruss et al., 2001; Gruss and Vernos, 2004]. A model for RCC1 function, the sole guanine nucleotide exchange factor for Ran, describes a coupled mechanism between

interaction with chromosomes and production of RanGTP [Li et al., 2003]. RCC1 binds to chromatin via its N-terminal region. It is possible that administration of a general DNA intercalator might inhibit the binding of RCC1 to chromatin thus impeding its function.

Many proteins that function during mitosis relocate to the mitotic apparatus after nuclear envelope breakdown. The prolonged arrest could be due to activation of the spindle checkpoint regulated by the BubR1 arm [Skoufias et al., 2001; Shannon et al., 2002]. The spindle checkpoint kinase BubR1 was detected at kinetochores during prometaphase while Mad2 levels at kinetochores appeared to be significantly reduced in actinomycin D treated cells. Mad2 is a highly conserved protein from yeast to vertebrates. This is consistent with its pivotal role in mitotic checkpoint signaling. The HORMA domain (for Hop1p, Rev7p and MAD2) was found to be conserved and homology has been established in yeast proteins involved in DNA repair. HORMA-domain-containing proteins share a common feature, that is, they are able to associate directly with chromatin [Aravind and Koonin, 1998]. In sharp contrast, protein-protein interaction seems to be responsible for BubR1 recruitment to the checkpoint signaling complex [Johnson et al., 2004; Bolanos-Garcia et al., 2005]. Recruitment of checkpoint proteins has to occur in a timely and orderly fashion and through RNAi studies, the protein kinase Bub1 is localized to the kinetochores prior to other components, which follows in the sequence CENP-F, BubR1, CENP-E and Mad2 [Johnson et al., 2004]. However, the structural details of how the various components interact with one another have yet to be elucidated and more effort is called for in order to gain a more wholesome understanding of the detailed mechanism.

The chromosomal passenger proteins show unique localization patterns throughout different phases of mitosis [Adams et al., 2001]. They regulate multiple and diverse aspects of mitosis. Aurora B provides the enzymatic core whilst survivin dictates localization of the chromosomal passenger complex [Lens et al., 2006]. Deletion mutants of survivin lacking the Baculovirus IAP Repeat (BIR) domain failed to localize properly to the inner centromere [Vader et al., 2006]. Therefore, in a way, RCC1, Mad2 and survivin can be considered chromosome-binding proteins and it is logical that intercalation of actinomycin D into DNA disrupts their binding and in turn, affect their biological activity.

Proteins are dynamic entities. The cell assembles its paraphernalia prior to entering mitosis. Proteins move in an orchestrated fashion to fulfill their respective designated roles. This highlights the importance of the ability of certain proteins to relocate to specific locations in the cell or to act in a timely fashion for execution of vital processes needed for activation of downstream effectors. We studied the kinetic behavior of several proteins using photobleaching techniques. RCC1 catalyzes the production of RanGTP, which has multiple functions in the mitotic cell. Thus, our results demonstrate that protein mobility has far reaching consequences, especially in cases where catalysis is the outcome. Changes in protein mobility accompany changes in conformation during the trans-activation of enzymes, which in turn strongly correlate with the microscopic rates of substrate turnover [Eisenmesser et al., 2002; Hoofnagle et al., 2001].

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DNA^{Q1} Damage During Mitosis Invokes a JNK-Mediated Stress Response That Leads to Cell Death

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*Division of Molecular and Cell Biology, School of Biological Sciences, College of Science, Nanyang Technological University, Singapore 637551, Singapore***ABSTRACT**

Mitotic catastrophe is a phenomenon displayed by cells undergoing aberrant mitosis to eliminate cells that fail to repair the errors. Why and how mitotic catastrophe would lead to cell death remains to be resolved and the answer will prove valuable in design of better therapeutic agents that specifically target such cells in mitosis. The antibiotic actinomycin D has been shown to induce chromosomal lesions in lower order organisms as well as in human interphase cells. Relatively few studies have been conducted to elucidate molecular events in the context of mitotic DNA damage. We have previously established a model of mitotic catastrophe in human HeLa cells induced by actinomycin D. Here, we show that actinomycin D induce cellular stress via DNA damage during mitosis. The higher order packing of chromosomes during mitosis might impede efficient DNA repair. γ H2AX serves as a marker for DNA repair and active JNK interacts with γ H2AX in actinomycin D-treated mitotic extracts. We believe JNK might be in part, responsible for the phosphorylation of H2AX and thereby, facilitate the propagation of a positive signal for cell death, when repair is not achieved. The mitotic cell activates JNK-mediated cell death response that progresses through a caspase cascade downstream of the mitochondria. In the mean time, remaining checkpoint signals may be sufficient to put a restraining hand on entry into anaphase and the cell eventually dies in mitosis. *J. Cell. Biochem.* 9999: 1–8, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: MITOSIS; MITOTIC CATASTROPHE; JNK; γ H2AX; ACTINOMYCIN D

Less is known on mitotic catastrophe although much effort has been directed towards understanding the molecular mechanisms behind this phenomenon. Defined loosely, mitotic catastrophe is a phenomenon displayed by cells that undergo aberrant mitosis. It has been associated with the abnormal activation of cyclin B/Cdk1 and cells that survived the selection process often emerge with abnormalities in nuclear structure and organization [Castedo et al., 2002, 2004]. It has become apparent in recent years that chemotherapy and radiation induce antiproliferative responses, cell death through mitotic catastrophe being one of them. Failure to arrest cell-cycle progression and eliminate these events can lead to chromosomal instability that is likely to contribute to oncogenesis.

The mitogen-activated protein kinase (MAPK) family member, c-jun N-terminal kinase (JNK1/2) is primarily activated by cytokines and environmental stress. JNK signaling has been implicated in the decision between cell survival and apoptosis. A new JNK substrate has been identified, that is phosphorylated in apoptotic cells—H2AX, a histone H2A variant [Lu et al., 2006]. The site of

phosphorylation on H2AX corresponds to a non-canonical site for MAPK phosphorylation. In response to DNA damage, phosphorylated H2AX accumulates at the site of double strand breaks (DSBs), where it is thought to restructure chromatin and assist in the recruitment of DNA repair and signaling factors. Lu et al. [2006] showed that JNK-mediated H2AX phosphorylation may be essential for DNA fragmentation in UV-stimulated cells. However, the bulk of the work was aimed at elucidating DNA damage in interphase cells. Relatively little was understood about the same events in a mitotic context.

Actinomycin D was the first antibiotic to display anticancer properties and have been indicated for treatment of a wide range of tumors, including retinoblastoma, Kaposi's sarcoma ecetera. Actinomycin D is an example of a cell-cycle-specific chemotherapeutic agent. It has been known to cause severe damage to genetic material. One study published four decades ago reported that actinomycin D treatment-induced formation of sticky chromatids, especially at the distal segments that seemed to have difficulties

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separating in anaphase; while separated proximal segments were stretched. When treated cells were grown in drug-free medium for several days, high frequencies of endoduplicated mitotic figures were found. In addition to that, chromosome and chromatid breaks and other aberrations were commonly observed [Pathak et al., 1975]. Another group observed that mitosis was delayed in the presence of actinomycin D in *Physarum* sp. [Vimala Nair and Indirabai, 1991]. These few, but insightful studies constituted the first evidence that actinomycin D led cells to undergo mitotic catastrophe, a term that was coined much later on in 1986.

We have previously established a model of mitotic catastrophe in the human cervical cancer cell line HeLa induced following treatment with the DNA intercalator actinomycin D [Ho et al., 2008]. In this report, we investigated the cellular stress response to DNA damage introduced by actinomycin D during mitosis, a unique time frame of the cell cycle whereby genetic material is condensed into rigid, tightly packed chromosomes. γ H2AX serves as a marker for DNA repair. The higher order packing of chromosomes during mitosis renders the repair mechanism inefficient. The mitotic cell activates JNK-mediated cell death response that progresses through a caspase cascade downstream of the mitochondria.

MATERIALS AND METHODS

ANTIBODIES

Antibodies to Actin, Cyclin B1, and Cdc2 p34 HRP were purchased from Santa Cruz (Beverly, MA). Antibodies to phospho-Histone H3 were from Calbiochem[®] (San Diego, CA), MAD2 were from Covance (Denver, PA), Tubulin-fluorescein isothiocyanate (FITC) were from Sigma (MO), AIM-1/Aurora B and BubR1 antibodies were from BD Transduction Laboratories (Palo Alto, CA), and Novus Biologicals (Littleton, CO). Cdc20 antibodies were from MBL International. Separase antibodies were from Abcam. γ H2AX, H2AX, phospho-Cdc2, Cleaved ICAD/DFP40 antibodies, and JNK Phosphoplu Kit were from Cell Signaling (Danvers, MA). Secondary antibodies conjugated with appropriate fluorophores were purchased from Invitrogen (Carlsbad, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Dakocytomation (Glostrup, Denmark).

CELL CULTURE AND DRUG TREATMENTS

HeLa cells were maintained in DMEM GlutaMax medium (Gibco, Invitrogen) containing 10% fetal calf serum (Hyclone), and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% carbon dioxide. For drug treatment, HeLa cells were treated with 600 nM nocodazole (Sigma). For actinomycin D (Sigma) treatment, cells were supplemented with 400 nM actinomycin D for the required periods. Time points were collected at 2 h intervals.

IMMUNOFLUORESCENCE STAINING

For immunofluorescence staining, cells were collected and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Primary antibodies in 4% bovine serum albumin in Tris-buffered saline (TBS) with 0.05% Tween-20 were incubated for 1 h at room temperature or overnight at 4°C. Cells were washed three

times and incubated with appropriate secondary antibodies for 1 h at room temperature. Slides were mounted in Prolong Gold Anti-Fade medium with DAPI (Invitrogen). Images were collected and analyzed on an Axiovert 200 M inverted fluorescence microscope (Carl Zeiss, Germany).

WESTERN BLOTTING

Mitotic cells were collected via shake-off and lysed in M-PER Mammalian Lysis Buffer (Pierce, Rockford, IL) and samples were boiled for 5 min before being subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to nitrocellulose membrane. HRP-conjugated secondary antibodies were from Dakocytomation. Detection was via chemiluminescence using ECL reagent from GE Healthcare (Little Chalfont, Buckinghamshire, UK) or Thermo-Scientific (Rockford, IL).

IMMUNOPRECIPITATION

HeLa cells were lysed with Pierce M-PER Mammalian Lysis Buffer. Lysates were clarified by centrifugation at 13,200 rpm for 10 min at 4°C. Clarified lysates were incubated with primary antibody at 4°C overnight. Rec. Protein G-Sepharose[®] 4B (Invitrogen) was then added to the lysates and the mixture was incubated for an additional 2 h. After that, immunoprecipitates were washed five times with wash buffer (1× TBS with 250 mM NaCl) and proteins were recovered by boiling the beads in sample buffer and analyzed by Western blotting.

RESULTS

HIGHLY COMPACTED MITOTIC DNA CAN BE DAMAGED IN THE PRESENCE OF ACTINOMYCIN D

We examined mitotic cells for acquired DNA lesions. HeLa cells were treated with nocodazole and actinomycin D and mitotic cells were collected by mitotic shake-off at the indicated time point in Figure 1A. More than 95% of cells isolated by this method were arrested in mitosis (Supplementary Fig. 1). Mitotic figures prepared from actinomycin D-treated cells showed obvious DNA damage at the chromosomal level such as fragmentation and formation of dicentric chromosomes. Control mitotic chromosomes, on the other hand, were compact and tightly condensed and displayed little spontaneous aberrations (Fig. 1B). Previously, it has been reported that actinomycin D indirectly induces DNA double-strand breaks in interphase chromatin, when a stalled topoisomerase I collides with a replication fork [Mischo et al., 2005]. We showed in this report that this antitumor agent has the ability to induce chromosomal lesions during mitosis, when the chromosomes are rigid and compact.

H2AX IS PERSISTENTLY PHOSPHORYLATED EVEN WHEN THE GENOTOXIC AGENT IS REMOVED

The histone H2A variant, H2AX is a well-known marker of double-stranded DNA breaks [Rogakou et al., 1998; Forand et al., 2004]. H2AX has an extended carboxyl terminal that is characterized by a unique Ser-Gln-Glu (SQE) motif. This sequence in the C terminus of H2AX is a target for post-translational modification during DNA damage, during which serine 139 is phosphorylated, giving rise to the γ H2AX moiety. γ H2AX accumulates around sites of DNA lesions

Fig. 1. A: Timeline showing experimental design. Cells were arrested using nocodazole before actinomycin D was introduced. Mitotic cells were collected via shake-off at 2-h intervals. B: Mitotic chromosomes exhibit chromosomal breaks and other aberrations upon treatment with actinomycin D. Bar, 5 μm. C: γH2AX recruitment to discrete foci on chromosomes in mitotic cells damaged by actinomycin D. Representative cells were displayed with DNA in blue and γH2AX in green, respectively. Bar, 10 μm. D: Mitotic cells with ≥3 discrete foci were categorized as γH2AX positive. Cell counting was done in a sample of at least 30 mitotic cells and the data were represented as a bar chart.

and is thought to recruit downstream repair proteins. γH2AX formed distinct foci on the chromosomes in mitotic cells induced to undergo mitotic catastrophe (Fig. 1C). Cells with three brightly stained foci were scored as γH2AX positive. Practically all actinomycin D-treated mitotic cells (100%) were decorated with this moiety, compared to ~13% in control mitotic cells (Fig. 1D). The Western blot findings corroborated with immunofluorescence data that γH2AX levels were dramatically upregulated in actinomycin D-treated mitotic lysate (Fig. 2A).

γH2AX facilitates post-replicative DNA repair by recruiting chromatin remodeling complexes at DSB lesions, which have been suggested to decondense chromatin and allowing access for other repair factors [Furuta et al., 2003]. The importance of γH2AX in DNA repair has been implied not only by γH2AX role as the player which kickstarts the DNA repair signaling cascade, but dephosphorylation of γH2AX is required to switch off the checkpoint signal [Nazarov et al., 2003; Chowdhury et al., 2005]. We were interested to know if removal of the death stimuli would furnish a chance for mitotic cells to repair their DNA. Mitotic chromosomes are highly condensed entities and are functionally and structurally different from interphase chromatin. It is therefore intriguing to think of how repair factors might get about performing their function in a compact environment. Cells were first arrested in mitosis before addition of actinomycin D, which was withdrawn after 2 h of treatment. Fresh growth medium with nocodazole was provided subsequently to keep the cells in mitosis. γH2AX profiles were similar to that obtained earlier (Fig. 2B,C). This data therefore

suggest that the repair pathway was not efficient when the cells were maintained in mitosis, which might be partly due to the compact organization of mitotic chromosomes.

JNK PATHWAY IS ACTIVATED AND TARGETS H2AX IN VIVO

The MAPKs are a family of proteins mediating distinct signaling cascades that are targets for a myriad of extracellular stimuli. It has been reported in the literature that JNK or stress-activated protein kinase (SAPK) is activated in response to a variety of stress factors. JNK activation is mediated by dual phosphorylation on Thr and Tyr residues by the MAPK kinases MKK4 and MKK7 [Ip and Davis, 1998]. It was previously reported that duration of JNK phosphorylation decides whether the outcome is cell-cycle regulation or apoptosis [Chen et al., 1996].

JNK was phosphorylated in actinomycin D-treated mitotic cells but not in control cells (Fig. 3B). We also observed that activation of JNK coincides with phosphorylation of histone H2AX and we proceeded to check if the two events are related. Although members of the PI3K family, including ATM, ATR, and DNA-PK were reported to be involved in mammalian DNA damage response, there is no direct data showing that these kinases phosphorylates H2AX in vivo [Burma et al., 2001; Ward and Chen, 2001; Bakkenist and Kastan, 2003; Park et al., 2003; Ward et al., 2004; Lu et al., 2006]. Furthermore, formation of γH2AX was observed in cell lines deficient in one or three of the PI3Ks [Fernandez-Capetillo et al., 2002; Koike et al., 2007]. JNK was previously proposed and proven to be the kinase responsible for phosphorylating the histone variant

Fig. 2. A: γ H2AX expression levels accumulated in actinomycin D-treated mitotic cells over time but not in control mitotic cells. B: Timeline showing experimental design. Cells were arrested in mitosis using nocodazole. Subsequently, actinomycin D was added for 2 h before washing out. Cells were collected in the continued presence of mitotic block via mitotic shake-off at 2-h intervals. C: Gamma H2AX expression persists even though actinomycin D had been removed.

H2AX to form γ H2AX in an array of in vitro and in vivo assays [Lu et al., 2006]. We did a pulldown using pJNK antibody and indeed, γ H2AX was precipitated together with pJNK in actinomycin D-treated mitotic lysate (Fig. 3C) but not in control. This indicated physical interaction between pJNK and γ H2AX and that JNK is a potential candidate as kinase for histone H2AX in the context of actinomycin D-triggered mitotic catastrophe.

The induction of γ H2AX foci in actinomycin D-treated cells demonstrated that DNA damage could occur in highly condensed DNA. It is a possibility that the signal for DNA damage is amplified by γ H2AX foci, which was created by the activity of phosphorylated JNK. If so, JNK must localize to chromosomes for it to be of close proximity with its target substrate. JNK is associated with centrosomes throughout the cell cycle and is largely found in the cytoplasm relative to the nucleus [MacCorkle-Chosnek et al., 2001]. We co-stained for phosphorylated JNK and spindle fibers after drug treatment. In actinomycin D-treated cells, we observed phosphorylated JNK to be localized on chromosomes as well as on the spindle apparatus, but not in control (Fig. 3A). This is reminiscent of the formation of γ H2AX foci on damaged DNA.

CASPASE ACTIVATION AND A POSSIBLE MITOCHONDRIAL-LINKED CELL DEATH

SAPKs, as their name implies, is primarily related to the relay of stress signals in apoptotic pathways. However, in a non-stress

Fig. 3. A: Phosphorylated JNK co-localized onto chromosomes and partially with the spindle apparatus. Cells were treated as illustrated in Figure 1A and immunofluorescence staining was done using antibodies against microtubules (green) and phosphorylated JNK (red). Samples were counter-stained with DAPI. Bar, 10 μ m. B: Mitotic cells treated with actinomycin D showed activation of JNK1/2 over time, but not in control. C: Phosphorylated JNK co-immunoprecipitated γ H2AX in actinomycin D-treated mitotic lysate but not in control lysate. Mitotic cells were collected 6 h (*) after of mitotic arrest by mitotic shake-off and subjected to immunoprecipitation with 4 μ g of anti-pJNK antibody and probed for γ H2AX and H2AX, respectively. Quantification was done using ImageJ software.

setting, SAPKs, in particular the JNK pathway, have been suggested to contribute to proliferative responses, as suggested by genetic and biochemical evidences. Current data indicate that signaling through the JNK pathway is important in the activation of the mitochondria-dependent apoptotic pathway (intrinsic pathway) but dispensable for apoptosis induced by activation of the death receptors (extrinsic pathway).

We asked whether mitotic catastrophe displays the most prominent biochemical apoptotic hallmark—caspase activation. Caspases execute the cell's decision to undergo apoptosis. Caspases are activated proteolytically and upstream-activated caspases cleave their downstream counterparts. Our data showed mitotic catastrophe to be dependent upon caspase activation. Mitotic cells treated with the DNA-damaging agent exhibited cleaved patterns of all the effector caspases, including caspases 3, 6, and 7. The downstream effector caspases cleave key proteins involved in apoptosis and they play non-redundant roles in the execution of apoptosis. Caspases 6 and 7 have been suggested to compensate for

the lack of caspase 3. For example, MCF-7 cells, which lack caspase 3, undergo mitochondrial-dependent apoptosis via caspase 7 activation [Twiddy et al., 2006]. Moreover, caspase 9, a constituent that makes up the apoptosome together with APAF-1 and cytochrome c to cleave caspase 3 downstream of the mitochondria in response to apoptotic signals is also activated (Fig. 4A). Assembly of the apoptosome leads to processing of procaspase 9 to yield the large and small subunits of caspase 9. Our data hints at a possible involvement of the intrinsic pathway via release of mitochondrial proteins. Control mitotic cells arrested with nocodazole did not show activation of these apoptotic parameters, ruling out the possibility that mitotically arrested cells display activation of these apoptotic variables per se.

INHIBITOR OF CASPASE-ACTIVATED DNase, DFF45 IS CLEAVED
DNA fragmentation factor (DFF) (human homologue of murine ICAD) is a heterodimeric protein composed of 45-kDa (DFF45) and 40-kDa (DFF40) subunits, a protein that mediates regulated DNA fragmentation and chromatin condensation in response to apoptotic signals [Widlak, 2000]. DFF45 is a specific molecular chaperone and an inhibitor for the nuclease activity of DFF40. Previous studies have shown that upon cleavage of DFF45 by caspase 3, the nuclease activity of DFF40 is relieved of inhibition [Liu et al., 1999]. The cleaved DFF45 fragments dissociate from DFF40, allowing DFF40 to oligomerize to form a large functional complex that cleaves DNA by introducing DSBs [Gu et al., 1999; Inohara et al., 1999].
DFF40 in UV-treated cells cleaved DNA strongly when H2AX was phosphorylated. Without H2AX phosphorylation, caspase 3/CAD (DFF40) cannot induce DNA fragmentation [Lu et al., 2006]. Cleaved DFF45 accumulated in actinomycin D-treated mitotic cells over time

but not in control (Fig. 4A). Thus, this gives us an indication that the function of γ H2AX might not be restricted to DNA damage repair but could play a part in regulation of apoptosis.

SUPPRESSION OF HISTONE H3 AND Cdc2 PHOSPHORYLATION UPON DNA DAMAGE

Phosphorylation of histone H3 is a marker of mitotic cells. Phosphorylation of histone H3 at Ser10 usually begins before prophase. This modification is added by chromosome passenger protein Aurora B kinase [Goto et al., 2002; Sugiyama et al., 2002]. The level of phosphorylated histone H3 increased in control mitotic cells treated with nocodazole with increasing time but remained at basal level in cells exposed to actinomycin D. Cdc2 activation is another indication of the cell's mitotic status. We observed that upon actinomycin D treatment, these mitotic cells lost their Cdc2 phosphorylation signature that was needed to form the active mitosis promoting factor (MPF) (Fig. 4B,C). Taken together, they indicate the mitotic cells are attempting to exit mitosis.

MITOTIC CHECKPOINT COMPLEX IS DISRUPTED

We wondered whether DNA damage would affect the spindle checkpoint components as it has been shown that an active spindle checkpoint is required for mitotic cell death induced by DNA damage [Nitta et al., 2004]. BubR1 and Mad2 are crucial members that constitute the spindle checkpoint complex, and activation of which serves to keep the anaphase promoting complex and its activator, Cdc20 in check. The mitotic checkpoint ensures accurate chromosome segregation during mitosis. Mutations in genes encoding essential checkpoint proteins such as BUB and MAD lead to chromosomal instability and promote cancer development [Kops

Fig. 4. A: Caspase cascades are activated in actinomycin D-treated mitotic cells, but not in control. Cell death occurs via a mitochondria-related pathway. Caspase substrate DFF45 was also cleaved. B: Histone H3 phosphorylation is suppressed in mitotic cells in the presence of actinomycin D. C: Levels of various mitotic regulators were checked in actinomycin D-treated mitotic cells. Cdc2 was dephosphorylated and separase cleavage occurred. D: Interactions within the mitotic checkpoint complex was disrupted. Immunoprecipitation reactions were done using 1.5 mg/ml mitotic lysate. Forty micrograms of lysate were loaded to check for protein expression.

et al., 2005]. BUB and MAD are negative regulators of the Anaphase Promoting Complex/Cyclosome (APC/C) during mitosis to prevent cells with unaligned chromosomes from entering anaphase prematurely.

We have performed endogenous immunoprecipitations of components of the mitotic checkpoint complex. Briefly, mitotic cells were collected after the relevant treatments and subjected to immunoprecipitation by various antibodies against Mad2, BubR1, and Cdc20, respectively. Cdc20 association with phosphorylated form of BubR1 appeared to be reduced in actinomycin D-treated mitotic cells. Reciprocal immunoprecipitations with a BubR1 antibody showed consistent result. It was noted that the BubR1 antibody did not pick up Mad2 as an interaction partner of BubR1. These data lend support to the hypothesis that there exists diffusible complexes of the mitotic checkpoint that is not dependent on kinetochore localization and this, in conjunction with our previous immunofluorescence data [Ho et al., 2008], could imply that the diffusible and kinetochore checkpoint complexes might complement each other's activity [Fang, 2002]. Meanwhile, interactions between freely diffusible Cdc20 and Mad2 did not demonstrate any major changes (Fig. 4D). All in all, these data further substantiate a weakened but still functional checkpoint signal and cooperative interaction between the two arms of the spindle checkpoint to maintain mitotic arrest preceding catastrophic cell death.

CLEAVAGE OF ANAPHASE REGULATOR SEPARASE

Separase initiates sister chromatid separation by cleaving the Scc1 subunit of cohesin and thereby resolving the glue that holds sister chromatids together. Activation of separase requires degradation of its inhibitor securin and removal of inhibitory phosphates [Holland and Taylor, 2006]. In human cells, separase activation at anaphase onset not only coincides with securin degradation and cohesin cleavage but also with cleavage of separase itself. It was shown that securin inhibits separase by blocking access of substrates to the active site of separase and securin degradation induces separase to undergo autocatalytic cleavage at one of three potential sites. However, separase cleavage is not essential for its activation [Waizenegger et al., 2002]. Separase was noted to undergo cleavage, possibly via autocatalytic cleavage (Fig. 4C). However, from our mitotic figures, sister chromatid pairs did not appear to have undergone separation (Fig. 1B). A recent publication showed an additional mechanism keeps separase inactive in *Xenopus* egg extracts displaying high Cdc2 activity. Inhibitory phosphorylation at a specific site functions independently of securin binding and complete activation of separase is proposed to require both securin removal and separase dephosphorylation [Stemmann et al., 2001; Huang et al., 2005]. Hence, arrested cells did not appear to have experienced anaphase or mitotic slippage prior to mitotic catastrophe.

DISCUSSION

In this report, we explored whether DNA breakage caused by actinomycin D is repairable during mitosis and we are interested in the downstream events incurred after detection of DNA lesions.

Understanding how DNA damage can lead to cell death will provide valuable information in the design of better therapeutic agents.

γ H2AX foci label damaged sites on DNA. It is widely accepted that γ H2AX foci recruit DSB repair proteins and dissolve when repair is completed. Actinomycin D-treated mitotic cells exhibited chromosomal abnormalities that accumulated over time. In a mitotic cell, the chromosomes are packed tightly into a highly compacted structure. DNA repair factors may have a tougher time to access damaged sites. Hence, homologous recombinational repair or even DNA end-joining cannot occur efficiently. γ H2AX levels increased and persisted, regardless of whether the damaging stimuli was maintained or removed from cells, indicating inability to repair the damage and to switch off the positive signal for downstream death signaling. The continuous propagation of this signal may lead to activation of downstream caspase-related cascades.

When cytotoxic drugs were first generated, they were believed to mainly target cancer cells because of their rapid proliferation rate. Today, we understand this view is simplistic and inadequate to explain the complex cellular processes that drive tumorigenesis. Cytotoxic agents owe their efficacy not only to the fact that they cause cellular injury directly, but also because of the stress response being set into motion as a secondary effect of the insult afflicted upon genetic material or cytoskeleton.

We have investigated the effect of MAPKs on the cellular decision to undergo catastrophic cell death. MAPKs exert broad range of effects on cellular activities. Among them, SAPK/JNK was activated by stress imposed on cells. The exact effects of JNK on cellular events have not been fully elucidated although JNK1 is mainly associated with the induction of apoptosis while JNK2 seems to have an effect on anaphase progression. Mitotic cells appear more "potentiated" than cells in other phases of the cell cycle in the sense that they have higher sensitivity towards stress stimuli. JNK phosphorylates H2AX at a non-canonical site for MAPKs. We show that active JNK co-immunoprecipitates γ H2AX in actinomycin D-treated mitotic extracts. It is tempting to speculate that JNK might be in part, responsible for the phosphorylation of H2AX and thereby, facilitate the propagation of a positive signal for cell death, when repair is not achieved.

In our model of cell death, cells try to exit mitosis by attempts to switch off the mitotic spindle checkpoint. However, we hypothesized that a functional checkpoint signal carried by both kinetochore-dependent and -independent complexes exist, complying with the notion that the spindle checkpoint is a pre-requisite to drive the cells toward mitotic catastrophe. Events such as dephosphorylation of histone H3 and Cdc2 as well as degradation of anaphase inhibitor separase point toward an attempt to exit mitosis. Histone H3 has been postulated to adopt a more closed configuration when it is phosphorylated on the N terminal tail as the negative charge on the phosphate group neutralizes the positive charge on the N terminal lysines. Dephosphorylation of histone H3 brings about global decondensation of chromosomes, which, is assumed to be a more practical setting for cells to repair damaged DNA.

Hereby, we propose a model of mitotic catastrophe in HeLa cells induced by actinomycin D whereby persistent DNA damage invoked stress responses mediated by the SAPK/JNK. The signal relayed by γ H2AX amplifies the initial insult and drives the cell to activate

downstream apoptotic pathways by the intrinsic mechanism. The cell attempts to exit mitosis as a default setting in the event of DNA damage, as uncondensed, relaxed chromatin is more accessible for repair factors. However, the remaining checkpoint signal carried by kinetochore-dependent and -independent mechanisms appear to be sufficient to put a restraining hand on entry into anaphase and the cell eventually dies in mitosis.

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