The regulation of the actin cytoskeleton and cell cycle progression

Heng, Yi Wen

2011

Heng, Y. W. (2011). The regulation of the actin cytoskeleton and cell cycle progression.
Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/46278>

<https://doi.org/10.32657/10356/46278>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**THE REGULATION OF THE
ACTIN CYTOSKELETON AND
CELL CYCLE PROGRESSION**

HENG YI WEN
SCHOOL OF BIOLOGICAL SCIENCES
2011

THE REGULATION OF THE ACTIN CYTOSKELETON AND CELL CYCLE PROGRESSION

HENG YI WEN

School of Biological Sciences

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

2011

ACKNOWLEDGEMENTS

The writing of this dissertation has been one of the most significant academic challenges I ever had to face. Although only my name adorns the cover of this dissertation, a great number of people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible and because of whom my graduate experience has been one that I will cherish forever. While many people have helped me with their insightful ideas and critique, I am fully responsible for any mistakes and flaws that this piece of work may contain.

Foremost, I would like to express deepest gratitude to my supervisor Dr. Koh Cheng Gee, Nanyang Technological University. Her intense knowledge and passion in research had inspired me greatly. I thank her for the patience, valuable guidance and tremendous encouragement from the initial to final, without which this dissertation would have been impossible. I am grateful for her trust and continuous belief in me, and in giving me ample freedom to experiment amidst critical comments; allowing me to develop such momentous enthusiasm for the subject. Her mentorship was paramount to develop me not only as an experimentalist and biologist, but also as an independent thinker. As a result, my learning journey has become smooth and extremely rewarding. I cannot imagine a better teacher than her.

I am greatly thankful to members of my thesis advisory committee, Dr. Li Hoi Yeung and Dr. Tan Suet Mien, Nanyang Technological University, for their insightful comments and constant advices in guiding me during my period of candidature. Their rigor and passion for research set great examples and role models for me of a world-class researcher.

Dr. Randy Poon, Hong Kong University of Science and Technology, and Dr Uttam Surana, Institute of Molecular and Cell Biology, A*Star, deserves many thanks for generously providing CDK1 constructs and indicible cell lines that laid the foundations for the work described herein Chapter 3.

I also owe thanks to Dr. Lim Chwee Teck, National University of Singapore, whose insights in the field of mechanobiology are second to none. I thank him for sharing his incomparably profound knowledge with me and his amicable personality. I truly enjoyed the discussions with him and I always felt wiser after speaking to him.

I express my gratitude to Dr. Zhong Shaoping, National University of Singapore, for providing brilliant assistance and critical troubleshooting for the micropipette aspiration

assays. I am deeply grateful to him for the long discussions that helped me sort out the technical details of my work. Without him, many imperative conclusions cannot be reached.

Similarly, Dr. Lim Hong Hwa, Institute of Molecular and Cell Biology, A*STAR, deserves mention for the collaborative work done on the kinase assay. I thank her for the logistics support and useful suggestions.

My dearest friends and mentors from the lab, both past and present, deserve mention for their wonderful company: Jayne, Xieyi, Gopal, Alison, Hongyuen, Agatha, Elynn, Meihua, Andrew, Anna, JingLing, Songjing, Sirong, Theresia and the many attachment students I cannot finish naming. It is their company that created such convivial environment that my study has become much pleasurable. Their support and care has helped me overcome the many setbacks I have had and I will always treasure the times we worked together.

My heartfelt thanks to Nanyang Technological University for the opportunity to undertake my PhD candidature, for providing conducive environment with excellent facilities for learning, as well as the generous scholarship support throughout the four years. In addition, I am grateful for the financial support from the Academic Research Fund, Ministry of Education, Singapore, and Biomedical Research Council, A*STAR, Singapore, that funded the research discussed in this dissertation.

Lastly, but surely the most, I thank my family for their constant and unflagging love for me.

Above all, my most esteemed father, Siam Leng, and my dearest mother, Bong Yeoh, as they are the most wonderful parents to me. They have worked industriously all their lives, and spared no effort in providing the best possible environment for me and my brothers to grow up. It is their unwavering faith in me that led me to where I am today. I thank them for being always supportive of me in chasing after my dreams. And my loving brothers, Jianxing and JinLiang, thanks for the laughter and love through our growing up years.

Finally, thanks to my husband Ronghui who had, over the years, lived and breathed this thesis as much as I have. Although the biology terms in this thesis seemed alien to him, he has been one particularly important person who made every word written in this thesis achievable. He is my constant source of strength and soul mate. I thank him for his tolerance, and the many nights he had sacrificed, accompanying me through those excruciating hours of overnight experiments. I dedicate this thesis to him and our beautiful daughter.

Yi Wen
September 2011

To my dearest family.

TABLE OF CONTENTS

Acknowledgements.....	i
List of figures.....	vii
List of tables.....	xi
Summary.....	xii
Abbreviations.....	xiv
 CHAPTER 1 INTRODUCTION.....	 1
1.1 The cell cycle and its regulation.....	1
<i>1.1.1 Stages of the cell cycle.....</i>	<i>1</i>
<i>1.1.2 Regulation of the cell cycle.....</i>	<i>2</i>
1.2 The actin cytoskeleton and its regulation.....	5
<i>1.2.1 The actin cytoskeleton.....</i>	<i>5</i>
<i>1.2.2 The Rho GTPase.....</i>	<i>7</i>
1.3 Actin cytoskeleton, myosin and the cell cycle.....	9
<i>1.3.1 Actin cytoskeleton in cell cycle control.....</i>	<i>9</i>
<i>1.3.2 Actin, myosin and the regulation of the mitosis.....</i>	<i>13</i>
1.4 Rho GTPases, their regulators and the cell cycle.....	16
<i>1.4.1 Rho GTPase in interphase.....</i>	<i>16</i>
<i>1.4.2 RhoA and partners in early M phase.....</i>	<i>18</i>
<i>1.4.3 RhoA and partners in late M phase.....</i>	<i>18</i>
<i>1.4.4 Cdc42 and partners in cell cycle.....</i>	<i>20</i>
<i>1.4.5 Cyclin-dependent kinase and actin in cell cycle.....</i>	<i>21</i>
1.5 Aim of the project.....	22
 CHAPTER 2 MATERIALS AND METHODS	 23
2.1 Materials.....	23
<i>2.1.1 Primary antibodies.....</i>	<i>23</i>
<i>2.1.2 Secondary antibodies.....</i>	<i>24</i>
<i>2.1.3 Others.....</i>	<i>24</i>

2.1.4	<i>Cloning primers</i>	24
2.1.5	<i>Site-directed mutagenesis primers</i>	25
2.1.6	<i>DNA sequencing primers</i>	26
2.1.7	<i>Stealth siRNA</i>	26
2.1.8	<i>Real-time PCR primers</i>	26
2.1.9	<i>Plasmids</i>	27
2.2	Methods	29
2.2.1	<i>Tissue culture, transfection and cell cycle arrest</i>	29
2.2.2	<i>General molecular cloning techniques</i>	30
2.2.3	<i>In vitro site-directed mutagenesis</i>	31
2.2.4	<i>Indirect immunofluorescence microscopy and live imaging</i>	33
2.2.5	<i>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot</i>	34
2.2.6	<i>Glutathione S-transferase pull-down assay</i>	34
2.2.7	<i>Fluorescence-activated cell sorting</i>	35
2.2.8	<i>RNA extraction, RT-PCR and real-time quantitative PCR</i>	35
2.2.9	<i>Micropipette aspiration assay</i>	36
2.2.10	<i>Bacterial expression and purification of proteins</i>	36
2.2.11	<i>Kinase Assay</i>	37
2.2.12	<i>Spindle orientation assay</i>	37
2.2.13	<i>Cell proliferation assay</i>	38
2.2.14	<i>Centrosome isolation</i>	38
2.2.15	<i>Statistical analysis</i>	39
CHAPTER 3	ACTIN AND THE MITOTIC TEMPO	40
3.1	Results	40
3.1.1	<i>Localisation pattern of actin, focal adhesions and cyclin B1 in interphase cells</i>	40
3.1.2	<i>Hydroxyurea-induced S phase arrest activates ATM/ATR kinase activity</i>	40
3.1.3	<i>Over-expression of CDK1(T14A/Y15F) in S-phase arrested cells caused forced expression of mitotic marker and morphology</i>	43

3.1.4	<i>Over-expression of active form CDK1 in U2OS cells does not accelerate cell cycle progression.....</i>	47
3.1.5	<i>Actin filaments disruption by cytochalasin D caused cytokinesis failure.....</i>	49
3.1.6	<i>Actin filament stabilisation using jasplakinolide caused mitosis delay.....</i>	53
3.1.7	<i>Actin filaments disruption by Y27632 and C3 transferase caused mitotic acceleration.....</i>	57
3.1.8	<i>Actin filaments disruption by Y27632 and C3 transferase caused cytokinetic defects.....</i>	61
3.2	Discussions.....	66
CHAPTER 4 ACTIN AND THE MITOTIC SPINDLES.....		76
4.1	Results.....	76
4.1.1	<i>Proper actin dynamics is required for control of metaphase astral microtubules and mitotic cortical rigidity.....</i>	76
4.1.2	<i>RhoA and ROCK may play a role in tubulin dynamics.....</i>	82
4.1.3	<i>Knockdown of LIMK1 and LIMK2 affects astral microtubules and cortical rigidity.....</i>	84
4.1.4	<i>LIMK2 localised to mitotic spindle and function downstream of ROCK in regulating mitotic astral microtubules and cortical rigidity.....</i>	87
4.1.5	<i>Moesin phosphorylation is downstream of Rho, but independent of ROCK and mDia, and may regulate metaphase astral microtubule and cortical rigidity.....</i>	92
4.1.6	<i>Cofilin does not function downstream of RhoA-ROCK pathway in regulation of metaphase astral microtubules.....</i>	96
4.1.7	<i>TPPP is a novel substrate for LIMK2.....</i>	99
4.1.8	<i>RhoA-ROCK-LIMK2 pathway controls TPPP localisation to the mitotic spindle which function to regulate astral microtubule formation.....</i>	100
4.1.9	<i>RhoA-ROCK-LIMK2-TPPP pathway controls metaphase spindle orientation.....</i>	109

4.2	Discussions.....	113
CHAPTER 5 ACTIN AND THE MITOTIC CENTROSOMES.....		120
5.1	Results.....	120
5.1.1	<i>RhoA and ROCK play a role in mitotic centrosome integrity.....</i>	120
5.1.2	<i>LIMK1 and LIMK2 may be involved in regulation of mitotic centrosome integrity.....</i>	121
5.1.3	<i>LIMK1 and LIMK2 knockdown does not affect cell's proliferation and division ability.....</i>	122
5.1.4	<i>LIMK1 and LIMK2 co-fractionate with centrosomal proteins.....</i>	124
5.1.5	<i>Specific localisation pattern of LIMK1 and/or LIMK2 phosphorylation during mitosis.....</i>	125
5.2	Discussions.....	128
References.....		130
Related publications.....		156

LIST OF FIGURES

Figure 1-1	Functional connections between proteins involved in the regulation of Rho GTPases, the actin cytoskeleton and cell cycle progression.....	17
Figure 3-1	Localisation pattern of actin, tubulin, focal adhesions and cyclin B in interphase cells.....	41
Figure 3-2	Degree of ATM/ATR kinase activity in hydroxyurea (HU)-treated cells.....	42
Figure 3-3	Over-expression of CDK1(T14A/Y15F) in hydroxyurea (HU)-arrested cells caused forced expression of mitotic markers.....	45
Figure 3-4	Over-expression of CDK1(T14A/Y15F) caused chromosomal condensation and actin filaments dissolution.....	46
Figure 3-5	Over-expression of CDK1(T14A/Y15F) in double thymidine arrested cells does not accelerate cell cycle progression.....	48
Figure 3-6	Cytochalasin D treatment caused dissolution of actin filaments.....	49
Figure 3-7	Cytochalasin D treatment disrupted 2N DNA peak recovery post mitosis.....	51
Figure 3-8	Cytochalasin D treatment caused cytokinesis failure.....	52
Figure 3-9	Jasplakinolide treatment caused altered actin filaments.....	53
Figure 3-10	Jasplakinolide treatment during early S phase caused mitotic delay.....	55
Figure 3-11	Jasplakinolide treatment during late G ₂ phase caused mitotic delay.....	56
Figure 3-12	Inhibition of Rho and ROCK caused disruption of actin filaments and loss of stress fibres.....	57
Figure 3-13	Inhibition of Rho and ROCK increased cell spreading in cells recovering from M phase arrest.....	58
Figure 3-14	Inhibition of Rho and ROCK accelerated recovery from nocodazole arrest.....	59
Figure 3-15	Inhibition of Rho and ROCK accelerated anaphase chromosome movement.....	60
Figure 3-16	Inhibition of Rho and ROCK caused cytokinesis and abscission defects.....	62

Figure 3-17	Inhibition of Rho and ROCK led to aberrant Aurora B staining in late M phase.....	64
Figure 3-18	Inhibition of Rho and ROCK led to aberrant Polo-like-kinase 1 (PLK1) staining in late M phase.....	65
Figure 4-1	Perturbation of actin dynamics caused increased metaphase astral microtubules.....	77
Figure 4-2	Perturbation of actin dynamics changed mitotic cortical rigidity.....	78
Figure 4-3	Alpha-actinin 1 co-localise to tip and length of filamentous actin.....	80
Figure 4-4	Blebbistatin treatment caused disruption of actin filaments and loss of stress fibres.....	80
Figure 4-5	Blebbistatin, concanavalin A and alpha-actinin 1 over-expression caused increased metaphase astral microtubules.....	81
Figure 4-6	Rho and ROCK inhibition caused increased microtubule polymerisation.....	83
Figure 4-7	Knock-down of LIMK1 and LIMK2 using siRNA.....	85
Figure 4-8	Knock-down of LIMKs affect metaphase astral microtubule and spindle poles.....	86
Figure 4-9	LIMK1 and LIMK2 activity causes increased filamentous actin formation.....	88
Figure 4-10	Rescue of Y27632 and C3 transferase-induced astral microtubules by LIMK2.....	89
Figure 4-11	LIMK2 co-localisation to the mitotic spindle is dependent on RhoA and ROCK activity.....	91
Figure 4-12	Rescue of Y27632-induced mitotic rigidity defect by LIMK2.....	92
Figure 4-13	Moesin phosphorylation is responsible for mitotic cortical rigidity and control of metaphase astral microtubules.....	93
Figure 4-14	Ezrin/radixin/moesin phosphorylation is downstream of Rho, but independent of ROCK and mDia.....	95
Figure 4-15	Cofilin does not function downstream of RhoA-ROCK pathway in the regulation of metaphase astral microtubules.....	97
Figure 4-16	Slingshot1 does not play a role in regulation of metaphase astral microtubules.....	98

Figure 4-17	TPPP is a substrate of LIMK1 and LIMK2.....	100
Figure 4-18	TPPP co-localises with LIMK2 at the mitotic spindle.....	101
Figure 4-19	TPPP localisation to the mitotic spindle is dependent on RhoA, ROCK and LIMK2, but independent of LIMK1.....	103
Figure 4-20	Knock-down of TPPP using siRNA.....	104
Figure 4-21	Knock-down of TPPP does not affect interphase and metaphase microtubule network.....	105
Figure 4-22	TPPP functions downstream of RhoA-ROCK-LIMK2 in the regulation of metaphase astral microtubules.....	106
Figure 4-23	Mutations of TPPP at threonine 14 and serine 18 does not affect metaphase astral microtubules formation.....	107
Figure 4-24	RhoA and ROCK participate in the regulation of metaphase spindle orientation.....	110
Figure 4-25	LIMK2 functions downstream of RhoA-ROCK in regulating metaphase spindle orientation.....	111
Figure 4-26	TPPP functions downstream of RhoA-ROCK-LIMK2 in regulating metaphase spindle orientation.....	112
Figure 5-1	RhoA and ROCK activity are required for centrosome integrity.....	121
Figure 5-2	LIMK1 and LIMK2 knock-down does not affect cell proliferation and division.....	123
Figure 5-3	LIMK1 and LIMK2 co-fractionate with centrosomal proteins.....	124
Figure 5-4	Phospho-LIMK1/2 localisation pattern during cell cycle progression.....	126
Figure 5-5	Phospho-LIMK1/2 at the spindle pole is dependent on Rho and ROCK activity.....	127

LIST OF TABLES

Table 1-1	CDK-cyclin complexes at different stages of the cell cycle.....	3
Table 1-2	Summary of different reagents used to perturb the actin cytoskeleton and their effects on cell cycle progression.....	10
Table 1-3	Summary of the roles of different proteins in actin cytoskeleton regulation and cell cycle progression.....	12
Table 1-4	The localisation of different actin cytoskeleton related proteins during interphase and mitosis.....	13
Table 2-1	Cycling parameters for QuikChange Site-Directed Mutagenesis.....	32
Table 2-2	Number of cycles required for different types of mutation.....	32

Summary

The actin filament network is one of the most crucial cytoskeletal structures within the cell that serves as a scaffold and provides cells' their tension support. It also participates in the regulation of a wide range of cellular functions including intracellular vesicular transport, morphogenesis, attachment properties and locomotion abilities. In adherent cell types, cells adhere to the substratum during interphase and assume a rounded morphology during cell division. After dividing, there is re-establishment and re-formation of focal contacts. In cells that are trypsinised, there is similar loss of focal contacts and stress fibres, accompanied by cell rounding. Such cells reform contact and spreading when re-plated. Mitotic cells, on the other hand, stay rounded until the completion of mitotic events. Clearly, there must be distinctive mechanisms between the different stages of the cell cycle that can promote or inhibit cytoskeletal events, or *vice versa*.

Our aim is to investigate the functional relationship between actin cytoskeleton regulation and cell cycle progression. In this report, we examined three aspects of actin cytoskeleton's relation to the cell cycle: namely the polymerisation status of actin cytoskeleton and cell cycle progression, the cortical actin network and the formation of mitotic spindles, and the cortical actin network and the mitotic centrosomes.

We found that ectopic expression of CDK1, a mitosis kick start kinase, in synchronised cells did not accelerate the timing of mitosis entry even though mitotic markers such as histone H3 phosphorylation could occur. Forced disruption of actin filaments in late G₁ phase did not affect cells' ability to undergo DNA replication and cells proceeded with cell cycle progression. However, these cells exhibited cytokinesis failure. On the other hand, the forced stabilisation of actin filaments by jasplakinolide was unable to overrule cytoskeletal dissolution signals during mitosis. Nonetheless, the enhanced stabilisation resulted in delayed onset of mitosis-specific events such as histone H3 phosphorylation, increased cyclin D1 expression and delayed mitotic exit. Inhibition of the Rho signalling pathway using C3 transferase and ROCK inhibitor resulted in accelerated anaphase and recovery from nocodazole arrest, suggesting that the molecular mechanics of actin played an important role in controlling the timing of events during mitosis. Our data suggest a potential link between the regulation of actin cytoskeleton and the control of cell cycle tempo.

In the investigation of actin cytoskeleton during early mitosis, we found that perturbation of the actin cytoskeleton prior to mitosis onset resulted in the increase of astral microtubules at the metaphase spindle. Increase in actin polymerisation or depolymerisation state during mitosis correlated with the cellular cortical rigidity. We found that the kinase activity of LIMK2 was essential in maintaining normal astral microtubules formation downstream of the RhoA-ROCK signalling pathway which was essential in controlling the spindle orientation angle in relation to the substratum. Knockdown of LIMK2 resulted in formation of metaphase spindles with excessive astral microtubules. This regulation is found to be mediated via a novel substrate of LIMK2, the TPPP, which regulates microtubules polymerisation. Our work suggests that the signalling between LIMK2 and TPPP forms the missing link between RhoA signalling and the metaphase spindle microtubule dynamics.

In addition to microtubules dynamics, we also demonstrated the importance of RhoA-ROCK signalling in maintaining the integrity of the spindle poles during mitosis. The regulation of centrosome focusing might be mediated by LIMKs as knockdown of LIMKs resulted in formation of diffused centrosomes (for LIMK1 and LIMK2 knockdown) and multiple polar spindles (for LIMK1 knockdown) in metaphase cells. LIMK1 and LIMK2 were found to co-fractionate with the centrosomes and phosphorylation of LIMK1/2 at the centrosome appeared to be mediated by RhoA-ROCK. Our current data are insufficient to conclude if LIMK directly affects the mechanical cohesion properties of centrosomal proteins at the spindle poles, or does it affect centrosome duplication at earlier cell cycle stages. Nevertheless, it is clear the LIMK1/2 can play a functional role at the centrosomes.

Abbreviations

ADF	Actin depolymerisation factor
APC	Adenomatous polyposis coli protein
ATM	Ataxia telangiectasia mutated
ATP	Adenosine-5'-triphosphate
ATR	Ataxia telangiectasia mutated and Rad3-related
BDM	2,3-Butanedione monoxime
BSA	Bovine serum albumin
CCD	Cytochalasin D
Cdc42	Cell division control protein 42 homolog
CDK	Cyclin-dependent kinases
CKI	Cyclin-dependent kinase inhibitor
CSPP	Centrosome/spindle pole-associated protein
DAPI	4',6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside 5'-triphosphate
DTT	Dithiothreitol
dUTP	2'-Deoxyuridine 5'-triphosphate
EB1	Adenomatous polyposis coli-binding protein 1
ECM	Extracellular matrix
ECT2	Epithelial cell transforming sequence 2 oncogene
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERM	Erzin/radixin/moesin
FAK	Focal adhesion kinase
FH	Formin homology
FACS	Fluorescence-activated cell sorting
F-actin	Filamentous-actin
FBS	Foetal bovine serum
G-actin	Globular-actin
GAP	GTPase-activating protein

GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
HDAC6	Histone deacetylase 6
HRP	Horseradish peroxidase
HU	Hydroxyurea
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ITSN2	Intersectin2
KID	Kinase inhibitory domain
LB	Lysogeny Broth
LIMK1	LIM domain kinase 1
LIMK2	LIM domain kinase 2
MAP	Microtubules associated protein
MAPK	p42/p44 Mitogen-activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
MKLP1	Mitotic kinesin-like protein 1
MLCK	Myosin light chain kinase
MPF	Mitosis promoting factor
MRCK	Myotonic dystrophy kinase-related CDC42-binding kinase
MTOC	Microtubules organising centre
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Myo-GEF	Myosin II-interacting-Guanine nucleotide exchange factor
NMII	Non-muscle myosin II
N-WASP	Neural Wiskott-Aldrich syndrome protein
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-Activated kinase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3 kinase

PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidyl 3,4,5 triphosphate
PIP5K	Phosphatidylinositol 4-phosphate 5-kinase
PLK1	Polo-like kinase 1
pRB	Retinoblastinoma protein
PRK	Protein-kinase-C-related kinases
PtdInsP3	Phosphatidylinositol triphosphates
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
RNA	Ribonucleic acid
ROCK	Rho-associated coiled coil containing kinase
Rpm	Revolution per minute
rtTA	Tetracycline trans-activation repressor
SDS	Sodium dodecyl sulphate
siRNA	Small interfering ribonucleic acid
Slik	Ste20-liked protein kinase
SOC	Super optimal broth with catabolic repression
SSH1	Slingshot1
TBP	TATA binding protein
TBS	Tris buffered saline
TdT	Terminal deoxynucleotidyl transferase
TGF- β	Transforming growth factor- β
TPPP	Tubulin polymerisation promoting protein
TUNEL	Terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labelling
UV	Ultraviolet
WASP	Wiskott-Aldrich syndrome protein
WAVE	Wiskott-Aldrich syndrome protein-family Verprolin-homologous protein
WT	Wildtype
ZIPK	Leucine zipper interacting kinase

CHAPTER 1 INTRODUCTION

1.1 The cell cycle and its regulation

1.1.1 Stages of the cell cycle

The mammalian cell cycle progression involves a series of highly defined and regulated process that encompasses two major activities; the duplication of cell's genetic material, and the segregation of the replicated chromosomes into two daughter cells. It is separated into two morphologically distinctive phases; the interphase where the cell expands, duplicate its DNA and prepare for division, and the Mitotic (M) phase where the actual division takes place. In most animal cells, the interphase consists of the Gap 1 (G_1), Synthesis (S) and Gap 2 (G_2) phases. Cells in G_1 can enter a resting state called G_0 before they commit to DNA replication. Cells in G_0 represent most part of the non-proliferating cells in an organism. The M phase is a relatively short process during the cell cycle and is further divided into two stages; mitosis where the chromosomes separate and then immediately followed by cytokinesis where the cytoplasm is divided into two daughter cells.

In eukaryotic organism, the genome is distributed into several chromosomes and each DNA fibre is divided into multiple replicating units termed replicons (Cairns, 1966; Huberman and Riggs, 1968). As DNA is tightly bundled with a large magnitude of proteins, the DNA replication machinery is able to dislodge or loosen these histones and non-histones chromosomal proteins and interact with the DNA for replication and this process often requires specific modifications such as the phosphorylation of the DNA-binding proteins (Elgin and Weintraub, 1975; Hewish, 1976). Apart from chromosomal DNA replication, cells also replicate their mitochondrial DNA, and this was found to occur at all times during the eukaryotic cell cycle (Bogenhagen and Clayton, 1976).

In most cell types, preparation for mitosis occurs mainly during G_2 phase. As the cell enters into mitosis, it re-organises its actin networks and rounds up (Sanger, 1975). The mitosis stage is further categorised into the stages prophase, prometaphase, metaphase, anaphase and telophase. During prophase, the chromatin condenses to form chromosome and molecular motors push the centrosomes apart (Varmark, 2004). During prometaphase, the nuclear envelope breaks down, allowing microtubules to invade into the nuclear matrix. The two

centrosomes now form the spindle poles which re-organise microtubules that serve to capture the kinetochores of chromosomes. The cell then progresses into metaphase whereby the chromosomes are aligned at the metaphase plate which is located at the cell equatorial region. This process occurs due to the counterbalance of pulling forces at both sides of the kinetochore microtubules (Zhou et al., 2002). Later, the cell proceeds into anaphase where proteins holding the chromatids are cleaved and allowing the sister chromosome to separate. This is followed by shortening of the kinetochore microtubules as the sister chromosomes are pulled towards their respective attached centrosomes (Draviam et al., 2004). During telophase, the chromosomes further separate and move towards the opposite ends. New nuclear envelope reforms around each set of separate sister chromosomes and the chromosomes unfold into chromatids. The mitosis stage is complete at this phase and the cell progresses to physically separate into two daughter cells, a process termed cytokinesis.

Preparation for cytokinesis occurs early during mitosis. Some time during late anaphase and telophase, the actin and myosin filaments re-organise to form the contractile ring at where the site of metaphase plate used to be. The site of contractile ring formation is dictated by the positioning of the mitotic spindle and central spindle (Barr and Gruneberg, 2007). Myosin then hydrolyses ATP and generates force that drives the constriction of the contractile ring, forming the cleavage furrow. Ingression continues to occur until the formation of the midbody structure. Finally abscission takes place which depends on septin filaments organised beneath the cleavage furrow. The remaining non-kinetochore microtubules then re-organised to form the new cytoskeleton in the daughter cells and the cell cycle returns to interphase (Glotzer, 2005).

1.1.2 Regulation of the cell cycle

The transition from one cell cycle stage to another is tightly regulated by proteins such as cyclin-dependent kinases (CDKs). CDKs are serine/threonine kinases belonging to the CMGC kinase family. Activities of CDKs are determined by their non-covalent association with different types of cyclins, their phosphorylation pattern and presence of inhibitors (Noble et al., 2004). So far, nine CDKs have been identified and of which five have been shown to play key roles in cell cycle regulation as they serve to induce downstream processes by phosphorylating their specific substrates (Morgan, 1995; Pines, 1995). On the other hand sixteen cyclins have been identified but not all participate in cell cycle related events

(Okamoto et al., 1994; Peng et al., 1998; Rickert et al., 1996). Cyclin levels rise and fall periodically during the cell cycle and different CDK-cyclin complexes are involved in regulating different stages of the cell cycle: CDK3-cyclin C for G₀/G₁ transition (Ren and Rollins, 2004), CDK4/6-cyclin D for G₁ progression (Ekholm and Reed, 2000; Pan et al., 1998), CDK2-cyclin E for G₁/S transition (Chen and Li, 1998), CDK2-cyclin A for S phase progression (Dynlacht et al., 1994; Guadagno and Newport, 1996), and CDK1-cyclin A/B for G₂/M transition (Draetta and Beach, 1988; Dunphy et al., 1988; Rhind et al., 1997; Riabowol et al., 1989). The different CDK-cyclin complexes required for cell cycle are summarised in table 1-1.

CDK	Cyclin	Cell cycle activity
CDK3	Cyclin C	G ₀ to G ₁ phase transition
CDK4	Cyclin D1, D2, D3	G ₁ phase progression
CDK6	Cyclin D1, D2, D3	G ₁ phase progression
CDK2	Cyclin E	G ₁ to S phase transition
	Cyclin A	S phase progression
CDK1	Cyclin A	G ₂ to M phase transition
	Cyclin B	Mitosis progression
CDK7	Cyclin H	CDK-activating kinase, all cell cycle phase

Table 1-1. CDK-cyclin complexes at different stages of the cell cycle.

In addition to binding to cyclins, CDK activities are also regulated by phosphorylation which occurs on conserved tyrosine and threonine residues. For example during S phase, CDK1 is phosphorylated at residues Thr¹⁴, Tyr¹⁵ and Thr¹⁶¹ and binds to cyclin B, forming an inactive mitosis promoting factor (MPF) complex. The phosphorylations at CDK bring about conformational changes and affect binding affinity to their cyclin partners (Jeffrey et al., 1995; Paulovich and Hartwell, 1995). This complex continues to accumulate through G₂ phase until at G₂/M transition, dephosphorylation of Thr¹⁴ and Tyr¹⁵ residues occur and the MPF complex becomes activated (Ducommun et al., 1991; Hayles et al., 1994; Rhind et al., 1997). The phosphorylation of CDK1 is precisely controlled by WEE1 and MYT1 kinases and CDK1 is dephosphorylated by members of the CDC25 phosphatase. WEE1 phosphorylates Tyr¹⁵ but not Thr¹⁴ (Parker and Piwnica-Worms, 1992) while MYT1 phosphorylates both residues (Booher et al., 1997). CDC25 phosphatase consists of three

isoforms CDC25A, B and C, with each functioning at specific cell cycle stages. CDK1 dephosphorylation is largely carried out by CDC25C during mitosis (Peng et al., 1997) and more recently also found to be carried out by CDC25A (Mailand et al., 2002). Activation of CDK1 then occurs rapidly via a positive feedback loop where the activated MPF complex further phosphorylates CDC25C, increasing its phosphatase activity (Strausfeld et al., 1994).

CDKs activities are also regulated by cell cycle inhibitory proteins. These proteins, called CDK inhibitors (CKI) can bind to CDK alone or CDK-cyclin complexes. Currently, two distinct class of CKI are known, the INK4 family and Cip/Kip family (Sherr and Roberts, 1995). The INK4 family specifically inactivates G₁ phase CDKs including CDK4 and CDK6, and comprises of p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}. Binding of these CKI to CDKs forms stable complexes and prevents CDKs association with cyclins (Carnero and Hannon, 1998). The Cip/Kip family of inhibitors consists of p21^{Waf1/Cip1}, p27^{Cip2} and p57^{Kip2}, and they bind and inhibit CDK-cyclin complexes (Harper et al., 1995; Lee et al., 1995; Polyak et al., 1994). p21 can also bind to proliferating cell nuclear antigen (PCNA) and inhibit DNA synthesis (Pan et al., 1995; Waga et al., 1997). CKIs are regulated by different mechanisms; the promoter of p21 contains a p53-binding site and is transcriptionally-regulated by p53 (el-Deiry et al., 1993), while the expression and activation of p15 and p27 is regulated by the presence of transforming growth factor- β (TGF- β) (Hannon and Beach, 1994; Reynisdottir et al., 1995).

Cell cycle progression is also regulated by the different cellular localisation of different cell cycle-regulating proteins. For example, cyclin B contains a nuclear exclusion signal and is mainly cytoplasmic. It continuously shuttles between the nucleus and cytoplasm during interphase (Hagting et al., 1998; Toyoshima et al., 1998) (Yang et al., 1998a). At the end of prophase, cyclin B rapidly translocates into the nucleus (Bailly et al., 1992; Hagting et al., 1999) and associates with the mitotic apparatus (Clute and Pines, 1999; Hagting et al., 1998; Pines and Hunter, 1991). WEE1, located in the nucleus during interphase, has been reported to inhibit the activity of cytoplasmically-activated CDK1 which was translocated into the nucleus (Heald et al., 1993) thus preventing premature activation of CDK1 before mitosis. MYT1, on the other hand, has been reported to localise to the endoplasmic reticulum and Golgi apparatus, which serves to inhibit CDK1-cyclin B complexes (Liu et al., 1997) which are also targeted at the Golgi complex (Jackman et al., 1995). In addition, the intracellular localisation pattern of some proteins is regulated by the 14-3-3 group of proteins. For

example, during interphase, CDC25 interacts with 14-3-3 and is held in the cytoplasm. Following DNA damage, CDK1-cyclin B complex is also sequestered in the cytoplasm by the 14-3-3 protein (Peng et al., 1997; Yang et al., 1999).

1.2 The actin cytoskeleton and its regulation

1.2.1 The actin cytoskeleton

The cell cytoskeleton is made up of three types of protein filaments; the actin microfilaments, intermediate filaments and microtubules. These protein filaments serve to provide cells their mechanical support, giving shape, generating force, providing transport and allowing cellular locomotion. The microfilaments are made up of actin subunits, one of the most abundant and highly conserved proteins found in the eukaryotic cells. Actin can exist in the cell in two states, the globular form (G-actin) and filamentous form (F-actin). The actin molecule consists of two domains, the large and small domain. The ATP and Ca^{2+} binding sites are located in the cleft between the two domains (Kabsch et al., 1990). Actin is bound to either ADP or ATP. The ATP bound to G-actin is hydrolysed by its ATPase domain during the formation of F-actin. The actin filaments are formed by two strands of actin globules that twist around each other forming a double helix. Actin monomers in the helix all orientate their cleft towards the same end, forming the minus end or pointed end while the opposite end is termed the plus end or barbed end (Depue and Rice, 1965). Actin monomer polymerisation and depolymerisation can occur at both ends, but the nett elongation occurs at the barbed end.

The actin filaments are frequently bound by motor proteins of the myosin family. Myosins are motor proteins playing important role in cellular processes that require force and translocation. A typical myosin contains a head domain, a neck domain and a tail domain; with the highly conserved head domain functioning to bind actin and move along actin filaments using energy generated from ATP hydrolysis through the powerstroke mechanism. Such movement of myosin molecules help “pulls” actin filaments and contribute to the acto-myosin contractile forces. The neck domain acts as a linker and lever arm for force transduction from the catalytic domain. It also provides the binding site for the myosin light chains which serves regulatory functions and forms part of the myosin macromolecule

complex. The tail domain, on the other hand, can serve different functions such as dimerisation and protein-protein interaction, depending on the classes of myosin.

As many as thirteen classes of myosin have been identified thus far. Myosin II, belongs to the class of conventional myosin and is one of the most widely studied. It forms the major contractile protein of cardiac, skeletal and smooth muscle, and is also present in all non-muscle eukaryotic cells (Clark et al., 2007; Conti and Adelstein, 2008; Krendel and Mooseker, 2005). Non-muscle myosin II (NMII) plays a fundamental role in processes that require cellular shaping, for example in adhesion, migration and cell division. Like muscle myosin II, NMII are comprised of two heavy chains, two regulatory light chains and two essential light chains (Vicente-Manzanares et al., 2009). The globular head of NMII contains a binding site for ATP and actin, while the neck domain contains binding site for the light chains. The tail region extends into a long α -helical domain that aids in the dimerisation of the two heavy chains. NMII activity is mainly regulated by the phosphorylation of its regulatory light chain on Ser¹⁹, resulting in an increase in the Mg²⁺-ATPase activity of NMII (Somlyo and Somlyo, 2003). Several kinases have been reported to phosphorylate the regulatory light chain of NMII, including myosin light chain kinase (MLCK) (Kamm and Stull, 1985), Rho-associated coiled coil kinase (ROCK) (Amano et al., 1996), citron kinase (Yamashiro et al., 2003), leucine zipper interacting kinase (ZIPK; DAPK3) (Murata-Hori et al., 1999) and myotonic dystrophy kinase-related CDC42-binding kinase (MRCK; CDC42BP) (Leung et al., 1998). These kinases can phosphorylate NMII regulatory light chain at residue Ser¹⁹ or at both Thr¹⁸ and Ser¹⁹. On the other hand, dephosphorylation of NMII regulatory light chain is mainly carried out by myosin phosphatase (Hartshorne et al., 2004).

The cross-linking of actin filaments with myosin forms the stress fibres in non-muscle cells. Stress fibres consists of bundles of acto-myosin filaments ranging from 10 to 30 filaments held together by cross-linking proteins such as α -actinin (McKenna and Wang, 1986), fascin (Kane, 1975), espin (Chen et al., 1999), filamin (Pavalko et al., 1989), and palladin (Mykkanen et al., 2001; Ronty et al., 2004). They are involved in cell adhesion, motility and morphogenesis. According to their sub-cellular localisation and interactions with focal adhesions, stress fibres in cultured mammalian cells are divided into three classes: ventral stress fibres which display periodic myosin-actin filaments cross linked with α -actinin and associated to focal adhesions at both ends (Small et al., 1998); transverse arc stress fibres which also contains periodic myosin- α -actinin cross-linking but are connected to substrate

via the dorsal stress fibres (Heath, 1983; Hotulainen and Lappalainen, 2006; Small et al., 1998); and the dorsal stress fibres which are attached to focal adhesion at one end and rise towards the dorsal section of the cell at the other end (Hotulainen and Lappalainen, 2006).

1.2.2 *The Rho GTPase*

Rho GTPase belongs to the super-family of Ras-related small GTPase found in all eukaryotic cells and they function mainly to regulate the assembly of actin filaments and their contractility. The most intensively studied Rho GTPases include the RhoA, Rac1 and Cdc42. Similar to other GTPases, Rho GTPases act as molecular switches that cycle between an active guanosine-5'-triphosphate (GTP)-bound state and inactive guanosine diphosphate (GDP)-bound state. The activity of Rho GTPases can be controlled by several ways: (1) the guanine nucleotide exchange factors (GEFs) that catalyse the exchange of GDP for GTP, activating the GTPases (Schmidt and Hall, 2002), (2) the GTPase-activating proteins (GAPs) that stimulates the intrinsic GTPase activity and catalyse the hydrolysis of GTP to GDP (Bernards, 2003), and (3) the guanine nucleotide dissociation inhibitors (GDIs) that binds to GDP-bound form of Rho GTPases and prevent activation and membrane localisation of the GTPases (Olofsson, 1999).

There are three isoforms of highly homologous Rho proteins, namely Rho A, Rho B and Rho C, and all of which induces the formation of stress fibres (Wheeler and Ridley, 2004). The functions of Rho proteins have been studied extensively through the use of clostridial enzyme C3 transferase, which modifies and inhibits all three isoforms. RhoA, the more widely studied isoform amongst the three proteins, acts upon two known effector proteins Rho-associate coiled coil containing protein kinase 1 and 2 (ROCK1 and 2) and mammalian diaphanous homologue (*Drosophila*) (mDia). Activation of both ROCK and mDia is required for the formation of stress fibres (Watanabe et al., 1999). ROCK phosphorylates the myosin light chain and controls the contractility of acto-myosin filaments. ROCK can also phosphorylate LIM domain kinase (LIMK), LIMK1 and LIMK2 at Thr⁵⁰⁵ and Thr⁴⁸⁴ residue respectively (Sumi et al., 2001), which in turn phosphorylates cofilin at Ser³ residue (Sumi et al., 1999; Yang et al., 1998b). This inactivates the actin-severing function of cofilin and hence stabilises actin filaments. In contrast, mDia contains a forming homology (FH2) domain that binds to profilin and accelerates actin polymerisation (Kovar et al., 2006; Li and Higgs,

2003). However, ROCK and mDia can also antagonise each other in cell shape control, membrane ruffles and adheren junctions formation (Sahai and Marshall, 2002).

The Rac subfamily protein contains Rac1, Rac2 and Rac3 members, which function to stimulate lamellipodium extension and induce membrane protrusions during phagocytosis (Jaffe and Hall, 2005). The Ras-related C3 botulinum toxin substrate 1 (Rac1) is the best studied isoform and is ubiquitously expressed (Didsbury et al., 1989). Rac1 activates Arp2/3 via the Wiskott-Aldrich syndrome protein-family Verprolin-homologous (WAVE) family proteins (Miki et al., 1998).

Cdc42 plays a conserved role in regulating cell polarity and actin cytoskeleton in many eukaryotic organisms. They are mainly responsible for the formation of actin-rich protrusions known as filopodia (Gupton and Gertler, 2007). Cdc42 directly binds to Neural Wiskott-Aldrich syndrome protein (N-WASP), which acts as a scaffold to recruit proteins required for actin polymerization such as actin, profilin and Arp2/3 complexes (Snapper et al., 2001). Cdc42 can also bind to mDia2 and mediates polymerisation of un-branched actin filaments (Peng et al., 2003). Cdc42 can also signal via p21-activated kinase (PAK) and LIMK and result in phosphorylation and inactivation of cofilin (Wang et al., 2007). In addition, Cdc42 was also reported to interact with MRCK α and β , resulting in phosphorylation of NMII light chain and actin reorganisation (Leung et al., 1998).

The activities of these RhoGTPases are frequently required in synchrony to ensure the proper signalling of actin dynamics for a single process. For example all three RhoGTPases, Rac, RhoA and Cdc42 have been shown to play a role in different aspects of phagocytosis. There are two main pathways involved in the uptake of opsonised micro-organisms during phagocytosis; CR3 binds to C3bi on complement-opsonised targets while Fc γ Rs bind to immunoglobulin G-coated targets (Allen and Aderem, 1996; Newman et al., 1991). In earlier reports, Rac1 and Cdc42 have been found to play a role in Fc γ R-mediated phagocytosis while RhoA functions in CR3-mediated phagocytosis (Caron and Hall, 1998; Cox et al., 1997; Massol et al., 1998). GTP-bound Rac1 and Cdc42 has been found to be enriched at the site of particle attachment mediated by Fc γ R (Castellano et al., 2000; Castellano et al., 1999). In addition, in cells expressing Fc γ RIIA receptors, co-injection of dominant negative Cdc42 and Rac blocked the formation of actin structures (Caron and Hall, 1998). On the other hand, only inhibition of RhoA, but not Rac and Cdc42, was found to inhibit actin changes in cells

expressing CR3 receptors (Caron and Hall, 1998). In later studies, both Rac2 and Cdc42, as well as their downstream effector PAK were demonstrated to be activated in both CR3- and Fc γ R-mediated phagocytosis in neutrophils (Forsberg et al., 2003).

1.3 Actin cytoskeleton, myosin and the cell cycle

1.3.1 Actin cytoskeleton in cell cycle control

Apart from widely regarded for its mechanical properties, actin also plays a role in chemical signal transduction during the cell cycle. Actin regulation during cell cycle was frequently thought to be “inside-out” where the cell cycle machinery dictates the state of actin organization with the cell (Wang, 1991; Yamashiro and Matsumura, 1991). Recent evidence has since indicated retrograde signalling where the state of actin organization within the cell also plays an important influence on the cell cycle progression (Assoian and Zhu, 1997; Thery and Bornens, 2006). Pharmacological disruption of actin architecture has caused cell cycle arrest in a wide variety of adherent mammalian cells (Bohmer et al., 1996; Bottazzi et al., 2001; Fasshauer et al., 1998; Huang et al., 1998; Iwig et al., 1995; Maness and Walsh, 1982; Ohta et al., 1985; Reshetnikova et al., 2000; Rubtsova et al., 1998; Takasuka et al., 1987; Tsakiridis et al., 1998) via several proposed mechanisms such as disruption of the p42/p44 mitogen-activated protein kinase (MAPK) pathway, down-regulation of cyclin D1 levels, activation of p53 pathway, phosphorylation of retinoblastoma protein (pRB) or down-regulation of cyclin dependent kinase inhibitor, p27^{KIP1}. Depolymerisation of actin using cytochalasin D and latrunculin B has also been reported to delay mitosis progression (Gachet et al., 2001; Lee and Song, 2007), suggesting that an intact actin network may be required for efficient onset of mitosis. On the contrary, forced expression of cyclin D1 was able to drive rounded quiescence cells from G₀ into S phase (Roovers and Assoian, 2003; Welsh et al., 2001), thus uncoupling the cell cycle from an organised cytoskeleton and spreading shape. A summary of the different drugs and chemicals and their effects on the actin cytoskeleton is listed in Table 1-2. Apart from actin disruption by pharmacological means, disruption of actin polymerisation by interfering with upstream signalling cascade has also been reported to cause cell cycle disruption. Over-expression of cofilin, a member of the actin depolymerisation factor (ADF)/cofilin family, caused more than 90 % of H1299 lung carcinoma cells to arrest at G₁ phase.

Drug	Target	Action	Phenotype(s) reported
<i>Clostridium difficile</i> toxin B	RhoA, Rac1, Cdc42	Glycosylates RhoA, Rac1 and Cdc42, thereby inactivating them.	G ₂ arrest
<i>Clostridium botulinum</i> exoenzyme C3 transferase	RhoA, RhoB, RhoC	ADP-ribosylation on asparagine 41 in the effector binding domain of the GTPase	Slows down p21 degradation, at mitosis caused re-spreading of prometaphase cells
Y-27632 rock inhibitor	ROCK I, ROCK II	Competitive binding with ATP to active site of ROCK I and ROCK II	Failure in centrosome separation
ML-7, ML-9	MLCK	Competitive binding with ATP to active site of MLCK	G ₁ arrest, affect spindle organisation
2,3-Butanedione monoxime (BDM)	Myosin	Myosin ATPase inhibitor	G ₁ arrest, inhibit kinetochore fibre elongation
Blebbistatin	Myosin II	Binds to Myosin-ADP-Pi with high affinity and interferes with phosphate release process	Failure in centrosome separation
Calyculin A	Protein phosphatases	Binds to active site of PP1 and PP2A protein phosphatases	Accelerate anaphase chromosome separation
CEP1347	PAK1	Targets PAK1 ATP-binding site	G ₁ arrest, aberrant spindle formation, delay in mitosis transition
WR-PAK18		Binds SH3 domain of PIX, blocking PAK-PIX interaction	
Cytochalasin D	Actin	Binds to the barbed, fast growing plus ends of microfilaments, inhibiting actin monomer assembly and disassembly	Mitosis delay, G ₁ arrest, inhibit kinetochore fibre elongation
Latrunculin A/B	Actin	Binds to actin monomers near their ATP-binding site, preventing actin polymerisation	Mitosis delay, G ₁ arrest, inhibit kinetochore fibre elongation, failure in centrosome separation
Jasplakinolide	Actin	Binds to F-actin, stabilizing and promoting actin polymerization	Cytokinesis defect

Table 1-2. Summary of different reagents used to perturb the actin cytoskeleton and their effects on cell cycle progression (Heng and Koh, 2010).

Besides actin depolymerisation, excessive actin polymerisation has also been linked to cell cycle defect. For example, transfection of a mutant WASP protein, or addition of jasplakinolide, both increase the amount of F-actin within the cells, resulted in multi-nucleated cells, suggesting a possible defect in cytokinesis (Moulding et al., 2007). Similarly, expression of mutant WASP^{I294T}, which mis-regulates the Arp2/3 complex and enhances actin polymerisation, results in accumulation of F-actin around the mitotic chromosomes and cytokinesis defect. The vast information from literature has demonstrated that actin participates in the regulation of cell cycle progression, suggesting a possible inter-regulatory signalling network that is in place and capable of controlling both the actin dynamics and cell

cycle. The various proteins that are known to function in both the regulation of actin cytoskeleton and the cell cycle progression are summarised in Table 1-3. In addition, many of these proteins changes their cellular localisation throughout different stages of the cell cycle (Table 1-4), suggesting that they can play multiple roles during cell cycle progression. Although a morphogenesis checkpoint has already been established in the budding yeast which is activated in response to actin perturbation (McMillan et al., 1998), a similar cytoskeletal checkpoint has yet to be established in mammalian cells. This is despite the vast data that disruption of actin cytoskeleton leading to cellular arrest. Nevertheless, the above reported data prompt for serious consideration that actin checkpoint may also be present in mammalian cells.

Group	Protein	Roles in actin cytoskeleton regulation	Roles in cell cycle progression regulation
Actin binding	Myosin II	Actin crosslinker; ATPase dependent actomyosin force generation.	G1-S progression; mitotic cell rounding; centrosome separation; mitotic spindle assembly; kinetochore microtubule formation; anaphase chromosome movement; cytokinesis.
	Myosin X	Unconventional actin crosslinker.	Mitotic spindle shortening.
	Anillin	Actin and microtubule bundling.	Spindle associated cleavage specification.
	Moesin	Actin binding; ERM protein.	Mitotic cell rounding; mitotic cortical rigidity; metaphase spindle stability.
	Cortactin	Recruitment of Arp2/3 to F-actin.	F-actin mediated centrosome separation.
Rho GTPase	RhoA	Stress fibres formation.	G1-S transition; mitotic cell rounding; mitotic cortical rigidity; cytokinesis.
	Rac1	Lamellapodia formation.	G1-S transition; cytokinesis
	Cdc42	Filopodia formation.	G1-S transition; kinetochore microtubule stabilisation; spindle biorientation; metaphase chromosome alignment; cytokinesis.
Rho GTPase Regulator	p190RhoGAP	RhoA GAP.	Mitotic cell rounding.
	Ect2	RhoA GEF, Cdc42 GEF during mitosis.	Contractile ring formation; contraction of contractile ring at cleavage furrow.
	GEF-H1	RhoA GEF; microtubule dynamics.	RhoA related cytokinesis.
	MgcRacGAP	Rac1 and Cdc42 GAP; RhoA GAP during mitosis.	Formation of ingression furrow; interacts with Ect2.
	Lfc	RhoA GEF.	Mitotic spindle assembly.
	MyoGEF	RhoA GEF; myosin II binding.	Localise RhoA and Ect2 to contractile ring.
Rho GTPase effector	ROCK	RhoA effector; myosin regulation; actin bundling.	S phase progression, mitotic cell rounding; centrosome separation; mitotic spindle assembly; cytokinesis.
	LIMK1	ROCK & PAK substrates;	Mitotic spindle orientation.
	LIMK2	cofilin regulation; actin bundling.	Regulate cyclin A and p27 expression.
	Cofilin	Downstream of RhoA pathway; actin severing.	G1-S progression; mitotic spindle orientation.
	mDia1	RhoA effector; actin nucleation and elongation.	Spindle formation in early mitosis through an "Lfc-RhoA-mDia1" pathway.
	PRK2/PKN2	RhoA and Rac effector.	Mitosis entry; mitosis exit.
	PAK1	Rac and Cdc42 effector; cell motility; focal adhesion turnover.	G1-S transition; G2-M transition; centrosome maturation; regulation of Plk1 and Aurora-A activity; regulate astral microtubule dynamics; spindle orientation.
	N-WASP	Rac and Cdc42 effector; Arp2/3 activator; actin nucleation.	Cytokinesis.

Table 1-3. Summary of the roles of different proteins in actin cytoskeleton regulation and cell cycle progression. (Heng and Koh, 2010)

Protein	Subcellular localisation by immunofluorescence staining	
	Interphase (adherent cell type)	M-phase
F-actin	Stress fibres; actin structures	Contractile ring
Myosin II	Stress fibres; actin structures	Contractile ring
Anillin	Ubiquitous	Contractile ring
Cortactin	Ubiquitous, actin structures	Centrosomes (phosphorylated form)
Ect2	Ubiquitous	Central spindle
GEF-H1	Ubiquitous	Mitotic spindle; midzone
LIMK1	Cell-cell contacts	Centrosomes; equatorial cortex; contractile ring
LIMK2	Ubiquitous	Mitotic spindle, contractile ring
MyoGEF	Ubiquitous	Central spindle
PAK1	Ubiquitous; actin structures; centrosomes	Centrosomes; contractile ring; mid-body
RhoA	Ubiquitous	Contractile ring
HEF1	Focal adhesions	Mitotic spindle; mid-body
Integrin-linked kinase (ILK)	Focal adhesions	Centrosomes
Focal adhesion kinase (FAK)	Focal adhesions	Centrosomes
Pyk2	Focal adhesions	Centrosomes
Paxillin	Focal adhesions	Centrosomes
Zyxin	Focal adhesions	Mitotic spindle; central spindle

Table 1-4. The localisation of different actin cytoskeleton related proteins during interphase and mitosis. (Heng and Koh, 2010)

1.3.2 Actin, myosin and the regulation of the mitosis

The microtubules have always been regarded as the major player during mitosis, with their beautifully arrays and precise choreographed functions from the organisation of the bipolar spindles to the capturing, alignment and accurate segregation of chromosomes. On the other hand, the study of actin and its regulators have almost been solely focus on its mechanical function during cytokinesis. However, recent findings have sparked interest in the function of actin in the biogenesis of mitotic spindle. The presence of actin filaments within the mitotic spindles provides evidence that actin may play a role in the mitotic apparatus (Woolner et al., 2008; Yasuda et al., 2005). Interfering with actin polymerisation and actin-myosin bundling at the cellular cortex during mitosis has been shown to affect centrosomes separation in mammalian cells, suggesting the requirements of actin network in early mitotic events. Treatment of cells with latrunculin or inhibition of myosin II by ROCK inhibitor – Y27632,

which prevents ROCK-mediated phosphorylation of myosin light chain phosphatase and eventually block myosin activity, also resulted in failure of centrosomes separation. These cells eventually present spindle formation defects. Similarly, inhibition of myosin II by blebbistatin which inhibits the ATPase activity of non-muscle myosin II, or the silencing of non-muscle myosin II heavy or light chain using RNA interference, also gives the same centrosomal defects (Rosenblatt et al., 2004). Furthermore, when the cortical flow of acto-myosin filaments is disrupted by cross-linking cell surface with concanavalin A lectin, centrosomes separation and movement are affected and lopsided spindles are observed (Rosenblatt et al., 2004). Thus a dynamic acto-myosin network at the cell cortex is essential for early mitotic events.

Additional reports have also suggested actin's importance in spindle function, especially in regulating spindle orientation. For example the activity of LIMK1 was found to be increased during mitosis which leads to inactivation of cofilin (Kaji et al., 2008). Down-regulation of LIMK1 by siRNA resulted in mis-localisation of cofilin and eventual mis-orientation of the mitotic spindle. Similarly, disruption of F-actin by latrunculin or expression of active cofilin mutant (S3A) also resulted in spindle mis-orientation (Kaji et al., 2008). Phosphorylated LIMK1 have also been found to colocalise with γ -tubulin at the spindle pole during early mitosis, suggesting that LIMK1-mediated actin regulation may play an important role in the proper formation and positioning of the mitotic spindle. Besides the cortical actin, other site of interaction between the astral microtubules and the acto-myosin network has also been recently reported. Myo10, a myosin isoform, has been found to interact with both microtubules and actin, and can localise to the spindle poles in *Xenopus* embryos (Woolner et al., 2008). Spindle fragmentation and lengthening were observed when Myo10 was knockdown, suggesting a role of Myo10 in force generation within the spindles. These observations suggest that actin, myosin and microtubules can all play intrinsic and synergistic roles in the assembly and positioning of the mitotic apparatus.

Besides spindle formation, actin and myosin also play important roles in the regulation of cytokinesis. Myosin light chain kinase (MLCK)-dependent phosphorylation of myosin II has been shown to be essential for actin bundling during formation of contractile ring and generation of contraction forces required for cleavage furrow formation (Mabuchi and Okuno, 1977). Apart from contractile ring formation, the MLCK has also been implicated in earlier mitotic events. Microinjection of the catalytic fragment of MLCK into prophase cells

resulted in delay in the transition from nuclear envelope breakdown and anaphase onset, but does not affecting timing from anaphase to mid cytokinesis. Unregulated MLCK activity is also found to affect spindle microtubule formation during prometaphase and metaphase (Fishkind et al., 1991). On the other hand, inhibition of MLCK using ML-7 or auto-inhibitory peptide 18 also affects the localisation of actin cap on metaphase II spindles, leading to defective cortical organisation activities (Deng et al., 2005).

In addition to actin and myosin, moesin is also found to be important for the mitotic cortical structures and spindle stability. Moesin is a member of the ezrin/radixin/moesin (ERM) family of actin-binding protein. It is phosphorylated by the Ste20-liked protein kinase (Slik) in *Drosophila* S2 cells during mitosis (Hipfner et al., 2004; Kunda et al., 2008). This results in cell rounding via cross-linking of actin to the cell membrane (Carreno et al., 2008). Suppression of either moesin or its upstream kinase Slik results in decreased mitotic cell rigidity, defective cortical organisation which leads to metaphase spindle instability. On the other hand, external cross-linking of surface membrane proteins using concanavalin A was able to rescue the lost rigidity and spindle structures, indicating that the maintenance of cell shape and rigidity is sufficient to stabilise the mitotic spindle (Kunda et al., 2008). The function of moesin in cell rounding appears to be independent of myosin II, as cells with active T559D mutant moesin but lacking myosin light chain were still able to round up during mitosis (Kunda et al., 2008).

Apart from cortical force maintenance for proper spindle formation, the acto-myosin structures have also been shown to be important for force generation to accomplish chromosome segregation during anaphase. Depolymerisation of actin filaments using drugs such as cytochalasin D and latrunculin A, or inhibition of myosin ATPase activity using BDM prevented spindle microtubule elongation and hindered anaphase (Forer et al., 2007). On the contrary, treating mitotic cells with non-specific inhibitor of myosin light chain phosphatase, calyculin A, resulted in stabilisation of myosin II phosphorylation and accelerated pole-ward movement of chromosomes during anaphase (Fabian and Forer, 2007).

It is clear that apart from cytokinesis, actin and myosin functions are essential for many other aspects of mitosis, such as rounding and maintenance of cortical rigidity, spindle formation and positioning and anaphase chromosome segregation. Actin reorganisation during mitosis helps generate the optimal cellular environment for the mitotic spindle to serve its function,

while disruption of the cortical actin network can severely affect spindle functions, leading to cell arrest. Although not widely accepted, it is highly possible that a morphogenesis checkpoint may exist in the mammalian cells. This actin cytoskeleton or a spindle checkpoint may hinge on the principle of functional interactions between microtubules and cortical actin network.

1.4 Rho GTPases, their regulators and the cell cycle

1.4.1 Rho GTPase in interphase

In addition to its actin regulating function, the Rho GTPase has also been found to regulate the cell cycle. Suppression of Cdc42, Rac1 and RhoA activities result in G₁ phase arrest (Olson et al., 1995; Yamamoto et al., 1993). In the human capillary endothelial cells, active RhoA causes increased expression of F-box containing protein, Skp2. Skp2 then mediates ubiquitin-dependent degradation of the CDK inhibitor p27^{Kip1} (Mammoto et al., 2004) which in turn binds to and inactivates the cyclin D1/CDK4 and cyclin E/CDK2 complexes. Absence of RhoA increases the level of p27^{Kip1} thus leading to G₁ cell cycle arrest. Active RhoA acts via the balance of its two downstream effectors, mDia and ROCK, to activate the Skp2-p27^{KIP1} pathway. Intact F-actin and RhoA activity have also been shown to be required for the degradation of another CDK inhibitor p21^{Waf/Cip1} (Coleman et al., 2006), thereby regulating cell cycle progression at G₁ phase. On the other hand, ROCK-mediated LIMK2 activity was found to regulate Ras/MAPK pathway and balance cyclin D and cyclin A levels (Croft and Olson, 2006).

PAK1 signalling has also been shown to control G₁/S phase transition via the regulation of cyclin D1. In Ras-transformed NIH 3T3 cells, addition of PAK inhibitors such as CEP-1347 and WR-PAK18 cause down-regulation of cyclin D1 levels and inhibited its malignant growth (Nheu et al., 2004). Consistently, blocking of PAK1 activity using PAK1-KID, or knockdown of PAK1 using siRNA results in decrease in cyclin D1 levels (Balasenthil et al., 2004). Direct linkage of Cdc42 or Rac1 activity to PAK has not been documented in these instances. However, Cdc42 activity has been shown to increase concomitantly with PAK1 (Oceguera-Yanez and Narumiya, 2006). Thus, PAK1 may exhibit its cell cycle regulation function in a GTPase-dependent or independent pathway. The different cell cycle pathways regulated by Rho GTPase are summarised in Figure 1-1.

Figure 1-1. Functional connections between proteins involved in the regulation of Rho GTPases, the actin cytoskeleton and cell cycle progression. (Heng and Koh, 2010).

1.4.2 RhoA and partners in early M phase

The functions of RhoA in mitotic events have been documented in several studies. In the Rat2 fibroblast cells, microtubule-associated RhoGEF, Lfc, has been implicated in spindle formation. Knockdown of Lfc or microinjection of Lfc antibodies experiments have shown to result in spindle assembly defects (Bakal et al., 2005), whereas both active RhoA and mDia are able to rescue the defects, suggesting that an “Lfc-RhoA-mDia1” pathway is required for spindle formation during early mitosis. In addition, covalent capturing of kinase-specific phosphopeptide has recently identified mDia1 to contain a CDK1 phosphorylation consensus sequence (Blethrow et al., 2008), suggestive of a possible direct association between CDK1 and mDia1 during mitosis.

Besides mDia and ROCK, another RhoA effector, PRK2/PKN2, has also been implicated in mitosis onset and cytokinesis (Schmidt et al., 2007). Knockdown of PRK2 in HeLa cells results in accumulation of bi-nucleated cells suggesting defects in cytokinesis. In addition, these cells also displayed delayed progression from G₂ to M phase. PRK2 activity leads to increase in CDC25B phosphorylation, resulting in the activation of cyclin B-CDK1 complex. The activated CDK1 complex in turn signals via a positive feedback loop and results in further activation of PRK2 which then participates in cytokinesis.

1.4.3 RhoA and partners in late M phase

The activity of RhoA has been reported to be required for several stages of cell cycle progression. In particular, RhoA has been found to be essential for proper and complete cytokinesis. The key function of RhoA during cytokinesis is to organise the assembly of contractile ring and drive the acto-myosin contractility of the cleavage furrow (Barr and Gruneberg, 2007). During mitosis, GEF-H1, a microtubule-regulated GEF for RhoA has been shown to be activated by cyclin B/CDK1 and Aurora-A/B kinases (Birkenfeld et al., 2007; Hara et al., 2006; Niiya et al., 2006). This in turn modulates RhoA activity during cytokinesis (Birkenfeld et al., 2007). Polo-like kinase (Plk1), originally thought to regulate only spindle assembly, has also been shown to control the localisation of ECT2 to the central spindle and RhoA at the equator during anaphase (Brennan et al., 2007; Burkard et al., 2007; Petronczki et al., 2007). Inhibition of Plk1 blocks the interaction of ECT2 with midzone anchor HsCyk4, preventing proper assembly of the contractile ring which leads to cytokinesis defects.

Recently, the centrosome/spindle pole-associated protein (CSPP) has been shown to target Myosin II-interacting GEF (MyoGEF) to the central spindle during anaphase. Knockdown of MyoGEF results in mis-localisation of ECT2 and RhoA during cytokinesis (Asiedu et al., 2009). In addition, centralspindlin which is localised to both the astral microtubules and the tips of astral microtubules near the equatorial cortex serves to recruit ECT2 to the central spindle (Nishimura and Yonemura, 2006). Knockdown of centralspindlin component, MKLP1, result in non-localisation of ECT2 at the equatorial cell cortex (Yuce et al., 2005).

Another Rho GTPase regulator, MgcRacGAP (also known as HSCyk4), has also been shown to regulate contractile ring assembly and cytokinesis. MgcRacGAP is a GTPase-activating protein for Rac and Cdc42, but is phosphorylated by Aurora-B during mitosis and serves as a GAP for RhoA instead (Minoshima et al., 2003). Cells transfected with MgcRacGAP using siRNA show no ingression of the cleavage furrow and fail to undergo cytokinesis (Zhao and Fang, 2005). In addition, MgcRacGAP have been shown to interact with ECT2 and thus may exert its effect via ECT2. Recently, the GAP activity of MgcRacGAP and GEF activity of ECT2 have been shown to promote the flux of RhoA activities concurrently, thus maintaining the RhoA localisation at the cell equator region (Miller and Bement, 2009) for the formation of contractile ring.

Another RhoA binding protein, anillin, has been shown to accumulate at the cleavage furrow in a RhoA-dependent manner (Piekny and Glotzer, 2008). Anillin was first isolated as an actin-binding and bundling protein in *Drosophila* embryo (Field and Alberts, 1995). Later, it was shown to interact with myosin regulatory light chain and septin (Konishi et al., 2002; Oegema et al., 2000; Straight et al., 2005). A recent report reveals that in *Drosophila* the RhoGEF, Pebble, controls the formation of a filamentous structure containing Rho1, anillin and septin at the cleavage furrow. It also regulates the interaction of this filamentous structure with the plasma membrane and microtubules (Hickson and O'Farrell, 2008). Interestingly, anillin was also reported to interact with RacGAP50C, a spindle associated protein that specifies the cleavage site (Gregory et al., 2008). Depletion of anillin results in the loss of RacGAP50C from the equator of cleavage and the collapse of the cleavage furrow. Thus anillin acts as a linker between the contractile ring and the spindle.

1.4.4 Cdc42 and partners in cell cycle

The levels of GTP bound form of Cdc42 constantly changes during the cell cycle. During pre-metaphase, the level of Cdc42-GTP is low but increases and peaks during metaphase. The Cdc42-GTP level then declines as the cell enters telophase. There is, however, no change in the level of Rac-GTP throughout the cell cycle (Oceguera-Yanez and Narumiya, 2006). On the other hand, the level of RhoA-GTP is found to peak during telophase. Over-expression of dominant negative mutant ECT2 and MgcRacGAP, as well as knockdown of ECT2 by siRNA, abolished the cyclic change in the levels of Cdc42-GTP during mitosis (Oceguera-Yanez and Narumiya, 2006). This suggests that ECT2 and MgcRacGAP can regulate both RhoA and Cdc42 activity at different stages of the cell cycle. More specifically, ECT2 was found to catalyse the formation of Cdc42-GTP at metaphase and MgcRacGAP increases the hydrolysis of Cdc42-GTP during prometaphase. The activity of Cdc42 has also been reported to be important for mitosis progression. When Cdc42 and other Cdc42-like GTPase were knockdown using siRNA, metaphase cells display misalignment of chromosomes (Yasuda et al., 2006). Cdc42-dependent bi-orientation of spindle was shown to be regulated via its downstream activation of mDia3, thereby stabilising the kinetochore-microtubule attachment (Yasuda et al., 2006). In addition, another formin mDia2 has also been implicated in the stabilisation of the microtubules, a function that is separate and independent from its actin nucleation activity (Bartolini et al., 2008). mDia2 can bind directly to microtubules, and to proteins located at the tip of microtubules such as EB1 and APC. In the regulation of cytokinesis, mDia2 has been found to be required for the stabilisation of actin scaffold at the site of contractile ring formation (Watanabe et al., 2008).

Cdc42 has also been known to regulate spindle orientation. In human intestinal epithelial Caco-2 cells, knockdown of Cdc42 caused mis-orientation of the mitotic spindle and subsequent mis-positioning of the apical surface within the cyst (Jaffe et al., 2008). Cdc42 regulates the spindle orientation in two distinct pathway; one via Cdc42-PAK2- β PIX while the other involving the phosphatidylinositol 3 kinase (PI3K) (Mitsushima et al., 2009). Inhibition of the PI3K results in reduction of phosphatidylinositol triphosphates (PtdInsP3) at the mid-cortex and induces spindle mis-orientation, while re-introduction of PtdInsP3 was able to direct dynactin accumulation at the midcortex and rescue spindle orientation (Toyoshima et al., 2007). Recently, the Cdc42-specific GEF, Intersectin2 (ITSN2) has been reported to localise to the centrosomes and activate Cdc42 during mitosis. Knockdown of

ITSN2 leads to mis-orientation of the mitotic spindle and hinders lumen formation in the three-dimensional Madin-Darby canine kidney model (Rodriguez-Fraticelli et al., 2010). The above data suggest that Cdc42 signalling plays a role in the orientation of the mitotic spindle and hence regulate plane of division and cell polarity.

The downstream effector of Cdc42 and Rac1, PAK, has also been shown to participate in cell cycle regulation, particularly in the transition from G₂ to M phase. It has been demonstrated that PAK1 can regulate Plk1 activity and that inhibition of PAK1 could lead to delay mitotic entry and abnormal spindle formation (Maroto et al., 2008). PAK1 has been shown to localise to the centrosomes during early mitosis via the PIX-GIT1 complex (Zhao et al., 2005). At the centrosomes, it serves to activate Aurora-A kinase, an important kinase required for the maturation of centrosomes (Maroto et al., 2008). In addition, PAK1 has also been implicated in the regulation of astral microtubules formation as over-expression of active PAK1 construct caused cells to exhibit multiple spindle orientations (Vadlamudi et al., 2000).

The functions of Rac1 in cell cycle progression are less reported. One study suggests that in *C. elegans*, the CYK-4/MgcRacGAP of the centralspindlin complex is required for inactivation of Rac1 to ensue proper cytokinesis (Canman et al., 2008). Depletion of Rac but not RhoA in CYK-4 GAP mutant cells was found to successfully rescue cytokinesis defect. This work suggest that during cytokinesis, there is parallel activation of RhoA and inhibition of Rac1 which serves to prevents Rac-dependent activation of Arp2/3 by WAVE or WASP, thus preventing the formation of redundant actin networks which may otherwise interferes with the formation of the contractile ring.

1.4.5 Cyclin-dependent kinase and actin in cell cycle

The onset of mitosis involves the activation of CDK1, leading to mitotic cytoskeletal changes. One of the major downstream effectors of CDK1 has been proposed to be p190 Rho GTPase activating protein (Maddox and Burrridge, 2003). Activated CDK1 phosphorylates p190 RhoGAP, down-regulating its activity and thus decreases GTP hydrolysis of RhoA. This triggers a signalling cascade through ROCK, myosin light chain phosphatase and regulates the cytoskeletal changes that are observed during mitosis (Amano et al., 1996; Kimura et al., 1996; Maddox and Burrridge, 2003). CDK1 has also been reported to phosphorylate caldesmon (Yamashiro et al., 1991), an actin- and myosin-binding protein

which is essential for stress fibres formation. Phosphorylation of caldesmon leads to its dissociation from stress fibres and the re-organisation of actin and focal adhesion proteins (Helfman et al., 1999).

1.5 Aim of the project

It is clear that cell cycle progression in metazoans proceeds through a series of highly regulated events which are dependent on each other via various checkpoint controls. *In vitro*, adherent cell types exhibit substratum adherence and a well spread shape during interphase, which collapse during mitosis to form rounded cells with increased cortical rigidity. After mitosis, there is re-establishment of attachment and cytoskeletal tension. Modulation of such drastic changes in cell shape in synchrony to cell cycle progression suggests tight signalling regulation between cytoskeleton dynamics and mitotic events. Apart from their actin regulating activities, the RhoGTPase and their associated proteins have also been shown to be implicated in various aspects of the cell cycle progression.

The emphasis of this project is to study actin and the proteins that are known to interact and regulate the actin cytoskeleton, as well as their implications in the cell cycle control. In particular, we examined the impact of the polymerisation status of actin and the actin cytoskeleton on (1) the temporal control of different stages of cell cycle, (2) the formation and orientation of the mitotic spindle, and (3) the mitotic spindle pole integrity.

CHAPTER 2 MATERIALS AND METHODS

The materials and methods employed in this study are summarised in this chapter.

2.1 Materials

2.1.1 Primary antibodies

<u>Antibody</u>	<u>Species</u>	<u>Clonal</u>	<u>Company</u>
Anti-Actin	mouse	monoclonal	Chemicon International
Anti-Aurora B	rabbit	polyclonal	Bethyl Laboratories
Anti-CDK1	mouse	monoclonal	BD Transduction Laboratories
Anti-Centrin 2	rabbit	polyclonal	Cell Signaling Technology
Anti-Cyclin A	rabbit	polyclonal	Santa Cruz Biotechnology
Anti-Cyclin B1	rabbit	monoclonal	Santa Cruz Biotechnology
Anti-Cyclin D1	mouse	monoclonal	Santa Cruz Biotechnology
Anti-Cyclin E	mouse	monoclonal	Santa Cruz Biotechnology
Anti-FAK	rabbit	polyclonal	Upstate
Anti-Flag	mouse	monoclonal	Sigma-Aldrich
Anti-Flag	rabbit	polyclonal	Sigma-Aldrich
Anti-GFP	rabbit	polyclonal	Invitrogen
Anti-GST	rabbit	polyclonal	Bethyl Laboratories
Anti-HA	rabbit	polyclonal	Zymed Laboratories
Anti-LIMK1	rabbit	polyclonal	ECM Biosciences
Anti-LIMK2	rabbit	polyclonal	Santa Cruz Biotechnology
Anti-Myosin IIA	rabbit	polyclonal	Sigma-Aldrich
Anti-p140mDia	mouse	monoclonal	BD Transduction Laboratories
Anti-Pericentrin	rabbit	polyclonal	Abcam
Anti-phospho-ATM/ATR substrates	rabbit	monoclonal	Cell Signaling Technology
Anti-phospho-CDK1 (pY15)	mouse	monoclonal	BD Transduction Laboratories
Anti-phospho-Ezrin(Thr567) /Radixin(Thr564)/Moesin(Thr558)	rabbit	polyclonal	Cell Signaling Technology
Anti-phospho-histone H3 (Ser10)	rabbit	polyclonal	Santa Cruz Biotechnology
Anti-phospho-LIMK1(Thr508)/ LIMK2(Thr505)	rabbit	polyclonal	Cell Signaling Technology

Anti-phospho-PAK1(Thr423)/

PAK2(Thr402)	rabbit	polyclonal	Cell Signaling Technology
Anti-phospho-Ser/Thr-Pro MPM2	mouse	monoclonal	Upstate
Anti-PLK1	mouse	monoclonal	Abcam
Anti-RhoA	rabbit	polyclonal	Santa Cruz Biotechnology
Anti-ROCKII/ROK α	mouse	monoclonal	BD Transduction Laboratories
Anti- α -tubulin	mouse	monoclonal	Sigma-Aldrich
Anti- β -tubulin	mouse	monoclonal	Sigma-Aldrich
Anti- γ -tubulin	rabbit	polyclonal	Sigma-Aldrich
Anti-vinculin	mouse	monoclonal	Sigma-Aldrich

2.1.2 Secondary antibodies

<u>Antibody</u>	<u>Company</u>
Goat anti-mouse IgG, HRP conjugated	Dako Cytomation
Goat anti-rabbit IgG, HRP conjugated	Dako Cytomation
Goat anti-mouse IgG, Alexa Fluor 488	Molecular Probes
Goat anti-mouse IgG, Alexa Fluor 546	Molecular Probes
Goat anti-rabbit IgG, Alexa Fluor 488	Molecular Probes
Goat anti-rabbit IgG, Alexa Fluor 546	Molecular Probes

2.1.3 Others

<u>Name</u>	<u>Company</u>
Phalloidin-FITC	Sigma-Aldrich
Phalloidin-Alexa 546	Sigma-Aldrich

2.1.4 Cloning primers

<u>Primer Name</u>	<u>DNA Sequence (5' to 3') – restriction enzyme sites underlined</u>
LIMK1 HindIII sense	GGC <u>AAG CTT</u> ATG AGG TTG ACG CTA CTT TGT
LIMK1 NotI antisense	ATT <u>GCG GCC GCT</u> GAG AAA TCT GGT
LIMK2 XhoI sense	ATG <u>CTC GAG</u> ATG GGG AGT TAC TTG TCA
LIMK2 NotI antisense	ATG <u>TGC GGC CGC</u> TGT GGA GAT ACA TT
ACTN1 HindIII sense	GGC <u>AAG CTT</u> ATG GAC CAT TAT GAT TCT CAG
ACTN1 NotI antisense	ATA <u>TGC GGC CGC</u> TGG ATT AGA GGT CAC

CFL1 WT HindIII sense	TAA <u>AGC TTA</u> TGG CCT CCG GTG TGG CTG TC
CFL1 S3A HindIII sense	TAA <u>AGC TTA</u> TGG CCG CCG GTG TGG CTG TC
CFL1 S3E HindIII sense	TAA <u>AGC TTA</u> TGG CCG AAG GTG TGG CTG TC
CFL1 NotI antisense	TTG <u>CGG CCG CTC</u> ACA AAG GCT TGC
TPPP HindIII sense	TCA <u>AGC TTA</u> TGG CTG ACA AGG CCA AGC C
TPPP XhoI antisense	TTC <u>TCG AGC</u> TAC TTG CCC CCT TGC ACC
SSH1 HindIII sense	TAA <u>AGC TTA</u> TGG TCC TGG TGA CCC TGC
SSH1 XhoI antisense	ACC <u>TCG AGT</u> CAG CTT TTG CTC ATC CA

2.1.5 Site-directed mutagenesis primers

<u>Primer Name</u>	<u>DNA Sequence (5' to 3') – mismatch sites underlined</u>
MSN T558D sense	GCC GAG ACA AAT ACA AGG <u>ACC</u> TGC GCC AGA TCC G
MSN T558D antisense	CGG ATC TGG CGC AGG <u>TCC</u> TTG TAT TTG TCT CGG C
MSN T558A sense	GCC GAG ACA AAT ACA AGG <u>CCC</u> TGC GCC AGA TC
MSN T558A antisense	GAT CTG GCG CAG GGC <u>CTT</u> GTA TTT GTC TCG GC
LIMK1 T508E sense	CGC AAG AAG CGC TAC <u>GAG</u> GTG GTG GGC AAC CCC
LIMK1 T508E antisense	GGG GTT GCC CAC CAC <u>CTC</u> GTA GCG CTT CTT GCG
LIMK1 D460A sense	AAC ATC ATC CAC CGA <u>GCC</u> CTC AAC TCC CAC AAC
LIMK1 D460A antisense	GTT GTG GGA GTT GAG <u>GGC</u> TCG GTG GAT GAT GTT
LIMK2 T484E sense	CGC AAG AAG CGC TAC <u>GAG</u> GTG GTG GGA AAC CC
LIMK2 T484E antisense	GGG TTT CCC ACC ACC <u>TCG</u> TAG CGC TTC TTG CG
LIMK2 D430A sense	GCA TCA TCC ACC GGG <u>CTC</u> TGA ACT CGC ACA A
LIMK2 D430A antisense	TTG TGC GAG TTC AGA <u>GCC</u> CGG TGG ATG ATG C
TPPP T14D/S18D sense	AGC TGC CAA CAG <u>GGA TCC</u> CCC CAA <u>GGA</u> CCC GGG GGA CCC C
TPPP T14D/S18D antisense	GGG GTC CCC CGG <u>GTC</u> CTT GGG GGG <u>ATC</u> CCT GTT GGC AGC T
TPPP T14E/S18E sense	GCT GCC AAC AGG <u>GAG</u> CCC CCC AAG <u>GAG</u> CCG GGG GAC CCC
TPPP T14E/S18E antisense	GGG GTC CCC CGG <u>CTC</u> CTT GGG GGG <u>CTC</u> CCT GTT GGC AGC
TPPP T14A sense	GCT GCC AAC AGG <u>GCG</u> CCC CCC AAG T
TPPP T14A antisense	ACT TGG GGG GCG <u>CCC</u> TGT TGG CAG C
TPPP S18A sense	CGC CCC CCA AGG <u>CCC</u> CGG GGG AC
TPPP S18A antisense	GTC CCC CGG GGC <u>CTT</u> GGG GGG CG

2.1.6 DNA sequencing primers

<u>Primer name</u>	<u>DNA Sequence (5' to 3')</u>
Check MSN mutant	CAG AGA AGA ATG AGC GTG TGC
Check LIMK1 T508E/D460A mutant	AGA CCT CAA CTC CCA CAA CTG C
Check LIMK2 T484E mutant	GTC ACG GCT CAT AGT GGA AGA
Check LIMK2 D430A mutant	TGG CAG CAG AAG GTC AGG TT
Check TPPP mutant	AGC TTA TGG CTG ACA AGG C
Check mCherry forward	ACA ACG AGG ACT ACA CCA TT
T7 promoter forward	TAA TAC GAC TCA CTA TAG GG

2.1.7 Stealth siRNA

<u>siRNA Name</u>	<u>RNA Sequence (5' to 3')</u>
LIMK1 siRNA sense	CAU CCU UUG UGA AGC UGG AAC ACU G
LIMK1 siRNA antisense	CAG UGU UCC AGC UUC ACA AAG GAU G
LIMK2 siRNA sense	UCC CUU CGU UGU UCC AAC AGC UAU U
LIMK2 siRNA antisense	AAU AGC UGU UGG AAC AAC GAA GGG A
TPPP siRNA(1) sense	UUA GCA AGA UCA AAG GGA AGU CUU G
TPPP siRNA(1) antisense	CAA GAC UUC CCU UUG AUC UUG CUG A
TPPP siRNA(2) sense	UCG CCA AGA AGC GAU UCA AAG ACA A
TPPP siRNA(2) antisense	UUG UCU UUG AAU CGC UUC UUG GCG A
Luciferase siRNA sense	ACA UCA CGU ACG CGG AAU ACU UCG A
Luciferase siRNA antisense	UCG AAG UAU UCC GCG UAC GUG AUG U

2.1.8 Real-time PCR primers

<u>Primer Name</u>	<u>DNA Sequence (5' to 3')</u>
RT LIMK1 sense	AGG TTG ACG CTA CTT TGT TGC A
RT LIMK1 antisense	GGC AAC TCG CTT CCT TCC T
RT LIMK2 sense	TGG CAG CAG AAG GTC AGG TT
RT LIMK2 antisense	CAT AGA GTG CAA ATA GGC CAT T
RT TPPP sense	TGA CCG TCA CTG ACG TGG ACA AT
RT TPPP antisense	CGC TGC TCT TGT CTT TGA ATC G
RT TBP sense	TGC ACA GGA GCC AAG AGT GAA
RT TBP antisense	CAC ATC ACA CTC CCC ACC A

2.1.9 Plasmids

<u>Plasmid</u>	<u>Tag</u>	<u>Name</u>	<u>Amino Acid</u>	<u>Mutation(s)</u>
pUHD-P1-Flag-CDK1	Flag	Cyclin-dependent kinase 1	1 to 297	
pUHD-P1-Flag CDK1 T14A/Y15F	Flag	Cyclin-dependent kinase 1	1 to 297	T14A and Y15F
pXJ-Flag-RhoA WT	Flag	Ras homolog gene family member A	1 to 193	
pXJ-Flag-RhoA G14V	Flag	Ras homolog gene family member A	1 to 193	G14V
pXJ-Flag-RhoA T19N	Flag	Ras homolog gene family member A	1 to 193	T19N
pXJ-Flag-ROCK2	Flag	Rho-associated coiled-coil containing protein kinase 2	1 to 1388	
pXJ-Flag-ROCK2 KD	Flag	Rho-associated coiled-coil containing protein kinase 2	1 to 432	
pXJ-Flag-DIAPH1	Flag	Diaphanous homolog 1	1 to 1256	
pXJ-Flag-DIAPH1 DN	Flag	Diaphanous homolog 1	261 to 571	
pXJ-Flag-DIAPH1 ΔGBD	Flag	Diaphanous homolog 1	261 to 1256	
pXJ-mCherry-ACTN1	mCherry	Actinin alpha 1	1 to 914	
pXJ-Flag-LIMK1	Flag	LIM domain kinase 1	1 to 647	
pXJ-mCherry-LIMK1 T508E	mCherry	LIM domain kinase 1	1 to 647	T508E
pXJ-mCherry-LIMK1 D460A	mCherry	LIM domain kinase 1	1 to 647	D460A
pXJ-LIMK1 T508E-GST	GST	LIM domain kinase 1	1 to 647	T508E
pXJ-LIMK1 D460A-GST	GST	LIM domain kinase 1	1 to 647	D460A
pXJ-Flag-LIMK2	Flag	LIM domain kinase 2	1 to 617	
pXJ-mCherry-LIMK2 T484E	mCherry	LIM domain kinase 2	1 to 617	T484E
pXJ-mCherry-LIMK2 D430A	mCherry	LIM domain kinase 2	1 to 617	D430A
pXJ-LIMK2 T484E-GST	GST	LIM domain kinase 2	1 to 617	T484E
pXK-LIMK2 D430A-GST	GST	LIM domain kinase 2	1 to 617	D430A

pEGFP-MSN	eGFP	Moesin	1 to 577	
pEGFP-MSN T558D	eGFP	Moesin	1 to 577	T558D
pEGFP-MSN T558A	eGFP	Moesin	1 to 577	T558A
pXJ-mCherry-CFL1	mCherry	Cofilin 1	1 to 166	
pXJ-mCherry-CFL1 S3A	mCherry	Cofilin 1	1 to 166	S3A
pXJ-mCherry-CFL1 S3E	mCherry	Cofilin 1	1 to 166	S3E
pGEX-CFL1	GST	Cofilin 1	1 to 166	
pXJ-mCherry-TPPP	mCherry	Tubulin polymerisation promoting protein / p25	1 to 219	
pXJ-mCherry-TPPP T14D/S18D	mCherry	Tubulin polymerisation promoting protein / p25	1 to 219	T14D and S18D
pXJ-mCherry-TPPP T14E/S18E	mCherry	Tubulin polymerisation promoting protein / p25	1 to 219	T14E and S18E
pXJ-mCherry-TPPP T14A/S18A	mCherry	Tubulin polymerisation promoting protein / p25	1 to 219	T14A and S18A
pGEX-TPPP	GST	Tubulin polymerisation promoting protein / p25	1 to 219	
pXJ-GST-TPPP	GST	Tubulin polymerisation promoting protein / p25	1 to 219	
pXJΔ2-eGFP-TPPP	eGFP	Tubulin polymerisation promoting protein / p25	1 to 219	
pXJ-mCherry-SSH1	mCherry	Slingshot homolog 1	1 to 1049	

2.2 Methods

2.2.1 *Tissue culture, transfection and cell cycle arrest*

U2OS cells expressing rtTA tetracycline repressor chimera and U2OS cells expressing rtTA tetracycline repressor chimera and CDK1 T14A/Y15F were maintained in Dulbecco's modified Eagle's medium high glucose (Sigma) supplemented with 10% (v/v) foetal bovine serum (FBS) and 50 µg/mL geneticin (G418). HeLa cells were maintained in Minimum Essential Medium Eagle (Sigma) supplemented with 10% (v/v) FBS. COS-7 cells were maintained in Dulbecco's modified Eagle's medium high glucose (Sigma) supplemented with 10% (v/v) FBS. All cells were cultured at 37°C humidified incubator supplemented with 5% CO₂.

U2OS cells expressing rtTA tetracycline repressor chimera, U2OS cells expressing rtTA tetracycline repressor chimera and CDK1 T14A/Y15F constructs were gifts from Dr Randy Poon, Hong Kong University of Science and Technology, Hong Kong. All other cells were purchased from ATCC.

For routine sub-culturing, cells were washed once with phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and incubated with 2 mL of 0.25% (w/v) trypsin (Sigma-Aldrich) in 0.53 mM EDTA at 37°C until cells round up. 8 mL of complete growth media was added to stop reaction and cells were gently flushed off culture dish. Cells were pelleted by centrifugation at 1,200 rpm for 2 minutes and re-suspended in fresh complete growth media. Cells were sub-cultured at a ratio of 1:6.

For cell preservation, cells were trypsinised and re-suspended in complete growth media supplement with 5% DMSO at 1.5×10^6 cells/mL densities. Cells were subjected to cooling at a rate of 1°C/min until -80°C and subsequently maintained in liquid nitrogen gaseous phase for long term storage. For thawing, frozen cells were quickly brought to 37°C and washed once using complete growth media at 37°C before culturing as described above.

For cell cycle arrest at early M-phase, cells were treated with 0.1 µg/mL nocodazole (Sigma) for 12 hours. For arrest at S-phase, cells were treated with 2 µM hydroxyurea (HU) (Sigma) for 24 hours. As the full cell cycle duration for a typical metazoan cell can range up to 24

hours, hydroxyurea treatment for 24 hours allowed cells that have already exited the S phase at the point of HU treatment to be captured at the subsequent early S phase. Alternatively, to arrest cells at G1/S junction, double thymidine block was employed. Cells were arrested with 2 mM thymidine (Sigma) for 17 hours, released into fresh media without thymidine for 12 hours and blocked again with 2 mM thymidine for 17 hours. Double thymidine block allowed a highly synchronised population of cells at the G1/S border compared to single thymidine block. At the end of first thymidine treatment, cells were arrested at the G1/S transition, as well as throughout the S phase due to the inhibition of DNA synthesis. During the release for 12 hours, cells that were arrested at the G1/S border progressed through the cell cycle into G2/M phase. On the other hand, cells that were arrested in late S phase during the first block progressed into G1 phase. During the second thymidine treatment, cells that have entered G2/M progressed into G1 and were blocked at G1/S transition. Cells that have already entered G1 will also be captured at the G1/S border, thus a highly G1/S synchronised cell population was achieved. Mitotic cells were harvested 12 hours after second release. All releases were performed by washing cells thrice with complete growth media.

Cells were transfected in 35-mm dishes with 1 µg of plasmid and/or 150 pmol of siRNA using 3 µL Lipofectamin2000 (Invitrogen) for 24 hours (plasmid) or 48 hours (siRNA). Reagents were scaled up or down according to culture plate surface area.

CDK1(T14A/Y15F) gene expression was induced with 1.5 µg/mL doxycycline hydrochloride (Sigma) for the duration as stated.

Unless otherwise stated, cells were treated 50µM jasplakinolide (Invitrogen), 0.5 µg/mL cytochalasin D (Sigma), 30 µM Y27632 (Calbiochem), 2.0 µg/mL cell permeable C3 transferase (Cytoskeleton Inc), 100 µM blebbistatin (Sigma) or 15 µg/mL concanavalin A (Sigma).

2.2.2 General molecular cloning techniques

cDNA containing desired DNA sequences were used as a template for amplification by polymerase chain reaction (PCR) using primers flanked by appropriate restriction enzyme recognition sites and using the TaKaRa Ex TaqTM polymerase (TaKaRa). Amplification reactions were purified using the QIAquick PCR Purification Kit (QIAGEN) according to

manufacturer's protocol. Purified parent vector plasmids and amplified inserts were digested with the appropriate corresponding restriction enzymes. Digested DNA was separated by gel electrophoresis on a 1% agarose gel. Gel regions containing desired DNA were excised using razor blade, taking care to minimise ultraviolet (UV) light exposure, and purified using QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer's protocol. Purified digested plasmid vector and insert were quantified by optical density (OD)_{260nm} and subjected to ligation reaction using T4 DNA ligase (New England Biolabs) at vector to insert ratio of 1:3 at 23°C for 1 hour.

Ligated plasmids were transformed into chemically competent *Escherichia coli* DH5α cells by heatshock at 42°C for 45 seconds, and recovered in 500 µL of Super Optimal broth with Catabolite repression (SOC) (20 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 8.56 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose, pH 7.0) at 37°C for 30 minutes with occasional shaking. Bacterial cells were pelleted by brief centrifugation, re-suspended to 50 µL volumes and plated on Lysogeny Broth (LB) (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 8.56 mM NaCl, pH 7.4) agar supplemented with 100 µg/mL ampicillin or 50 µg/mL kanamycin and incubated at 37°C for about 16 hours. Colonies were picked and incubated in 5 mL of LB broth, with appropriate antibiotics selection, at 37°C for at least 16 hours with shaking at 200 rpm. Plasmids were purified using QIAprep Spin Miniprep Kit (QIAGEN) or AxyPrep™ Plasmid Miniprep Kit (Axygen) as described by manufacturer's protocol.

Purified plasmid preparations were digested with appropriate restriction enzymes and separated by electrophoresis on 1% agarose gel to check for positive ligation. DNA sequences were verified by sequencing and positive clones were selected for large scale purification using HiSpeed Plasmid Maxi Kit (QIAGEN) according to manufacturer's protocol.

2.2.3 *In vitro site-directed mutagenesis*

Moesin, LIMK and cofilin mutants were generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's protocol. Briefly, parental DNA was isolated from *dam*⁺ *E. coli* DH5α competent cells. Mutagenic oligonucleotide primers were designed to contain the desired mutation, flanked by unmodified nucleotide sequence, consist between 25 and 45 bases, terminate in one or more C or G bases, and has melting

temperature (T_m) greater than or equals to 78°C. Melting temperature was calculated using the formula, $T_m = 81.5 + 0.41 (\% \text{ GC}) - 675 / N - \% \text{ mismatch}$, where N represents primer length in bases; optimal % GC is 40. Mutant strands were synthesised using thermal cycling parameters as below.

Table 2-1. Cycling parameters for QuikChange Site-Directed Mutagenesis.

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

* Number of cycles depends on type of mutation desired

Table 2-2. Number of cycles required for different types of mutation.

Type of mutation desired	Number of cycles
Point mutations	12
Single amino acid changes	16
Multiple amino acid changes	18

Following thermal cycling reaction, methylated parental DNA template were digested by 10 U/ μ L of *Dpn* I at 37°C for 1 hour. 1 μ L of the *Dpn* I digested-DNA was used for transformation into XL1-Blue competent cells and plated on suitable antibiotic selection agar plates and incubated at 37°C for at least 16 hours. Positive colonies were picked and incubated in 5 mL LB broth with suitable antibiotics, shaking at 37°C for about 16 hours. Plasmids were purified using QIAprep Spin Miniprep Kit (QIAGEN) or AxyPrep™ Plasmid Miniprep Kit (Axygen) as described by manufacturer's protocol, sequenced (1st BASE or Suprenom) to verify for mutation and positive constructs were selected for large scale purification using HiSpeed Plasmid Maxi Kit (QIAGEN) according to manufacturer's protocol.

2.2.4 *Indirect immunofluorescence microscopy and live imaging*

Cells cultured on coverglass were fixed in 4% paraformaldehyde for 20 minutes, permeabilised with 0.2% Triton X-100 in PBS for 10 minutes and quenched with 10% bovine serum albumin (BSA) in PBS for 10 minutes. Coverglass were incubated with 100 µL of primary antibodies at 37°C humidified chamber for 2 hours, or at 4°C humidified chamber for overnight. Slides were then incubated with 100 µL of Alexa Fluor 488- or Alexa Fluor 546-labelled secondary antibodies (Invitrogen) and/or phalloidin (Sigma) at 25°C humidified chamber for 1 hour. All washings were performed with 0.1% Triton X-100 in PBS. Coverglass were dried briefly and mounted using Vectashield mounting medium with DAPI (Vector Laboratories).

For LIMK2 and TPPP visualisation, cells were first extracted with cytoskeleton buffer (10 mM MES pH 6.1, 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, 0.32 M sucrose) at 4°C for 10 minutes and fixed with methanol at -20°C for 10 minutes, followed by permeabilisation and subsequent steps as described above.

Fluorescence images were acquired using Axio Observer microscope (Carl Zeiss) with Ziess EC Plan-neofluar 40x/1.3 oil (for interphase cells) or Zeiss Plan-Apochromat 100x/1.4 oil (for mitotic cells) objective lens coupled to CoolSNAP HQ² camera (Photometrics) and analysed using MetaMorph software (Molecular Devices). Immunofluorescence images were processed in red/green/blue (RGB) colour mode and printed in cyan/magenta/yellow/key black (CMYK) mode. Print versions may be unable to represent colours that are out of gamut.

For centrosome staining intensity representation, integrated intensity was measured in an area of 50 by 50 pixel box surrounding each individual centrosome. Intensities were corrected for signal to noise ratio.

For astral microtubule quantification, fixed immunofluorescence images were taken. Astral microtubule fluorescence intensities were calculated by subtracting background-corrected fluorescence intensities of kinetochore/polar microtubules from the total microtubule fluorescence.

For phase-contrast live-imaging, cells were cultured in glass-bottom chambers and images were acquired using Axiovert 200 microscope (Carl Zeiss) with Zeiss LD Plan-neofluar 40x/0.6 Corr Ph2 objective lens coupled to CoolSNAP HQ camera (Photometrics). Cells were maintained in a humidified chamber at 37°C supplemented with 5% CO₂ throughout imaging. Temperature was controlled using tempcontrol 37-2 *digital* (Carl Zeiss) and CO₂ regulated by CO₂-controller (Carl Zeiss). Stacked images were analysed using MetaMorph software.

2.2.5 *Sodium dodecyl sulphate-polyacrylamide gel electrophoration and western blot*

For western immunoblot analysis, cells were lysed in SDS sample buffer (0.06 M Tris-HCl, 1.7% SDS, 4.3% glycerol) and cleared by centrifugation at 15,000 rpm at 4°C for 10 minutes. Supernatants were normalised at OD_{595nm} using Bradford reagent assay (BioRad). Lysates were heated at 100°C for 10 minutes and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoration (SDS-PAGE) and transferred to nitrocellulose membrane 0.45µm (BioRad) at 80 V, 4°C for 2 hours. Membrane was quenched with 5% skim milk in PBS at 25°C for 1 hour and blotted with primary antibodies at 25°C for 2 hours or 4°C for overnight. Membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse (DakoCytomation) or anti-rabbit (Amersham Biosciences) secondary antibodies at 25°C for 1 hour. All antibodies were prepared in 3% skim milk in PBS. Washing steps were performed with 0.05% Tween 20 in PBS. Membranes were subjected to chemiluminescence detection using ECL Plus Detection Kit (Amersham) according to manufacturer's protocol and exposed onto Super RX Fuji medical X-ray film (Fuji) and developed using Kodak X-OMAT ME-1 processor.

2.2.6 *Glutathione S-transferase pull-down assay*

For Glutathione S-transferase (GST) pull-down assays, cells were lysed with protein lysis buffer (25 mM Hepes pH 7.5, 0.3 M NaCl, 1 mM MgCl₂, 1 mM EGTA, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 10 mM sodium fluoride, 5% glycerol, 0.5% Triton-X, 5 mM DTT, protease inhibitor tablet) and cleared by centrifugation at 15,000 rpm at 4 °C for 10 minutes. For total lysate analysis, 10% supernatant were snap frozen using liquid nitrogen a stored at -80°C until further analysis. The remaining supernatant was incubated with 100 µL of slurry Glutathione Sepharose[®] 4B beads (Amersham Biosciences) while rolling at 4°C

for 2 hours. Beads were spun at 1,000 rpm for 1 minute and washed thrice each with 1 mL GST purification buffer (50mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄, 0.5 mM MgCl₂, 0.1% Triton-X 100) rolling at 4°C for 10 minutes. Proteins were recovered from beads by adding 50 µL of SDS sample buffer to beads and heated at 100°C for 10 minutes. Supernatants were analysed by SDS-PAGE and western blot.

2.2.7 Fluorescence-activated cell sorting

Cells were trypsinised and washed once with PBS. Cell pallets were re-suspended in 1 mL PBS at 4°C and fixed by adding 4 mL of ice-cold absolute ethanol drop-wise while vortexing cells. Cells were fixed for at least 4 hours. For fluorescence activated cell sorting (FACS) analysis, cells were washed once with PBS and re-suspended in 300 µL of 0.1% Triton X-100 in PBS, 1 mg/mL RNase A type IIIA (Sigma). DNA was stained with 0.15 mg/mL propidium iodide (Sigma) for at least 2 hours. Samples were subjected to flow cytometry analysis and 30,000 events were counted, without gating, using the FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using CellQuest software (Becton Dickinson).

2.2.8 RNA extraction, RT-PCR and real-time quantitative PCR

Cells were trypsinised and washed once with PBS. Cell pallets were snap frozen using liquid nitrogen. RNA was extracted using the RNeasy Mini Kit (QIAGEN) according to manufacturer's protocol. Purified RNA was quantified by OD_{260nm} and purity checked by OD_{260nm}/OD_{280nm}. First strand cDNA was synthesised using 2 µg of total RNA with random primers using the Superscript VILO cDNA Synthesis Kit (Invitrogen) according to manufacturer's protocol. cDNA samples were diluted ten-fold and 2 µL was used for quantitative PCR analysis. Samples were run on a StepOne Plus real time PCR machine (Applied Biosystems) using the default settings for Fast SYBR green reactions. Gene expression levels were calculated by the $\Delta\Delta$ CT method and were normalized using TATA binding protein (TBP) as an endogenous control and ROX passive reference dye for calibration of initial fluorescence. Real time PCR was carried out in triplicate for every sample. No template controls and no reverse transcription (RT) controls were performed to test for DNA contamination. To check for primer specificity and contamination, melting curve analysis and gel electrophoresis on a 4% agarose gel were performed on PCR end products.

2.2.9 Micropipette aspiration assay

Cells were cultured to confluence in 25 cm² culture flasks. Mitotic cells were collected by shake-off and re-suspended in 200 µL Leibovitz's L-15 Media (Gibco) supplemented with 10% (v/v) FBS at 37°C. Cell suspensions were suspended between a coverglass chamber and subjected to micropipette aspiration.

Borosilicate glass capillaries (World Precision Instruments, USA) with outer and inner diameters of 1.0 mm and 0.75 mm respectively, were used to produce micropipettes with inner diameters ranging from 7.8 µm to 13.1 µm using micropipette puller and microforge (ALA Scientific Instruments, Inc., USA). Micropipettes were coated with 1% BSA and stored at 4°C until ready to use. Mitotic cells were brought into contact with micropipette by applying gentle suction. Cells were then subjected to increasingly suction pressure and images were captured at 9 seconds interval using 100x objective brightfield microscope (Leica). Suction pressures were digitally controlled using a precision pump (Cole-Parmer) with a flow rate between 20 to 60 ml/hr. Fine movements of micropipettes were controlled using micromanipulator (Eppendorf AG, Germany).

Cortical deformation was measured using MetaMorph software (Molecular Devices). Cortical rigidity was represented using the Young's Modulus, E , in pN/µm², and was calculated using the formula, $\Delta P = (2\pi / 3) E (L_p / R_p) \Phi$, where ΔP represents change in pressure calculated using formula, $\Delta P = \rho g \Delta h$; ρ represents water density, 1000 kg/m³; g represents gravitational force, 9.8 m/s²; Δh represents change of liquid height in mm; Φ represents ratio of pipette wall thickness to lumen radius, approximately 2.1, L_p represents deformation length in µm; and R_p represents pipette radius in µm.

2.2.10 Bacterial expression and purification of proteins

Full length cofilin and TPPP were cloned into carboxyl-terminus of GST protein in pGEX 4T-1 expression vector. Plasmid was transformed into chemically competent *Escherichia coli* BL-21 cells. For protein expression, cells were cultured shaking overnight at 37°C. Subsequently saturated cultures were diluted ten-fold and cultured until OD_{600nm} reaches 0.6. Following, cultures were induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours at 25°C while shaking.

For protein purification, induced bacterial cells were pelleted and lysed with B-PER Protein Extraction Reagent (Pierce, Thermo Fisher Scientific, New York), supplemented with 0.1 mg/mL lysozyme, 5 U/mL DNase I and protease inhibitor tablet (Roche), at a ratio of 4 mL B-PER per gram of cell pellet and incubated at room temperature for 15 minutes. Homogenates were sonicated and cell lysates were centrifuged at 15,000 rpm at 4°C for 10 minutes. Supernatant were subjected to affinity purification using 100 µL of slurry Glutathione Sepharose® 4B beads (Amersham Biosciences) while rolling at 4°C for 1 hour. Beads were washed thrice with GST purification buffer and proteins were recovered by addition of 500 µL reduced glutathione to beads and rolling at 4°C for 30 minutes. Purified proteins were subjected to dialysis in 500x sample volume of PBS using 20K MWCO Slide-A-Lyzer G2 Dialysis Cassette (Pierce) for overnight at 4°C. Protein samples were snap-frozen in 10% glycerol, using liquid nitrogen and stored at -80°C until further use.

2.2.11 Kinase Assay

Kinase active and kinase dead clones of PXJ-LIMK1-GST and PXJ-LIMK2-GST were transiently transfected into three confluent 100-mm dishes of COS-7 cells for 36 hours. Cell lysates were harvested and kinases were purified by glutathione S-transferase pull-down purification. For kinase assay, immunoprecipitates on beads were washed three times with kinase buffer (50 mM HEPES-NaOH, pH7.5, 25 mM β-glycerolphosphate, 5 mM MgCl₂, 5 mM MnCl₂, 10 mM NaF, 1 mM Na₃VO₄), and then incubated in 30 µl kinase buffer containing 50 µM ATP, 5µCi [³²P]γ-ATP (6000 Ci/mM) and 6 µg of substrate for 20 minutes at 30°C. Substrates used included bacterially expressed GST (negative control), GST-cofilin (positive control), and GST-TPPP. Reactions were terminated by heating at 100°C for 3 minutes in SDS sample buffer and samples were subjected to SDS-PAGE.

2.2.12 Spindle orientation assay

Cells were cultured on fibronectin (Sigma)-coated coverglass with a coating density of 3 µg/cm². After various treatments, cells were fixed and immuno-stained with anti-pericentrin and DAPI and imaged. DNA staining was used as a reference for cell cycle stage. Z-stack images of metaphase cells were captured at 1 µm apart. Spindle orientation was calculated using inverse trigonometry function, $\alpha = \tan^{-1} (A / B)$, where α represents the metaphase

spindle angle, in degrees, in relation to the substratum; A represents z axis distance between spindle poles in μm ; and B represents x-y axis distance between spindle poles in μm .

Spindle angles were presented using box-and-whiskers diagram. Box represents upper quartile, median and lower quartile respectively; whiskers represent maximum and minimum, black dots represent mean; and circles represent outliers. Outliers were defined as data points deviating more than one-and-half times of interquartile range from the upper or lower quartile values.

2.2.13 Cell proliferation assay

Cells were grown on 60-mm culture dish and transfected with appropriate siRNAs. Eight hours post-transfection, cells were trypsinised and cultured into 24-wells culture plates at appropriate and equal densities. Plates were first harvested for proliferation assay 24 hours post-transfection and every 24 hours thereafter. For proliferation assay, cells were treated with 0.5 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and incubated at 37°C humidified incubator with 5% CO₂ for 2 hours. At intervention, media were gently removed and 200 μL of DMSO were added into each wells and incubated at room temperature for 15 minutes while shaking. Optical densities were measured at 570 nm, with background subtraction at 690 nm. Each sample was measured in triplicate wells, and individual experiment was performed in triplicate. Percent growth was represented by fold increase in absorbance, and was calculated over 5-day period.

2.2.14 Centrosome isolation

Approximately 1×10^9 cells were cultured in large flasks. Mitotic populations were collected via shake-off, and remaining adherent cells were trypsinised and collected as interphase populations. Cell pellets were washed sequentially and rapidly with 1x TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl), 0.1x TBS and 8% sucrose in 0.1x TBS. Washed pellets were resuspended in 2 mL 8% sucrose in 0.1x TBS, followed by adding 8 mL of lysis buffer (1mM Hepes pH 7.2, 0.5% NP-40, 0.5 mM MgCl₂, 0.1% β -mercaptoethanol, protease inhibitor tablet) and vortexed for 30 minutes at 4°C. Lysates were cleared by centrifugation at 2,500 rpm for 10 minutes at 4°C. Supernatants were collected and adjusted to 10 mM HEPES and 1 $\mu\text{g/mL}$ DNaseI final concentration and incubated for 30 minutes at 4°C.

Lysates were loaded onto an ultracentrifuge clear tube and underlaid with 1.5 mL of 60% (w/w) sucrose in gradient buffer (10mM PIPES pH 7.2, 0.1% Triton-X, 0.1% β -mercaptoethanol). Centrosomes were sedimented onto the sucrose cushion by centrifugation at 25,000 g for 15 minutes at 4°C. After centrifugation, the top 8 mL of solution was discarded and the remaining solution was mixed gently. The mixture was loaded onto 7 mL discontinuous sucrose gradient dissolved in gradient buffer, with 70% (w/w) sucrose at the bottom and 40% (w/w) sucrose on top. Tubes were centrifuged at 120,000 g for 1 hour at 4°C. Fractions were collected from the bottom of the tubes. Centrosomes from each fraction were pelleted by addition of 10 mL of 10 mM PIPES pH 7.2 and centrifugation at 20,000 g for 15 minutes at 4°C. Pellet proteins were solubilised in SDS buffer and analysed by SDS-PAGE and western blot.

2.2.15 Statistical analysis

Unpaired student's t-test was used for all statistical analysis. $P < 0.05$ was considered to be statistically significant. Unless otherwise stated, results are represented by mean $\pm$ standard deviation of at least three independent experiments.

CHAPTER 3 ACTIN AND THE MITOTIC TEMPO

The actin cytoskeleton is known to be highly versatile during the course of cell cycle progression. Under this chapter, we will examine the effects of ectopic expression of mitotic kinase CDK1 on cells with interphase morphology. In addition, we will also look into the effects of actin perturbation on the cell cycle progression.

3.1 Results

3.1.1 *Localisation pattern of actin, focal adhesions and cyclin B1 in interphase cells*

To study the roles of actin on the cell cycle regulation, we first examined the localisation pattern of the cell cytoskeletal network and focal adhesion complexes in unperturbed cells. U2OS human osteosarcoma cell line was selected because these cells display dynamic and thick stress fibres (Hotulainen and Lappalainen, 2006). Wildtype U2OS cells were examined for actin, tubulin, paxillin, vinculin, focal adhesion kinase (FAK) and cyclin B1 localisation (figure 3-1). Immunostaining showed that at interphase, U2OS cells expressed filamentous tubulin and rigid actin stress fibres pattern throughout the cytoplasm, maintaining a well-spread shape. Paxillin, vinculin and FAK localised to the distal tip of filamentous actin fibres; with paxillin and vinculin also displaying strong peri-nuclear localisation (figure 3-1, white arrowheads). Paxillin and vinculin were commonly reported to localise to the perinuclear region of cells (Norman et al., 1998). Paxillin was reported to be targeting to perinuclear region by the ARF-GAP domain of GIT family proteins (Kondo et al., 2000) and subsequently recruited to focal complexes via activity of ARF1 (Norman et al., 1998). Cyclin B1 was evenly distributed throughout the cytoplasm during interphase as expected (Clute and Pines, 1999).

3.1.2 *Hydroxyurea-induced S phase arrest activates ATM/ATR kinase activity*

To establish early G₁ phase cell cycle arrest by DNA-replication block, U2OS cells were treated with hydroxyurea (HU). HU inhibits ribonucleotide reductases that catalyses the reduction at the 2'-carbon from the ribose 5'-phosphate to form 2'-deoxyribose 5'-phosphate, thus depleting the pool of 2'-deoxynucleoside 5'-triphosphate (dNTP) within the cell and block DNA synthesis (Atkin et al., 1973; Sneed and Loeb, 2004). Cells were treated with

varying concentration of HU for 24 hours and the degree of genotoxic stress was examined by immunostaining of phosphorylated ataxia telangiectasia mutated (ATM)/ ATM and Rad3-related (ATR) substrates (figure 3-2). Results showed that at 2 mM HU, degree of ATM/ATR activity was weak. At 5 mM concentration, most cells showed high level of ATM/ATR substrates phosphorylation. Similar ATM/ATR activities were observed at 5 mM and 10 mM concentration HU. Phalloidin staining demonstrates cell remain adherent with morphologically intact actin structures up to 10 mM HU treatment for 24 hours.

Figure 3-1. Localisation pattern of actin, tubulin, focal adhesions and cyclin B in interphase cells. Wildtype U2OS cells were stained with anti- α -tubulin, anti-paxillin, anti-vinculin, anti-FAK or anti-cyclin B1 (green). White arrows pointing to perinuclear concentration for paxillin and vinculin. Cells were co-stained with phalloidin (red) and DAPI (blue). Merged images shown on right panel. Bar, 25 μ m.

Figure 3-2. Degree of ATM/ATR kinase activity in hydroxyurea (HU)-treated cells. U2OS were treated with solvent control, 2 mM, 5 mM or 10 mM HU and stained with anti-phosphorylated ATM/ATR substrates (green), phalloidin (red) and DAPI (blue). Ratios on figure indicate number of cells stained positive for phosphorylated ATM/ATR substrates over number of cells scored. Merged images shown on right panel. Bar, 25 μ m.

3.1.3 Over-expression of CDK1(T14A/Y15F) in S-phase arrested cells caused forced expression of mitotic marker and morphology

As the dephosphorylation of CDK1 has been found to be essential for its activation during late G₂ phase (Ducommun et al., 1991; Hayles et al., 1994; Rhind et al., 1997), we examined the effects of ectopic activity of CDK1 during S phase. We obtained a CDK1(T14A/Y15F) construct that represents non-phosphorylatable mutant form and hence constitutively active CDK1 and examined the effect of its over-expression in late G₁/ early S phase arrested cells.

In the control set, cells were arrested at early S phase by HU. FACS analysis showed at least 80% of cells were successfully arrested with 2N DNA content (figure 3-3A). Over a period of 2 to 6 hours post-release, cells underwent DNA replication as indicated from the gradual shift of DNA content peak from 2N to 4N. By 8 hours post-release, most cells have completed DNA replication and entered into G₂ phase. At 14 to 16 hours post-release, cells began to enter M phase as 2N DNA peak started to increase while the 4N peak declined (figure 3-3A), signifying the successful completion of division and cytokinesis. Various cell cycle dependent protein levels were then tracked and showed a similar temporal profile from the FACS data (figure 3-3B). Western immunoblot data showed that during interphase, the cells maintained a steady expression level of phosphorylated CDK1. As the cells enter into mitosis at 12 to 16 hours post-release, the level of phosphorylated CDK1 decreased while unphosphorylated CDK1 increased. Levels of phosphorylated histone H3, a marker specific for mitosis, also peaked at 14 to 16 hours post-release, a pattern coinciding with FACS data. Cyclin B1 levels started to decline in late mitosis of 16 hours post-release. Levels of cyclin D1, cyclin E and cyclin A were at their expected patterns as the cell progress through the cell cycle (figure 3-3B).

For cells that were constantly kept arrested by HU, DNA profiling by FACS showed that cells did not progress into the cell cycle and DNA content remained at 2N throughout the entire 18 hours (figure 3-3C). However, it was noted that there was a gradual spread of the 2N peak towards the sub G₁ phase as the cell progressed from 2 to 18 hours of tracking, suggesting that prolonged HU treatment may cause DNA fragmentation and cell death in U2OS cells. Hydroxyurea is a commonly used chemotherapy agent used in a wide range of cancers such as melanoma, chronic myelocytic leukaemia, ovarian cancer and some primary squamous cell carcinoma. It is known to cause cell death via apoptosis in a dose-dependant

manner (Huyghe et al., 2004; Johnson et al., 1992). Western immunoblot data showed a constant level of protein expression for cyclin B1, cyclin D1, cyclin E and cyclin A throughout. In addition, CDK1 remains phosphorylated in these cells and phosphorylated histone H3 level was undetectable (figure 3-3D).

Next we examined the effects of over-expression of constitutively active CDK1(T14A/Y15F) that is controlled under the tetracycline trans-activation repressor (rtTA) system (Gossen and Bujard, 1992) in HU arrested cells. In our experiment a more stable tetracycline analogue, doxycycline, was used to induce transgene expression. Force expression of CDK1(T14A/Y15F) in such arrested cells showed that, similar to un-induced cells (figure 3-3C), there are no significant effects on cell cycle progression as these cells remain arrested in late G₁ phase with 2N DNA content (figure 3-3E). Western immunoblot data revealed that Flag-CDK1(T14A/Y15F) expression was first detectable 4 to 6 hours post-release, which coincided with increased levels of phosphorylated histone H3. In addition, cyclin B1, cyclin E and cyclin A levels (figure 3-3F) were similar to those of the un-induced cells (figure 3-3D). However, it was noted that the levels of cyclin D1 decreased upon the induction of CDK1(T14A/Y15F) expression (figure 3-3F). This could be attributed prolonged growth inhibition under the influence of both hydroxyurea and doxycycline. Decreased cyclin D1 expression has been frequently associated to prolonged drug-induced growth inhibition in many cancer cells (Christov et al., 2003; Joe et al., 2002). Transient expression of CDK1(T14A/Y15F) in wildtype U2OS cells caused the collapse of stress fibres and chromosomal condensation (figure 3-4), a morphology frequently associated with mitosis.

Our data indicates that phosphorylation of histone H3, despite being widely recognised as a sufficient and rapid indicator of mitosis (Tapia et al., 2006), can be observed in cells which have not undergone DNA replication. In addition, our data suggest that the mitotic phosphorylation of histone H3 and its associated morphological changes, in particularly actin re-organisation and chromosomal condensation, may be under the direct regulation of CDK1 activity.

Figure 3-3. Over-expression of CDK1(T14A/Y15F) in hydroxyurea (HU)-arrested cells caused forced expression of mitotic markers. U2OS cells were arrested with 2 μ M HU for 24 hours and released into fresh media (**A** and **B**), continued arrest for another 18 hours (**C** and **D**), or continued arrest for another 18 hours coupled with CDK1(T14A/Y15F) induction 5 hours prior to first sample collection (**E** and **F**). Cells were harvested every 2-hourly for a period of 18 hours and subjected to FACS DNA profiling (**A**, **C** and **E**) and western immunoblotted with various cell cycle markers (**B**, **D** and **F**). Asyn, asynchronised cells; M, M phase reference sample; * upper and lower band represents phosphorylated and unphosphorylated CDK1 respectively.

Figure 3-4. Over-expression of CDK1(T14A/Y15F) caused chromosomal condensation and actin filaments dissolution. U2OS cells were transfected with GFP vector control or pUHD-P1-Flag-CDK1(T14A/Y15F) for 24 hours and induced with doxycycline for 8 hours. Cells were stained with phalloidin (red), anti-flag (green) and DAPI (blue). Merged images shown on right panel. Bar, 25 μ m.

3.1.4 Over-expression of active form CDK1 in U2OS cells does not accelerate cell cycle progression

We next examined the effects of premature CDK1 activity on the timing of entry into different phases of cell cycle. As a control, U2OS cells were first synchronised using double thymidine block and released into fresh media and the cell cycle progression timings were tracked. FACS data revealed that at 4 to 10 hours post-release, the 2N DNA peak shifted gradually from 2N to 4N, indicating the progression of S phase DNA replication. At 12 to 14 hours post-release, the 4N DNA peak gradually decreased and returned to 2N, indicating cells have successfully completed mitosis and entered into the next cell cycle (figure 3-5A). Western immunoblot data showed that under block-and-release situation, CDK1 dephosphorylation occurred at 12 hours post-release onwards, as coincided with FACS data signifying entry into M phase. Phosphorylated histone H3 levels also peaked during the expected M phase occurrence at 12 to 14 hours post-release. In addition, cyclin B1 levels started to decline at 14 hours post-release, coinciding with the entry into late mitosis. Levels of cyclin D1, cyclin E and cyclin A were as expected throughout (figure 3-5B).

In cells that were induced with doxycycline 5 hours and 17 hours prior to release, CDK1(T14A/Y15F) levels were detectable as early as 2 to 4 hours post-release (figure 3-5D and F). However, although the activity of CDK1 was prematurely elicited in these cells, FACS data showed that there was no change in the shift pattern of DNA profiles, indicating that cells undergone DNA replication and M phase at about similar period to the un-induced cells (figure 3-5C and E). Forced expression of active CDK1 by doxycycline induction at 5 and 17 hours prior release, however, did resulted in a slight acceleration in the peak of histone H3 phosphorylation to 10 to 12 hours and 8 to 10 hours respectively (figure 3-5D and F). Thus our data suggest that although CDK1 can elicit premature histone H3 phosphorylation, its activity is insufficient to bring about acceleration of the cell cycle.

Figure 3-5. Over-expression of CDK1(T14A/Y15F) in double thymidine arrested cells does not accelerate cell cycle progression. U2OS cells were synchronised by double thymidine block and released into fresh media (**A** and **B**), released into fresh media coupled to CDK1(T14A/Y15F) induction 5 hours prior release (**C** and **D**) or 17 hours prior release (**E** and **F**). Cells were harvested every 2-hourly for a period of 18 hours and subjected to FACS DNA profiling (**A**, **C** and **E**) and western immunoblotted with various cell cycle markers (**B**, **D** and **F**). Asyn, asynchronised cells; * upper and lower band represents phosphorylated and unphosphorylated CDK1 respectively.

3.1.5 Actin filaments disruption by cytochalasin D caused cytokinesis failure

To study the effect of filamentous actin on the control of various cell cycle stages progression, we disrupted the actin structures using cytochalasin D and tracked the cell cycle from late G₁ to M phase completion. Cytochalasin D is a cell-permeable fungal toxin that binds to the barbed end of actin filaments, inhibiting both the association and dissociation of actin subunits, thus resulting in disruption of actin filaments and inhibition of actin polymerisation(Cooper, 1987). We first examined the effects of cytochalasin D on the actin structure. Cell staining revealed that cell treated with cytochalasin D displayed a marked reduction of actin filaments and presented with aggregation of blotched actin staining in the cytoplasm (figure 3-6). Tubulin network remained intact otherwise.

Figure 3-6. Cytochalasin D treatment caused dissolution of actin filaments. U2OS cells were treated with DMSO control or cytochalasin D and stained with phalloidin (red) and anti- α -tubulin (green). Bar, 20 μ m.

To examine the effects of cytochalasin D on cell cycle progression, U2OS cells were synchronised with double thymidine block and released into media supplemented with cytochalasin D. In the control cells that were synchronised and released into fresh media, cell cycle progressed at the expected timing (figure 3-7A and B). In cells released into cytochalasin D, FACS data showed that cells were able to proceed into S phase as the DNA content peak gradually shifted from 2N to 4 N at similar timings compared to the control cells (figure 3-7C). However, at 14 hours post-release, there was a gradual decrease in the 4N DNA peak, while the 2N DNA peak remained low (figure 3-7C). Western immunoblot analysis showed that in these cells, histone H3 phosphorylation occurred at the similar timing as control cells and cyclin B1 expression declined at a timing coinciding with late M phase. Our data suggest that disruption of actin filaments by cytochalasin D does not affect cells ability to undergo DNA synthesis and subsequent display of histone H3 phosphorylation. However these cells do not return to the next subsequent G₁ phase after mitosis, indicating a possible defect in cytokinesis.

To examine if cytochalasin D-treated cells can enter and exit M phase, U2OS cells were synchronised using double thymidine block and released into media supplemented with cytochalasin D and observed via phase contrast microscopy (figure 3-8). Control cells that were released in fresh media displayed expected morphology (figure 3-8A). They remained adherent and displayed cytoplasmic spreading throughout interphase which subsequently collapsed and rounded up as the cells enter into mitosis. During mitosis, metaphase alignment of sister chromatids and anaphase separation occurred as expected. During late anaphase, ingression begins at the cleavage site and the cell pinches to form two daughter cells. Post mitosis, these cells re-established contact with the substratum and formed lamellapodia-like cytoplasmic projections (figure 3-8A, black arrows) as the cells regained its well-spread shape. For cells treated with cytochalasin D, cells similarly round up at the onset of mitosis and alignment of chromosomes at the metaphase plate and anaphase separation of sister chromatids were observed. However, there was no obvious formation of the cleavage furrow and the separation of the two daughter cells (figure 3-8B). Instead, after anaphase, the cells quickly flattened and re-attached to the substratum by the formation of long slender protrusions (figure 3-8B, white arrows).

Taken together, our data showed that cytochalasin D treatment used at the concentration herein, does not affect cell cycle progression from late G₁ phase to mitosis entry, suggesting

that an intact actin structure is dispensable for cell cycle progression up to anaphase stage. However, cells cannot undergo proper cytokinesis and enter into the subsequent G₁ phase.

Figure 3-7. Cytochalasin D treatment disrupted 2N DNA peak recovery post mitosis. U2OS cells were synchronised using double thymidine block and released into fresh media (**A** and **B**) or media supplemented with cytochalasin D (**C** and **D**). Cells were harvested every 2-hourly for a period of 18 hours and subjected to FACS DNA profiling (**A** and **C**) and western immunoblotted with various cell cycle markers (**B** and **D**). Asyn, asynchronous cells; * upper and lower band represents phosphorylated and unphosphorylated CDK1 respectively.

Figure 3-8. Cytochalasin D treatment caused cytokinesis failure. U2OS cells were synchronised with double thymidine block and released into fresh media (A), or media supplemented with cytochalasin D (B). Cells were subjected to phase-contrast live-imaging. Black arrows pointing to membrane rufflings; white arrows pointing to finger-like projections. Numbers on images represent elapsed time in hours and minutes. Bars, 25 μ m.

3.1.6 Actin filament stabilisation using jasplakinolide caused mitosis delay

Next, we examined the effects of forced stabilisation of actin filaments on cell cycle progression by treatment of cells with jasplakinolide and tracking cell cycle progression post-thymidine release. Jasplakinolide is a marine sponge cyclic peptide which binds to the interface of three actin subunits. It decreases the amount of sequestered actin by lowering the critical concentration required for actin polymerisation, thereby augmenting nucleation and stabilisation of actin filaments (Bubb et al., 2000). As jasplakinolide binds competitively with phalloidin, thus we transfected U2OS cells with mCherry-actin for visualisation of actin. Transfected U2OS cells were treated with varying concentrations of jasplakinolide. Cells treated with 20 nM and 30 nM jasplakinolide displayed thicker and more robust actin filaments compared to control cells treated with DMSO (figure 3-9). These cells also displayed prominent actin aggregation at the peri-nuclear region (figure 3-9, white arrows) but cell shape is otherwise preserved. At higher concentration of 50 nM and 100 nM, large actin clumps were observed and the cell losses its normal morphology and is accompanied by cytoplasmic blebbings (figure 3-9).

Figure 3-9. Jasplakinolide treatment caused altered actin filaments. U2OS cells were transfected with pXJ-mCherry-actin (red) for 24 hours and treated with DMSO control, 20 nM, 30 nM, 50 nM or 100 nM of jasplakinolide for 3 hours. Cells were co-stained with DAPI (blue). White arrows pointing to perinuclear actin aggregate. Bar, 25 μ m.

To examine the effects of forced stabilisation on cell cycle progression, U2OS cells were synchronised by double thymidine block and released into media containing various concentration of jasplakinolide. In the control cells, FACS and western immunoblot data revealed that at 2 to 10 hours post-thymidine release, cells underwent DNA replication as DNA content shifted from 2N to 4N. At 12 to 14 hours post-release, the cells entered mitosis, as seen from the increase in phosphorylated histone H3, dephosphorylation of CDK1 and recovery of 2N DNA peak (figure 3-10A and B). For cells released into 20 nM of jasplakinolide, the 2N DNA peak recovery was only seen at 14 hours post-release (figure 3-10C), a delay of 2 hours from the control cells. Western immunoblot data showed that histone H3 phosphorylation peak was similarly delayed for 2 hours, appearing only at 12 to 16 hours post-release (figure 3-10D). In addition, cyclin D1 level was slightly increased as the cells entered mitosis (figure 3-10D). As treatment of cells with 20 nM jasplakinolide seemed to delay mitosis onset by about 2 hours, we thus examined if treatment at higher concentration of 50 nM jasplakinolide would result in enhanced delay. FACS data showed that indeed, in these cells, although DNA replication occurred similarly from 2 to 10 hours post-release, the recovery of 2N DNA peak was only obvious at 16 hours post-release (figure 3-10E). The above data suggest that the forced stabilisation of actin filaments does not seem to affect the timing of S phase progression, but rather delayed the exit of cells from mitosis. Although we cannot be sure if this delay of exit was due to a delay in the onset of mitosis or that the mitotic cells were held at mitosis for a longer period.

To further confirm that jasplakinolide treatment does affect the release of cells from mitosis, and this phenotype is not a “carried over” effect from defective G₁ and S phase, if any, we added jasplakinolide to the cells at 9 hours post-release, presumably when the cells are in late G₂ phase. DNA profiles collected for this cells displayed similar pattern (figure 3-11B and C) as compared to the previous set where cells were subjected to jasplakinolide immediately post-release (figure 3-10B and C). In addition, live-cell imaging of Jasplakinolide-treated cells showed no significant changes in the general morphology of mitotic processes compared to control-treated cells (data not shown). Thus our data demonstrated that the timing of mitotic progression is dependent on the state of actin architecture within the cell during the period of mitosis.

Figure 3-10. Jasplakinolide treatment during early S phase caused mitotic delay. U2OS cells were synchronised with double thymidine and released into fresh media (**A** and **B**), media supplemented with 20 nM jasplakinolide (**C** and **D**) or media supplemented with 50 nM jasplakinolide (**E**). Cells were harvested every 2-hourly for a period of 18 hours and subjected to FACS DNA profiling (**A**, **C** and **E**) and western immunoblotted with various cell cycle markers (**B** and **D**). Asyn, asynchronised cells; * upper and lower band represents phosphorylated and unphosphorylated CDK1 respectively. Note: Figure 3-10 **A** and **B** control data used were similar to Figure 3-5 **A** and **B**, as both sets of experiments were performed in parallel.

Figure 3-11. Jasplakinolide treatment during late G_2 phase caused mitotic delay. U2OS cells were synchronised with double thymidine block and released into fresh media (**A**), supplemented with 20 nM jasplakinolide 9 hours post-relapse (**B**) or supplemented with 50 nM jasplakinolide 9 hours post-release. Cells were harvested every 2-hourly for a period of 18 hours and subjected to FACS DNA profiling (**A**, **B** and **C**). Asyn, asynchronised cells.

3.1.7 Actin filaments disruption by Y27632 and C3 transferase caused mitotic acceleration

In order to examine the effects of actin disruption on mitotic progression, HeLa cells were treated with Y27632 or C3 transferase, which inhibits ROCK and Rho respectively, and examined as the cells progress through mitosis. HeLa cells were used for experiments on this point forward as the effects of drugs and mitotic events were better studied and documented. Treatment of asynchronised HeLa cells with Y27632 or C3 transferase showed that actin filaments can be disrupted within 30 minutes of treatment (figure 3-12) compared to control HeLa cells that usually display thick and robust stress fibres. In order to study the effects of Y27632 and C3 transferase on mitotic events, HeLa cells were synchronised in early mitotic phase by treatment of nocodazole. Nocodazole depolymerises microtubules *in vivo* and are routinely used in the reversible arrest of cells at early mitosis (De Brabander et al., 1981; Zieve et al., 1980). We found that cells subjected to either Y27632 or C3 transferase treatment was able to assume a well spread adherent shape faster than non-treated cells after nocodazole release (figure 3-13A). Quantification showed that at 60 minutes post-nocodazole release, the number of spread cells increased from 24 % in control group to 33 to 34 % in the drugs-treated groups (figure 3-13B), and that the mean surface area of each cell doubled from $110 \mu\text{m}^2$ in the control group to 260 to $270 \mu\text{m}^2$ in the drugs-treated groups (figure 3-13C).

Figure 3-12. Inhibition of Rho and ROCK caused disruption of actin filaments and loss of stress fibres. Asynchronised HeLa cells were treated with control, Y27632 or C3 transferase for 30 minutes and stained with phalloidin (green) and DAPI (blue). Bar, 20 μm .

Figure 3-13. Inhibition of Rho and ROCK increased cell spreading in cells recovering from M phase arrest. (A) HeLa cells synchronised by nocodazole for 12 hours and released into fresh media, Y27632 or C3 transferase for 60 minutes. Cells were imaged for phase-contrast. Scale bar, 100 μm . (B) HeLa cells were treated as in (A) and the mean percentage of cells that were spread were plotted. Error bars represent standard deviation. * $p = 0.0024$, ** $p = 0.0056$. Experiment was performed in triplicate; $n \geq 597$. (C) HeLa cells were treated as in (A) and the mean surface area of each spread cells were calculated and plotted. Error bars represent standard deviation. * $p < 0.0001$. Experiment was performed in triplicate; $n \geq 69$.

We then examined the effects of Y27632 and C3 transferase on the progression timing of each mitotic stage. Our data showed that at 30 minutes post-nocodazole release, there were more cells at metaphase in the drug treated cells compared to control cells (figure 3-14), suggesting that cells with disrupted actin filaments were able to recover from nocodazole-induced microtubules disruption faster. By 90 minutes post-release, most Y27632 and C3 transferase-treated cells have progressed to cytokinesis while there were still a large percentage of control cells at telophase stage. By 120 minutes post-release, cells in all three groups have completed mitosis and displayed interphase morphology (figure 3-14).

Figure 3-14. Inhibition of Rho and ROCK accelerated recovery from nocodazole arrest. HeLa cells were synchronised by nocodazole for 12 hours and released into fresh media, Y27632 or C3 transferase. Cells were imaged for phase-contrast at regular intervals of 30, 60, 90 and 150 minutes post-release. Mean percentages of cells in each M phase stages were scored and plotted. Error bars represent standard deviation. Experiment was performed in triplicate; $n \geq 94$.

As anaphase occurred for only a brief period of time relative to the entire mitosis process, we studied the speed of sister chromatids separation in the presence of Y27632 and C3 transferase. We treated HeLa cells with actin disruption drugs and measured the distance between the two sets of sister chromatids at regular intervals (figure 3-15A). Cell treated with both Y27632 and C3 transferase showed a marked increase in the speed of chromosome movement of 2.3 and 2.5 $\mu\text{m}/\text{min}$ respectively, compared to control cells of 1.8 $\mu\text{m}/\text{min}$ (figure 3-15B and C).

Figure 3-15. Inhibition of Rho and ROCK accelerated anaphase chromosome movement. HeLa cells were treated with control, Y27632 or C3 transferase for 3 hours. (A) Metaphase cells were imaged by phase-contrast live-imaging throughout anaphase at 30 seconds interval. Bar, 10 μm . (B) Boxed region in (A) were enlarged. Red lines represent edge of separating chromatids. (C) The mean anaphase chromosome movement speed was calculated and plotted. Error bars represent standard deviation. * $p = 0.024$; ** $p < 0.0001$. Experiment was performed in triplicate; $n = 6$.

Our data have shown that the state of actin organisation during mitosis is essential in the control of the timing of progression of mitosis. In particular, the recovery of microtubule structures from nocodazole treatment, which is essential for chromosome alignment during metaphase and the dynamics of chromosome separation during anaphase.

3.1.8 *Actin filaments disruption by Y27632 and C3 transferase caused cytokinetic defects*

RhoA and its effector ROCK has been reported to be essential for the organisation of the contractile ring and induction of the actin-myosin-driven contraction at the cleavage furrow (Barr and Gruneberg, 2007). We examined the effects of Y27632 and C3 transferase on late cytokinesis (figure 3-16). In the control cell and drugs-treated cells, mitotic cells were able to round up and align its chromosome at the metaphase plate at approximately similar timing. However, drug-treated cells stayed for a longer period in metaphase compared to control cells, although subsequently during anaphase, the speed of sister chromatids separate was faster in the drug-treated cells compared to control (figure 3-15).

Time-lapse phase-contrast microscopy showed that in control cell, there is initial formation of cleavage furrow ingression and midbody (figure 3-16A, black arrowhead), and eventually the cell undergoes abscission and separates into two individual daughter cells (figure 3-16A). In Y27632-treated cell, ingression of the cleavage furrow was observed (figure 3-16B, black arrowhead), as well as the formation of the midbody (figure 3-16B, white arrowhead), however, daughter cells remained attached to each other via the midbody structure for as long as three hours after the initiation of mitosis. In C3 transferase-treated cells, furrow ingression was not observed (figure 3-16C, black arrowhead), and the cell subsequently fused to form a multi-nucleated daughter cell (figure 3-16C, white arrowhead).

(A)

(B)

(C)

Figure 3-16. Inhibition of Rho and ROCK caused cytokinesis and abscission defects. HeLa cells were synchronised by double thymidine block and released into fresh media (A), media supplemented with Y27632 at 8 hours post-release (B), or media supplemented with C3 transferase at 8 hours post-release (C). Cells were imaged by phase-contrast live-imaging from 10 hours to 15 hours post-release. Numbers on frames represent elapsed times in minutes. (A), black arrow pointing to site of abscission; (B), black arrow pointing to site of cleavage furrow ingression, white arrow pointing to failure in abscission; (C), black arrow pointing to absence of cleavage furrow formation, white arrow pointing to binucleated cell. Bars, 20 μ m.

To further examine the effects of RhoA and ROCK inhibition on cytokinesis, we examined the localisation pattern of Aurora B (figure 3-17) and polo-like kinase (PLK1) (figure 3-18), two important kinases implicated in the development of cleavage furrow. Immunofluorescence staining in control cells showed at metaphase, cells are round and myosin IIA stains strongly in a ring at the cell cortex while Aurora B co-localised with chromosomes at the metaphase plate. As the cell progress into anaphase, the cell retains its strong cortical localisation of myosin IIA while Aurora B localised to the central spindle. Eventually, the concentration of myosin IIA at the cortical ring becomes diffused and concentrates at the midzone where the cleavage furrow regressed while Aurora B localised at the midzone region and finally at the midbody structure (figure 3-17A). In the Y27632-treated cells, myosin IIA does not concentrate at the cell cortex but remains diffused throughout the entire cell. During telophase, there is also no localisation to the midzone and midbody structure. On the other hand Aurora B localised normally at the metaphase plate, central spindle region, spindle midzone and midbody structures as the cell progresses through mitosis. However, Aurora B continues to co-localise between the two non-separating daughter cells (figure 3-17B). In C3 transferase-treated cells, there is similar diffused myosin IIA staining throughout the entire cell as the cell progresses through mitosis. Aurora B staining was similar to control during early mitosis. As the cell progresses through late anaphase, the cleavage furrow does not form, and Aurora B localised diffusely along the midzone area. As the midbody structure do not form in these cells and daughter cells eventually fused together to form bi-nucleated cell, Aurora B staining at the supposed site of cleavage slowly tapered off and appeared as weak staining on either site of the joint segment (figure 3-17C).

Figure 3-17. Inhibition of Rho and ROCK led to aberrant Aurora B staining in late M phase. HeLa cells were treated with control (A), Y27632 (B), or C3 transferase (C) for 3 hours. Cells were immunostained with anti-MHCIIA (red), anti-Aurora B (green) and DAPI (blue) and cells at various progressive stages of M phase were imaged. Merged images shown on right panel. Bars, 10 μ m.

PLK1 staining pattern was similar to Aurora B. In the control and Y27632-treated cells, PLK1 localised to the midzone and midbody of the separating cells (figure 3-18A and B), while in C3 transferase-treated cells, there is no distinct localisation pattern at the midzone and the cleavage of daughter cells does not occur (figure 3-18C).

Figure 3-18. Inhibition of Rho and ROCK led to aberrant Polo-like-kinase 1 (PLK1) staining in late M phase. HeLa cells were treated with control (A), Y27632 (B) or C3 transferase (C) for 3 hours and stained with anti-PLK1 (green) and DAPI (blue). Cells at cleavage furrow formation and abscission stages were imaged. Merged images shown on bottom panel. Bar, 10 μ m.

Here, our data show that inhibition of RhoA and ROCK causes defect in cytokinesis, namely a failure to undergo cleavage furrow regression and failure to undergo abscission respectively. Similarly, RhoA activity has been reported to be essential for the assembly and organisation of the contractile ring and induces the acto-myosin-driven constriction of the cleavage furrow (Barr and Gruneberg, 2007).

Discussion

In this study we have explored the regulatory links between actin and the control of cell cycle. We investigated the effects of actin drugs such as cytochalasin D (CCD), jasplakinolide, ROCK inhibitor and C3 transferase on the cell cycle progression. The success of proper formation of contractile ring, cleavage furrow ingression and abscission is largely dependent on the actin network at the stage of cytokinesis, and can be perturbed by actin-depolymerising drugs such as cytochalasin D, C3 transferase and Y27632. Apart from regulating cytokinesis, RhoA and ROCK may also play a role in regulating earlier mitotic events during metaphase and anaphase. We report here that excessive actin polymerisation during mitosis can lead to a delay in mitosis exit. Our observations suggest that the state of actin organisation during mitosis plays a crucial role in the commanding the pace of cell division. Such knowledge serves important fundamentals in the understanding of various cellular processes where proper coordination of cell division timing is absolutely essential; such as during embryonic development, or cancer growth.

In the present study, we have induced the expression of active CDK1 at interphase to investigate if active CDK1 can drive the interphase cells into mitosis prematurely. Ectopic expression of non-phosphorylatable form of CDK1 has been reported to stimulate DNA replication, histone H3 phosphorylation and cell division even after DNA damage (Chow et al., 2003). Our preliminary data show that over-expression of CDK1(T14A/Y15F) in DNA-replication blocked cells was unable to abolish the G₁-phase arrest induced by hydroxyurea treatment (figure 3-3E). However, these cells exhibited histone H3 phosphorylation (figure 3-3F) that was otherwise not seen in DNA-replication blocked cells without CDK1(T14A/Y15F) induction (figure 3-3D). Our results thus created a new paradigm that mitosis specific phosphorylation of histone H3 could occur even when cells have not undergone DNA replication. In addition, our data also showed that prolonged over-expression of CDK1(T14A/Y15F) caused chromosomal condensation that was associated with apparent loss of stress fibres and actin rearrangement (figure 3-4). Our observations suggest although the CDK1 (T14A/Y15F) mutant was reported to be active; its activity cannot override G₁ arrest induced by DNA-replication block. Nonetheless, some mitotic events such as cell rounding or phosphorylation of histone H3 can occur independently from the cell cycle.

In agreement with our data, previous studies have shown that the expression of non-phosphorylatable form of CDK1 at normal levels may have little effects on the cell cycle progression through S phase or mitosis in human cells (Amon et al., 1992; Hagting et al., 1998; Jin et al., 1996; Sorger and Murray, 1992). In addition, the expression of this non-phosphorylatable CDK1 in DNA damaged cells showed no significant decreased in the lag time of mitosis initiation even at high levels of cytoplasmic CDK1-cyclin B activity (Jin et al., 1998). This suggests that forced expression of active-form CDK1 may not drive cell into mitosis. In accordance, our results also showed that premature expression of CDK1(T14A/Y15F) in synchronised cells were unable to accelerate the onset of mitosis based on the DNA content profiles (figure 3-5A, C and E). However, phosphorylation of histone H3 was found to occur 2 hours to 4 hours earlier than expected (figure 3-5B, D and F), providing another example of premature phosphorylation of histone H3, which shows independence from mitosis onset. Recent data have however proposed uncertainties in the role of inhibitory effects of CDK1 phosphorylation in DNA damaged and DNA-replication blocked cells. Inhibitory phosphorylation of CDK1 in response to DNA-replication block is abolished in ATR knockout mammalian cells, however replication checkpoint remains intact in such cells. This suggests that incomplete DNA replication can prevent mitosis entry independently of Thr¹⁴/Tyr¹⁵ phosphorylation of CDK1 (Brown and Baltimore, 2003). The precise expression level and duration control of CDK1 activity may determine if cell can bypass DNA checkpoint to enter mitosis or commit to apoptosis upon prolonged DNA-replication block.

It was previously thought that CDK1-cyclin B localises to the cytoplasm until at prophase, it accumulates in the nucleus where it plays a role in nuclear envelope breakdown (Pines and Hunter, 1991). Recently, data have pointed that the complex actually shuttles between the nucleus and the cytoplasm during interphase and the cytoplasmic localisation of CDK1-cyclin B during interphase is due to rapid export of CDK1-cyclin B complex from the nucleus, rather than cytoplasmic retention of the cyclin B subunit as previously proposed (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). Early observations that cyclin B localises to cytoplasm during interphase in cells arrested in G₂ phase by DNA damage suggested that the temporal control of CDK1-cyclin B localisation plays a role in checkpoint function (Jin et al., 1996; Smeets et al., 1994). It was found that expression of a constitutively nuclear-localised cyclin B caused a significant reduction in the G₂ delay due to DNA damage (Jin et al., 1998) while co-expression of CDK1(T14A/Y15F) and a

constitutively nuclear-localised cyclin B in cells with DNA damage led to premature mitosis (Hagting et al., 1998). As observed, the forced expression of CDK1(T14A/Y15F) in our study was not balanced with an increased in cyclin B1 levels (figure 3-3F), and this could explain the inability of cells to bypass the G₁ checkpoint induced by hydroxyurea. Together, these data suggest that partition control of CDK1-cyclin B complex between the cytoplasm and nucleus determine the role of CDK1 in cell cycle. In this context, it may be useful to look into concomitant expression of a constitutively nuclear-localised cyclin B with CDK1(T14A/Y15F) over-expression to study if CDK1 mutations at residues Thr¹⁴/Tyr¹⁵ can activate MPF activity and allow cells to bypass G₁ phase arrest induced by DNA-replication block. It seems likely that despite the relieve from inhibitory phosphorylation, CDK1 activity cannot be disengaged from the formation of the intact MPF complex with cyclin B. Cyclin B may be critical in ensuring proper localisation of CDK1 to its targets during M phase.

Although we were able to observe increase in phosphorylation of histone H3, it does not conclude that CDK1(T14A/Y15F) can elicit premature mitosis. Such observations of chromosomal condensation and cell rounding are frequently observed in many other cellular processes, one of which is apoptosis. Indeed, CDK1 activity has been previously associated to apoptosis. In earlier studies, analysis of CDK1-cyclin B complex in HL60 human promyelocytic leukaemia cells during apoptosis showed an increase in CDK1 kinase activity shortly after treatment with a variety of apoptosis inducing agents (Shimizu et al., 1995). In another example, roscovitine, an isopropylpurine derivative that inhibits CDK1 and CDK2 was found to reduce apoptosis in human hepatoma cell lines (Hsu et al., 1999), although the relative importance of CDK1 and CDK2 in apoptosis was not established. In rat brain slice, CDK1 expression and activity level was up-regulated when treated with low potassium chloride solution to induce apoptosis, even though these cells were not dividing (Zha et al., 1996). Investigations also revealed potential phosphorylation sites in residue Ser¹²⁸ of the pro-apoptotic protein Bad by CDK1 in apoptotic cells. The phosphorylation status of Bad was found important for its interaction with 14-3-3 proteins, sequestering it away from its targets at the mitochondria, permitting the interaction with protein partners at the mitochondrial surface to induce apoptosis (Konishi et al., 2002; Zha et al., 1996). It might thus be interesting to investigate the phosphorylation pattern of Bad, if any, during mitosis.

Further association of CDK1 activity in apoptosis was supported when YAC lymphoma cells were induced by fragmentin-2 and perforin to enter apoptosis, an increase in CDK1 kinase

activity was observed (Shi et al., 1994). Another experiment on Jurkat cells have shown that cells induced to enter apoptosis displayed increased CDK1 kinase activity that was associated with cyclin A, rather than cyclin B (Shi et al., 1996). It may be possible that in our studies, when U2OS cells were treated with HU to induce DNA-replication block, downstream genotoxic sensors are activated and result in down-regulation of endogenous CDK1 and cyclin B transcriptional activity (Crawford and Piwnica-Worms, 2001; Taylor et al., 1999). The forced over-expression of CDK1(T14A/Y15F) was not balanced with adequate corresponding increase in the amount of cyclin B, hence CDK1(T14A/Y15F) may complex with cyclin A. This tipped the balance of CDK1-cyclin B and CDK1-cyclin A complexes ratio, and disturbed the precise spatiotemporal localisation of the two complexes, which may be associated with apoptotic responses. Investigation of specific caspase activity assay and terminal deoxynucleotidyl transferase (TdT)-mediated 2'-deoxyuridine 5'-triphosphate (dUTP) nick end labelling (TUNEL) assay to validate the apoptotic status of cells (Gavrieli et al., 1992) would be useful. Additionally, co-immunoprecipitation assays, glutathione S-transferase (GST) pull-down assays and localisation studies may be useful to study the differential role of CDK1-cyclin A and CDK1-cyclin B complexes in mitotic and apoptotic response.

While the initiation of mitosis is dependant on CDK1-cyclin B activity, subsequent entry into late mitosis requires the critical destruction of CDK1-cyclin B activity at the metaphase/anaphase checkpoint. (King et al., 1996; Pines and Hunter, 1991). We do not rule out the possibility that prolonged expression of CDK1(T14A/Y15F) may cause cell death at metaphase/anaphase checkpoint rather than G₂/M checkpoint. It would be interesting to couple transgene expression induction with nocodazole treatment in DNA-replication blocked cells to investigate this possibility. Nocodazole depolymerises microtubules and hence disrupt mitotic spindle and result in metaphase arrest. If DNA-replication blocked cells were initiated into early mitosis by CDK1(T14A/Y15F) over-expression, nocodazole treatment may allow capturing of such cells before they enter the metaphase/anaphase arrest cell death. The dual role of CDK1 in mitosis and apoptosis thus creates a paradigm that CDK1 may be controlled under a precise switch, triggering different effects under different conditions; possibly in relation to specific balance in dosage and binding to cyclin A or cyclin B, or the specific threshold dosage or spatiotemporal control of CDK1 activity.

Our data shows that U2OS cells synchronised with double thymidine block and released from late G₁ phase arrest into media containing CCD were able to recover from arrest and proceed with DNA replication as observed by FACS data (figure 3-7C). The cytokinesis failure as observed (figure 3-8B) was as expected due to the failure in formation of the contractile ring that is responsible for ingression of mid-body and abscission. As opposed, such similar disruption of actin by pharmacological means has been reported to caused G₁ phase arrest in different cell types (Bohmer et al., 1996; Bottazzi et al., 2001; Fasshauer et al., 1998; Huang et al., 1998; Huang and Ingber, 2002; Iwig et al., 1995; Lohez et al., 2003; Maness and Walsh, 1982; Ohta et al., 1985; Reshetnikova et al., 2000; Rubtsova et al., 1998; Takasuka et al., 1987; Tsakiridis et al., 1998). The majority of these studies focused on cells ability to enter G₁ phase from quiescence, suggesting that cytoskeletal integrity may indeed be crucial for the transit through the G₀/G₁ border via down-regulation of p27^{KIP1}, induction of cyclin D levels and activation of p24/p44 MAPKs. Indeed, when continuously cycling cells were synchronised by mitotic shake-off method and released into media containing CCD, such cells exit mitosis and passage normally through subsequent G₁ phase (Margadant et al., 2007).

The excess amount of thymidine used in cultures were known to form thymidine dinucleotides, pTpT, which mimick the small DNA fragments released during excision repair mechanism (Pedeux et al., 1998), causing the cells to arrest at the G₁/S border. Our data shows that in U2OS cells arrested in late G₁ phase by excess thymidine, cell cycle progression is cytoskeleton-independent. Cells released into cytochalasin D or jasplakinolide were able to transgress S phase and into mitosis (figures 3-7, 3-10 and 3-11). It was previously demonstrated that G₁ phase progression can be divided into two phases; the initial coincident requirements for adhesion, mitogens and cytoskeletal integrity phase, and a subsequent stage of anchorage-, cytoskeletal- and mitogen-independence (Bohmer et al., 1996). The transition of this mitogen-independent growth is closely mapped to the timing of transition to cytoskeleton-independent phase of the cell cycle progression, possibly at the restriction point, R. Our observations support that in late G₁ phase, or at least at the border of G₁/S phase transition, cell cycle is cytoskeleton-independent.

It appeared that a variety of mammalian cells exhibit a concentration-dependent response to CCD treatment. In studies where low drug concentration were used, for example 0.5 µg/ml used herein and by Margadant *et al.* (2007), cells do not exhibit G₁ phase arrest and proceed into S phase while higher concentrations of 0.6 µg/ml (Takasuka et al., 1987) and 1.0 µg/ml

(Huang et al., 1998) resulted in G₁ phase arrest. Indeed, in one study, the concentration-dependent effects of CCD on cell cycle progression were determined where treatment with 0.1 to 1.0 µg/ml of CCD resulted in progression from G₁ to S phase but subsequent arrest at G₂/M phase while concentrations above 1.0 µg/ml resulted in G₁ phase arrest as determined by flow cytometry (Bohmer et al., 1996). Despite the ability to fully disrupt actin microfilaments integrity at such low CCD concentrations of 0.5 µg/ml (figure 3-6; (Ingber et al., 1995), our studies suggest that cells may indeed respond differently to CCD in a concentration-dependent manner. Our conclusion that cell cycle progression through late G₁ phase is cytoskeleton-independent should thus be substantiated by releasing synchronised cells into media at higher CCD concentrations in future studies.

Despite many data pointing to requirements of cytoskeleton integrity in early G₁ phase progression, a minority community has shown that cytoskeletal integrity can be uncoupled from cell cycle progression in quiescence cells (Roovers and Assoian, 2003; Welsh et al., 2001). In such experiments, forced over-expression of cyclin D was found to drive quiescence cells with disorganised actin structures into S phase. Certainly, cyclin D has been recognised as the main rate-limiting step in the G₀/G₁ transition (Assoian and Zhu, 1997; Baldin et al., 1993; Margadant et al., 2007). It is tempting to conclude that cytoskeleton integrity is dispensable for cell cycle progression. However, it is to note that such experiments exhibited an isolated situation demonstrating the dominant effect of cyclin D for G₁ phase progression. There is, however, no data presenting that quiescence cells can progress through G₁ in the absence of cytoskeleton integrity when induced with other forms of agents apart from cyclin D. While reports have shown that the arrest at G₁ phase following actin disruption is highly dependent on pRb activity (Lohez et al., 2003), the requirements for cyclin D in cell cycle has also been shown to be pRb-dependent and Rb-null fibroblasts losses their requirement for cyclin D during cell cycle (Lukas et al., 1995; Lukas et al., 1994). In addition, several reports have found that phosphorylation of pRb is cell-anchorage-dependent (Kang and Krauss, 1996; Schulze et al., 1996; Zhu et al., 1996). Coupled to the idea that extracellular matrix (ECM) and cytoskeleton integrity can affect the transcriptional and translational activity for cyclin D1 (Bohmer et al., 1996; Margadant et al., 2007; Zhu et al., 1996), and that forced stabilisation of actin filaments by jasplakinolide resulted in increased cyclin D1 expression (figure 3-10D), it becomes clear that cytoskeleton and attachment requirements are upstream control mechanism for cell cycle progression which affects cyclin D levels and downstream phosphorylation control of pRb, a candidate critical

substrate for the restriction point regulation in quiescence cells. Hence, one can expect the forced over-expression of cyclin D1 to rescue actin disrupted cells to enter S phase, rather than the ability to uncouple cell cycle progression from an intact actin network. Correspondingly in non-adherent NIH-3T3 cells, forced expression of cyclin D1 rescued phosphorylation of pRb and allowed cell to progress into S phase (Schulze et al., 1996; Zhu et al., 1996), further enforcing that ECM participates in cell cycle control upstream of cyclin D1 regulation.

Jasplakinolide treatment has been frequently used to stabilise actin filaments *in vitro*. Jasplakinolide treatment for 48 hours has been reported to cause growth inhibition of PC-3, LNCaP, and TSU-Pr1 cells (Senderowicz et al., 1995). In accordance, our data shows that at 20nM of jasplakinolide treatment, mitosis onset is delayed by 2 hours while 50nM jasplakinolide delays onset by about 4 hours (figure 3-10A, C and E). The jasplakinolide concentrations used in this study was unable to maintain cells at the interphase well-spread shape during mitosis, as these cells were able to progress normally through the cell cycle with completion of the cytokinesis process. However, it is noted that the effects of jasplakinolide renders a stress on mitotic dissolution of actin cytoskeleton, resulting in delay in mitosis onset (figure 3-10A, C and E) which is associated with a delay in the onset of mitosis-specific phosphorylation of histone H3 (figure 3-10B and D). Cytoskeleton associated proteins such as HEF1 (Dadke et al., 2006; Pugacheva and Golemis, 2005), zyxin (Hirota et al., 2000) and Ajuba (Hirota et al., 2003) have been found to cross signal with mitotic events. It is likely that jasplakinolide treatment resulted in enhanced actin stability which consequently delayed dissolution of focal contacts and thus the observation of impediment in the onset of such molecular mitotic events. Pharmacological treatment of cells by jasplakinolide seemed unable to overrule the signals of cytoskeletal dissolution that is activated during mitosis.

The dissolution of actin and focal contact structures and the maintenance of cleavage activity during mitosis are largely dependent on the activity of RhoA (Amano et al., 1996; Kimura et al., 1996). Inhibition of RhoA by C3 transferase has been reported to cause re-spreading of prophase cells (O'Connell et al., 1999) while RhoA inhibition by C3 or inhibition of ROCK, a RhoA effector, using Y27632 has also been reported to cause flattening of cells arrested in mitosis (Dadke et al., 2006). Similarly, our data have shown that cells recover faster from nocodazole arrest after treatment with Y27632 and C3 transferase, with a higher percentage of cells appearing well-spread at 60 minutes post-nocodazole release (figure 3-13). Further

examination into different M phase stages revealed that Y27632- and C3 transferase-treated cells have accelerated metaphase to telophase transition (figure 3-14). Acceleration during anaphase may be attributed to the increase in chromosome separation (figure 3-15).

Interestingly, it appeared that Y27632 and C3 transferase treatment did not cause cytokinesis defects in cells released from nocodazole treatment (Figure 3-14). This is contrary to normal situations where cells displayed cytokinetic defects (Figure 3-16). It was also previously shown that suspended cells in cultures appeared to have a higher RhoA-GAP activity (Ren et al., 1999). In addition, several microtubule-depolymerising drugs have been shown to induce formation of actin stress fibres and focal adhesion (Bershadsky et al., 1996) in a Rho-dependant manner (Enomoto, 1996; Liu et al., 1998; Zhang et al., 1997). Similarly, weakly adherent nocodazole-treated fibroblasts were reported to express irregular cortical ingression when cells start to increase attachment during re-spreading under C3 transferase conditions (O'Connell et al., 1999). Thus it appeared that the effects of C3 transferase are dependent on cell adhesion and an intact microtubules network.

Our data suggests that the condition of actin dynamics within the cell matrix can contribute to factors dictating the mitotic molecular mechanics. Although, microtubules and their associated motor proteins are generally understood to play major role in generating anaphase force movement (O'Connell and Khodjakov, 2007; Rogers et al., 2004; Yang et al., 2007), other studies have provided evidence that spindle matrix provide forces required for poleward movement of chromosomes during anaphase. For instance when a kinetochore microtubule is severed during metaphase, the pole-ward force exerted on the chromosomes remains unaffected (Maiato et al., 2004). Similarly during anaphase, severing of kinetochore microtubules did not affect chromosome separation (Forer et al., 2003). These data suggests apart from microtubules, the spindle matrix contains other factors and/or structural elements responsible for force generation during anaphase. In fact, several previous studies have clearly shown the presence of actin and myosin in spindles of a variety of cells (Espreafico et al., 1998; Royou et al., 2002; Sampson and Pickett-Heaps, 2001; Silverman-Gavrila and Forer, 2000; Silverman-Gavrila and Forer, 2003; Simerly et al., 1998; Woolner et al., 2008). Both actin depolymerising drugs such as cytochalasin D, cytochalasin J and latrunculin B (Fabian and Forer, 2007; Forer and Pickett-Heaps, 1998; Snyder et al.), and actin-polymerising drugs such as jasplakinolide (Xie and Forer, 2008), has been found to alter anaphase chromosome movement, further supporting our idea that the alterations of actin

structures by addition of Y27632 and C3 transferase may alter force distribution with the cell that is required for proper timing of chromosome separation.

Apart from early mitotic events, Rho and ROCK also plays important role in the regulation of cytokinesis, in particular the formation and regression of the cleavage furrow. We have shown that addition of Y27632 and C3 transferase can cause cytokinesis defect (figure 3-16). Similar biochemical inhibition of RhoA has also been shown to prevent furrowing during telophase (Piekny et al., 2005). Additional evidence that the RhoA protein may play a role in cytokinesis is clearly demonstrated when active RhoA accumulates at a narrow strip of zone which marks the presumed site of furrow formation (Bement et al., 2005; Yuce et al., 2005). After the RhoA-dependent remodelling of cell shape by the acto-myosin ring, the cell undergoes SNARE-dependent membrane fusion to control the final abscission (Jahn and Scheller, 2006).

Although it is widely understood that RhoA-mediated actin remodelling is required for cytokinesis, the underlying molecular mechanism leading to the accumulation of RhoA at the site of furrowing is only beginning to surface. Studies have shown that the central spindle controls the spatial cue for transportation of RhoGEF such as Ect2 (Nishimura and Yonemura, 2006), and RhoGAP proteins such as MgcRacGAP (Zhao and Fang, 2005), required for regulation of RhoA activity at the equatorial region of the cell. In addition, we have shown that in Y27632 and C3 transferase-treated cells, there is a lack of strong cortical localisation of myosin ring during metaphase and anaphase as oppose to control cells (figure 3-17). This suggests that the inhibition of the RhoA pathway does not only affect the formation of myosin bundling at the equatorial region, but may also mis-regulate cortical myosin. In parallel, a model was previously proposed whereby the astral microtubules can negatively regulate cortical myosin, inhibiting myosin accumulation at the poles where there are high astral microtubules as oppose to regions of low density at the cell equator, thereby leading to high contractility at the equatorial zone (Glotzer, 2004; Werner et al., 2007). Indeed, in agreement to the proposed model, the RhoA-ROCK pathway may also play secondary roles in regulating astral microtubules, thus leading to cytokinesis defects (see chapter 4 later; on RhoA-ROCK regulation of the mitotic astral microtubules). As the understanding of microtubules in the positioning of the plane of cleavage furrow begins to take shape, two distinct but non-mutually exclusive models have emerged, suggesting that both the central spindle microtubules and astral spindle microtubules can play a role in such regulation.

Studies have also shown that cleavage formation was possible in fly cells lacking centrioles and astral microtubules (Basto et al., 2006), while mutants of *C. elegans* nematode lacking the central spindle were also able to form functional cleavage furrow (Jantsch-Plunger et al., 2000), arguing against the absolute need of either microtubule populations for proper cytokinesis. It appears that the spatial regulation of RhoA activity, acto-myosin ring, central spindle microtubules and astral microtubules can all play subtle roles in ensuing cytokinesis.

CHAPTER 4 ACTIN AND THE MITOTIC SPINDLES

To this end, we have shown that actin can play an important role in regulating cell cycle timing and cytokinesis. In this chapter, we will examine the effects of cortical actin perturbation on early mitotic events, notably the metaphase astral microtubules formation and spindle orientation.

4.1 Results

4.1.1 Proper actin dynamics is required for control of metaphase astral microtubules and mitotic cortical rigidity

To examine the effects of actin dynamics on mitotic spindle formation, asynchronised HeLa cells were treated with F-actin stabilising drug jasplakinolide, or actin-depolymerising drugs such as cytochalasin D, Y27632 or C3 transferase for not more than 3 hours, then stained and examined for metaphase astral microtubules (figure 4-1A). The short drug treatment duration ensures that perturbation of actin architecture occurred in late G₂ or early mitosis. Thus any phenotypes examined were not effects carried over from defects, if any, in previous cell cycle stages. In control metaphase cells, microtubules formed two compact crescent-shaped spindles with chromosomes aligned at the metaphase plate and little visible astral microtubules. In cells treated with actin drugs, the crescent-shaped mitotic spindle appeared less defined and presented with abundant astral microtubules radiating from the spindle pole towards the cell cortex. Quantification of astral microtubules was done by subtracting background-corrected fluorescence intensity of kinetochore/polar microtubules from the total microtubules fluorescence (figure 4-1B). Our data show that compared to the control cells (intensity = 88 ± 16 s.d.), cells treated with jasplakinolide (intensity = 116 ± 22 s.d.), cytochalasin D (intensity = 121 ± 31 s.d.), Y27632 (intensity = 128 ± 33 s.d.) and C3 transferase (intensity = 136 ± 32 s.d.) showed a marked increase in the integrated astral microtubules intensities (figure 4-1C). In addition, the increase in astral microtubules after Y27632 and C3 transferase treatment was also accompanied by an increase in EB1 staining at the tip of the microtubules (figure 4-1D).

Figure 4-1. Perturbation of actin dynamics caused increased metaphase astral microtubules. **(A)** HeLa cells were treated with control, jasplakinolide, cytochalasin D, Y27632 or C3 transferase for 3 hours and stained with anti- α -tubulin (green) and DAPI (blue). Bar, μm . **(B)** Schematic representation of astral microtubules quantification. **(C)** The mean background-corrected integrated astral microtubules fluorescence intensities from **(B)** were calculated and plotted. Error bars represent standard deviation. * $p < 0.0001$. Experiment was performed in triplicate; $n = 20$. **(D)** HeLa cells were treated with control, Y27632 or C3 transferase for 3 hours and stained with anti-EB1 (green) and DAPI (blue). White bar, $10 \mu\text{m}$. Bottom panel represents enlarged image of boxed region. Black bar, $2 \mu\text{m}$.

The drug-treated cells were then assayed for cortical rigidity using the micropipette aspiration assay (figure 4-2A). As expected, mitotic cells treated with actin polymerising enhancing drug, jasplakinolide, displayed increased cortical rigidity ($E = 393 \pm 179$ s.d.) compared to control cells ($E = 240 \pm 65$ s.d.). Conversely, cells treated with actin depolymerising drugs such as cytochalasin D, Y27632 and C3 transferase decreases the mitotic cortical rigidity ($E = 42 \pm 18$ s.d., 76 ± 16 s.d. and 47 ± 47 s.d. respectively) (figure 4-2B).

Figure 4-2. Perturbation of actin dynamics changed mitotic cortical rigidity. **(A)** HeLa cells were treated with various drugs for 3 hours and mitotic cells were collected via shake-off and subjected to increasing suction pressure to measure cortical rigidity. **(B)** Mean values of Young's modulus, E , were calculated and plotted. Error bars represent standard deviation.

In addition, we examined the effect of perturbation of actin cytoskeleton by other means such as addition of blebbistatin, concanavalin A and over-expression of α -actinin 1, on metaphase astral microtubules and cortical rigidity. Blebbistatin is a small molecule inhibitor that binds specifically to myosin IIA and blocks acto-myosin cross-linking, resulting in less actin filaments within the cell (Kovacs et al., 2004). Interphase cells treated with blebbistatin for 3 hours showed rapid dissolution of filamentous actin (figure 4-3). Alpha-actinin 1 is an actin-binding and cross-linking protein that serves to bundle actin microfilaments. Cells over-expressing α -actinin 1 showed localisation at regions of actin stress fibres and sites of focal adhesion (figure 4-4). Concanavalin A is a lectin extracted from jack-bean *Canavalia ensiformis* that binds specifically to surface glycoproteins and glycolipids. Addition of concanavalin A to cells serve to cross-linking surface molecules, thereby increasing cortical rigidity of the cell from the “outside” (Carreno et al., 2008; Gibson et al., 1975). Our results showed that after treatment with blebbistatin or concanavalin A, or over-expression of α -actinin 1, metaphase cells displayed increased astral microtubules (figure 4-5A and B) and altered mitotic cortical rigidity (figure 4-5C). These findings indicate that F-actin may be required for the maintenance of cortical rigidity in rounded mitotic HeLa cells. The perturbation of actin dynamics, regardless of the increase or decrease of F-actin polymerisation, can result in the increase of metaphase astral microtubules.

Figure 4-3. Alpha-actinin 1 co-localise to tip and length of filamentous actin. HeLa cells were transfected with pXJΔ2-eGFP-α-actinin (green) and stained with phalloidin (red) and DAPI (blue). Merged images shown in right panel. White arrowhead points to untransfected cell. Bottom panel represents enlarged image of boxed region. Bars, 10 μm.

Figure 4-4. Blebbistatin treatment caused disruption of actin filaments and loss of stress fibres. HeLa cells were treated with control or blebbistatin for 3 hours and stained with phalloidin (red), anti-α-tubulin (green) and DAPI (blue). Merged images on right panel. Bar, 10 μm.

Figure 4-5. Blebbistatin, concanavalin A and alpha-actinin 1 overexpression caused increased metaphase astral microtubules. HeLa cells were treated with control, blebbistatin or concanavalin A for 3 hours or transfected with α -actinin for 24 hours and stained with anti- α -tubulin (green) and DAPI (blue). Merged images on bottom panel. Bar, 10 μ m. **(B)** background-corrected integrated astral microtubules fluorescence intensities from **(A)** were calculated and plotted. Error bars represent standard deviation. * $p = 0.0003$; ** $p < 0.0001$. Experiment was performed in triplicate; $n = 20$. **(C)** Treated mitotic cells were collected via shake-off and subjected to micropipette aspiration assay. Mean Young's modulus value were calculated and plotted. Error bars represent standard deviation. * $p = 0.0004$; ** $p < 0.0001$. Experiment was performed in triplicate; $n \geq 7$.

4.1.2 *RhoA and ROCK may play a role in tubulin dynamics*

As the inhibition of Rho and ROCK appears to have an effect on metaphase microtubules, we examined if Rho and ROCK can play a role in affecting the microtubule dynamics. To visualise microtubules, we constructed mCherry tagged-CLIP-170 under the expression control of a weak promoter. CLIP-170 is a microtubule-binding protein that allows tracking of the growing plus-end of microtubules (Perez et al., 1999; Schuyler and Pellman, 2001). Cells transfected with CLIP-170 displayed prominent localisation of CLIP-170 to the microtubule network (figure 4-6A). Using live imaging microscopy, we tracked the nett elongation rate of microtubules via visualisation of a single point CLIP-170 fluorescence over a period of several seconds (figure 4-6B). Our data show that after treatment with Y27632 and C3 transferase, the nett microtubule elongation rate was increased to $24.4 \mu\text{m}/\text{min} \pm 2.1 \text{ s.d.}$ and $25.2 \mu\text{m}/\text{min} \pm 2.8 \text{ s.d.}$ respectively compared to the control with an elongation rate of $19.4 \mu\text{m}/\text{min} \pm 2.1 \text{ s.d.}$ (figure 4-6B and C). This finding indicates that inhibition of RhoA and ROCK activity may cause an increase in microtubule polymerisation, thus provide possible explanation on the increase in astral microtubules seen during metaphase. Although C3 transferase can inhibit RhoA, RhoB and RhoC, we observe similar phenotypes using C3 transferase and Y27632. Hence, it is reasonable to assume that the inhibition of RhoA is primarily linked to the phenotype.

Figure 4-6. Rho and ROCK inhibition caused increased microtubule polymerisation. **(A)** CLIP-170 localise to plus-end of microtubule. HeLa cells were transfected with pXJΔ2-mCherry-CLIP-170 (red) and stained with anti- α -tubulin (green) and DAPI (blue). Merged images on right panel. White bar, 10 μ m. Bottom panel represents enlarged image of boxed region. Black bar, 2 μ m. **(B)** HeLa cells were transfected with pXJΔ2-mCherry-CLIP-170 and treated with control, Y27632 or C3 transferase for 3 hours and subjected to live-fluorescence microscopy. Bottom panel represents enlarged image of boxed region. White bars, 10 μ m. Images were collected at 2 seconds interval and single CLIP-170 fluorescence point was tracked. Black bar, 5 μ m. **(C)** Mean nett microtubule elongation rate was calculated and plotted. Error bars represent standard deviation. * $p < 0.0001$. Experiment was performed in triplicate; $n \geq 7$.

4.1.3 Knockdown of LIMK1 and LIMK2 affects astral microtubules and cortical rigidity

As the inhibition of RhoA and ROCK led to excessive astral microtubules formation during metaphase, we set to examine one of its effectors the LIM kinases on the astral microtubules. To examine the role of LIMK in mitosis, we used RNA interference to knockdown endogenous LIMK1 and LIMK2. HeLa cells were transfected with siRNA targeting LIMK1 and LIMK2 for 48 hours, and the mRNA and protein levels were determined. Real-time PCR data showed that siRNA transfection successfully decreased LIMK1 transcript levels by 85% while LIMK2 transcript was decreased by 60% (figure 4-7A), and that the siRNA targeting was relatively specific for each gene. Although there was some increase in LIMK2 transcript levels when the more dominantly expressed LIMK1 was knocked down, this is presuming due to compensation effect. At the protein level, LIMK1 siRNA showed more than 95% decrease while LIMK2 siRNA showed a lower knockdown efficiency of approximately 70% only when a higher amount of 150 pmoles of siRNA was used (figure 4-7B). Both siRNAs were specific for their target. siRNA targeting luciferase was used as a control. The siRNA transfected cells were stained and examined for metaphase microtubules and the spindle pole (figure 4-8A). LIMK1 knockdown caused the formation of multi-polar spindles while LIMK2 siRNA transfected cells displayed mostly bipolar spindles with increased astral microtubules. In cells with higher amount of astral microtubules, the centrosomal material appeared to be diffused around the spindle pole (figure 4-8A). Cells co-transfected with both LIMK1 and LIMK2 siRNA resulted in multiple centrosomes and multi-aster formation. The phenotypes were counted and scored in figure 4-8B.

Next we examined if the loss of spindle integrity is associated with changes in cortical rigidity. Mitotic cells treated with LIMK siRNA were assayed by micropipette aspiration (figure 4-8C). LIMK1 knockdown resulted in decreased cortical rigidity ($E = 68 \pm 18$ s.d.) compared to luciferase control ($E = 189 \pm 31$ s.d.). Knockdown of the less dominant LIMK2 in HeLa resulted in only a slight but significant decrease in cortical rigidity ($E = 136 \pm 25$ s.d.), while LIMK1 and LIMK2 double knockdown did not further aggravate the lost of cortical rigidity ($E = 75 \pm 23$ s.d.). The slight decrease in cortical rigidity observed after LIMK2 knockdown could be due to the lower knockdown efficiency of the LIMK2 siRNAs (figure 4-7B). The above data show that LIMK1 and LIMK2 are required for maintenance of mitotic cortical rigidity and the perturbation of this rigidity is consistently correlated with increase in the metaphase astral microtubules.

Figure 4-7. Knock-down of LIMK1 and LIMK2 using siRNA. HeLa cells were transfected with luciferase siRNA, LIMK1 siRNA, LIMK2 siRNA or double-transfect with LIMK1 and LIMK2 siRNAs for 48 hours. **(A)** RNA were extracted and subjected to RT-PCR and quantitative real-time PCR and relative quantity of LIMK1 and LIMK2 transcripts were plotted. Error bars represent standard deviation. **(B)** Cell lysate were harvested and subjected to SDS-PAGE and western blotted with anti-LIMK1, anti-LIMK2 and anti- α -tubulin.

Figure 4-8. Knock-down of LIMKs affect metaphase astral microtubule and spindle poles. HeLa cells were transfected with luciferase siRNA, LIMK1 siRNA, LIMK2 siRNA or double transfected with LIMK1 and LIMK2 siRNAs. **(A)** After 48 hours, cells were stained with anti- α -tubulin (green), anti-pericentrin (red) and DAPI (blue). Merged images on bottom panel. Bar, 10 μm . **(B)** Cells were treated as in **(A)** and the mean percentages of metaphase cells in each category were scored. CS: centrosomes; MT: microtubules. Error bars represent standard deviation. Experiment was performed in triplicate; $n = 100$. **(C)** HeLa cells were transfected as in **(A)** and mitotic cells were collected via shakeoff and subjected to micropipette aspiration assay. Mean values of Young's Modulus, E , were plotted. Error bars represent standard deviation. * $p = 0.0045$; ** $p < 0.0001$. Experiment was performed in triplicate; $n = 7$.

4.1.4 *LIMK2 localised to mitotic spindle and function downstream of ROCK in regulating mitotic astral microtubules and cortical rigidity*

Because the inhibition of RhoA, ROCK and silencing of LIMK during early mitosis gave rise to increased astral microtubules and decreased cortical rigidity, we examined if LIMK function downstream of RhoA/ROCK in modulating these events. We constructed mCherry-tagged LIMK1 and LIMK2 and their kinase-active (T508E and T484E for LIMK1 and LIMK2 respectively) and kinase-inactive (D460A and D430A for LIMK1 and LIMK2 respectively) forms and assessed the ability of these mutants in rescuing the defects seen in Y27632- and C3 transferase-treated cells. We have used cDNA encoding for LIMK2 transcript isoform 2b, whose mimetic-mutants of kinase active and kinase dead are at residues T484 and D430 respectively, homologous to sites on LIMK1 (figure 4-9A). HeLa cells transfected with both active LIMK1 and active LIMK2 constructs displayed more prominent and robust actin stress fibres staining compared to adjacent un-transfected cells; while both the kinase-dead mutant-transfected cells showed no difference in the stress fibres staining (figure 4-9B), indicating that they do not have detectable effect on the actin dynamics and does not act as dominant negative mutant forms in regulating F-actin formation.

HeLa cells were transfected with mCherry vector control or LIMK mutants for 24 hours, and treated with either Y27632 or C3 transferase for 3 hours, and examined for metaphase spindle. In both drug-treatment situations, only LIMK2 T484E construct-transfected cells displayed a significant rescue of the increased metaphase astral microtubule phenotype (figure 4-10A, white arrows). Astral microtubules fluorescence intensity was scored in figure 4-10B. This suggests that LIMK2, but not LIMK1, function downstream of RhoA and ROCK in the regulation of proper mitotic spindle formation; and that the rescue is dependent on the kinase activity of LIMK2.

(A)

LIMK1	FAKDIASGMAYLHSMNIIHR ⁴⁶⁰ DLNSHNCLVRENKNVVVADFGLARL
LIMK2_1	FAKGIASGMAYLHSMCI ⁴³⁰ IHRDLNSHNCLIKLDKTVVVADFGLSRL
LIMK2_2a	FAKGIASGMAYLHSMCI ⁴⁵¹ IHRDLNSHNCLIKLDKTVVVADFGLSRL
LIMK2_2b	FAKGIASGMAYLHSMCI ⁴³⁰ IHRDLNSHNCLIKLDKTVVVADFGLSRL
LIMK1	MVDEKTQP-----EGLRSLKKPDRKKRY ⁵⁰⁸ TVVGPNPYWMAPEMING
LIMK2_1	IVEERKRAPMEKATTKKRTL ⁴⁸⁴ RKNDRKKRYTVVGPNPYWMAPEMLNG
LIMK2_2a	IVEERKRAPMEKATTKKRTL ⁵⁰⁵ RKNDRKKRYTVVGPNPYWMAPEMLNG
LIMK2_2b	IVEERKRAPMEKATTKKRTL ⁴⁸⁴ RKNDRKKRYTVVGPNPYWMAPEMLNG

(B)

Figure 4-9. LIMK1 and LIMK2 activity causes increased filamentous actin formation. (A) Sequence alignment of LIMK1 and the three isoforms of LIMK2 coding sequencing; highlighting sites of mutation for kinase active and kinase dead constructs. (B) HeLa cells were transfected with mCherry-tagged LIMK1 or LIMK2 kinase active or kinase dead mutant constructs (red) for 24 hours. Cells were stained with phalloidin (green) and DAPI (blue). Merged images on right panel. Bar, 20 μ m.

Figure 4-10. Rescue of Y27632 and C3 transferase-induced astral microtubules by LIMK2. **(A)** HeLa cells were transfected with vector control, LIMK1 active, LIMK1 kinase dead, LIMK2 active or LIMK2 kinase dead constructs for 24 hours and co-treated with Y27632 or C3 transferase for 3 hours. Cells were stained with anti- α -tubulin (green) and DAPI (blue). Merged images on right panel. Bar, 10 μ m. **(B)** The mean background-corrected integrated astral microtubules fluorescence intensities from **(A)** were calculated and plotted. Error bars represent standard deviation. * $p < 0.0001$. Experiment was performed in triplicate; $n = 20$.

As LIMK2 has previously been shown to localise to the mitotic spindle (Sumi et al., 2006), we examined if this localisation is affected by the addition of Y27632 and C3 transferase. HeLa cells were treated with either Y27632 or C3 transferase for 3 hours and fixed with cytoskeleton buffer (see methods) and stained for LIMK2 and α -tubulin (figure 4-11A). The treatment of cells with cytoskeleton buffer allows removal of cytosolic LIMK2 proteins and better visualisation of the low abundant LIMK2 that is associated to the spindle during mitosis. In the majority of control-treated metaphase cells, LIMK2 co-localised with α -tubulin to the mitotic spindle ($87.9\% \pm 2.7$ s.d.; figure 4-11B), which formed well define double crescent structure with little astral microtubules (figure 4-11A, top panel). In both the Y27632- and C3 transferase-treated metaphase cells, LIMK2 localisation to the mitotic spindle was flawed ($40.4\% \pm 3.9$ s.d. and $36.1\% \pm 8.1$ s.d. respectively; figure 4-11B), and the spindles appeared diffused with significant increased in astral microtubules (figure 4-11A, middle and bottom panels respectively), suggesting the localisation of LIMK2 to the mitotic spindle is dependent on the activity of RhoA and ROCK.

Next we examined the effect of LIMK mutant constructs on cortical rigidity in Y27632- and C3 transferase-treated mitotic cells. Micropipette aspiration assay revealed that only the kinase-active LIMK2 T484E construct, but not its kinase-inactive mutant or LIMK1 mutants, was able to rescue the decreased in cortical rigidity caused by Y-27632 (figure 4-12). However, in the case of C3 transferase-treated mitotic cells, none of the LIMK1 or LIMK2 mutant constructs was able to rescue the diminished cortical rigidity. Taken together, our data suggest that RhoA and ROCK activities are required to ensure proper localisation of LIMK2 to the mitotic spindle; whose kinase activity is eventually crucial in the regulation of astral microtubules formation. The maintenance of mitotic cortical rigidity requires the activity of both RhoA and ROCK, and which LIMK2 mediates cortical rigidity downstream of ROCK. However it appears that Rho-mediated mitotic cortical rigidity is independent of the ROCK/LIMK pathway. As C3 transferase inhibits all three isoforms of Rho proteins, it may be possible that RhoB and RhoC can play a role in maintaining mitotic cortical rigidity.

Figure 4-11. LIMK2 co-localisation to the mitotic spindle is dependent on RhoA and ROCK activity. **(A)** HeLa cells were treated with control, Y27632 or C3 transferase for 3 hours, fixed with cytoskeleton buffer and stained with anti-LIMK2 (green), anti- α -tubulin (red) and DAPI (blue). Merged images on right panel. Bar, 10 μ m. **(B)** Quantification of LIMK2 localisation to metaphase spindle. Error bars represent standard deviation. * $p < 0.0001$. Experiment was performed in triplicate; $n \geq 163$.

Figure 4-12. Rescue of Y27632-induced mitotic rigidity defect by LIMK2. HeLa cells were transfected with vector control, LIMK1 active, LIMK1 kinase dead, LIMK2 active or LIMK2 kinase dead constructs for 24 hours and co-treated with Y27632 or C3 transferase for 3 hours. Mitotic cells were collected via shakeoff and subjected to micropipette aspiration assay. Mean values of Young's Modulus, E , were plotted. Error bars represent standard deviation; $n \geq 6$.

4.1.5 *Moesin phosphorylation is downstream of Rho, but independent of ROCK and mDia, and may regulate metaphase astral microtubule and cortical rigidity*

As moesin phosphorylation is reported to be essential for mitotic cortical rigidity (Kunda et al., 2008), we examined if ezrin/radixin/moesin (ERM) proteins may contribute to cortical rigidity downstream of Rho and ROCK pathway. We constructed phospho-mimic (T558D) and non-phosphorylatable form of moesin (T558A) which has been shown to represent active moesin and dominant negative moesin respectively and examined its effect on mitotic cells cortical rigidity (figure 4-13A). Cells that were transfected with either wildtype, active or inactive moesin were subjected to micropipette aspiration assay. As expected, cells over-expressing wildtype and phospho-mimic form of moesin has increased cortical rigidity ($E = 393.62 \pm 69.09$ s.d. and 438.48 ± 68.81 s.d. respectively) while cells with dominant negative moesin displayed decreased mitotic cortical rigidity ($E = 154.57 \pm 13.58$ s.d.) compared to cells transfected with vector control ($E = 240.49 \pm 30.11$ s.d.). Next, we studied the effects of these moesin mutants on the metaphase astral microtubules organisation (figure 4-13B). In accordance with our previous observation, in either situation of increasing or decreasing the mitotic cortical rigidity by alteration of the moesin phosphorylation could result in the increase of metaphase astral microtubule (figure 4-13B and C).

Figure 4-13. Moesin phosphorylation is responsible for mitotic cortical rigidity and control of metaphase astral microtubules. **(A)** HeLa cells were transfected with vector control, moesin wildtype (WT), moesin T558D or moesin T558A for 24 hours. Mitotic cells were collected via shakeoff and subjected to micropipette aspiration assay. Mean values of Young's Modulus, E , were plotted. Error bars represent standard deviation. * $p = 0.0013$; ** $p = 0.0004$; *** $p = 0.0002$. Experiment was performed in triplicates; $n \geq 5$. **(B)** Cells were transfected as in **(A)** and stained with anti- α -tubulin (green) and DAPI (blue). Merged images on right panel. Bar, 10 μm . **(C)** The mean background-corrected integrated astral microtubules fluorescence intensities from **(B)** were calculated and plotted. Error bars represent standard deviation. * $p < 0.0001$. Experiment was performed in triplicate; $n = 20$.

Since activity of RhoA, ROCK and the phosphorylation of moesin are important in contributing to the rigid state of cell cortex during mitosis, we next examined for regulatory links between RhoA and ROCK activity and ERM phosphorylation. As there are no phospho-specific antibodies specific for each of ezrin, radixin or moesin, we have used a phospho-specific antibodies that detects all three members of the protein complex. We found that C3 transferase, but not Y27632, resulted in reduction of the levels of phospho-ERM present in mitotic cells (figure 4-14A). Immunofluorescence staining of mitotic cells also confirmed the reduction of phospho-ERM at the cell periphery after treatment of C3 transferase (figure 4-14B). This data suggest that phosphorylation of ERM is dependent on the activity of Rho, but independent of ROCK activity.

Next, we examined if over-expression of wildtype, constitutively active or dominant negative RhoA, full-length and truncated ROCK (containing only the active kinase domain), full-length mDia, constitutively active (Δ GBD) or dominant negative (DN) mDia constructs can have an effect on the phosphorylation status of ERM in mitotic cells (figure 4-14C). From the western blot data, we found that the level of phospho-ERM cannot be increased by wildtype or constitutively active ROCK and mDia constructs nor is reduced by any of the dominant-negative constructs. Our data represent a novel indication that the GTPase activity of RhoA is required for moesin phosphorylation, yet such regulation is independent of its established effectors pathway of ROCK and mDia. We suspect the phosphorylation may be mediated by synergistic effect of RhoA, RhoB and/or RhoC, since C3 transferase can inhibit all three forms of Rho, but the expression of dominant negative RhoA cannot diminish phospho-ERM in similar capacity to C3 transferase treatment. This may also explain why active LIMK2 cannot rescue the reduction of mitotic cortical rigidity caused by C3 transferase treatment (figure 4-12).

Figure 4-14. Ezrin/radixin/moesin phosphorylation is downstream of Rho, but independent of ROCK and mDia. **(A)** Asynchronised HeLa cells were treated with control, Y27632 or C3 transferase for 3 hours. Mitotic cells were collected via shake-off and cell lysates were western blotted with anti-phospho-ERM and anti- α -tubulin. **(B)** Cells were treated with control, Y27632 or C3 transferase and stained with anti-phospho-ERM (green) and DAPI (blue). Merged images on right panel. Bar, 10 μ m. **(C)** Asynchronised HeLa cells were transfected with RhoA wildtype (WT), RhoA active (G14V), RhoA dominant negative (T19N), ROCK wildtype (WT), ROCK kinase domain (KD), mDia1 full-length (FL), mDia1 dominant negative (DN) or mDia1 active (Δ GBD) constructs for 24 hours. Mitotic cells were collected via shake-off and cell lysates were western blotted with anti-phospho-ERM and anti- α -tubulin.

4.1.6 Cofilin does not function downstream of RhoA-ROCK pathway in regulation of metaphase astral microtubules

Since LIMK2 is responsible for the regulation of astral microtubules during metaphase, we examined if its only known substrate, cofilin (Sumi et al., 1999), may play a role in this regulation. LIMK phosphorylate cofilin at serine 3 and inhibits its activity (Arber et al., 1998; Sumi et al., 1999; Yang et al., 1998). We constructed wildtype cofilin and an active (S3A) and inactive (S3E) mutants of cofilin and examined its effect on astral microtubules. At interphase, over-expression of wildtype and S3A of cofilin were able to decrease the amount of F-actin while the inactive phospho-mimic cofilin S3E did not cause significant changes to the interphase actin structures (figure 4-15A). None of the constructs could significantly affect interphase or metaphase microtubules organisation (figure 4-15A), neither could over-expression of cofilin WT and S3A constructs rescue the increased in astral microtubules caused by addition of Y27632 and C3 transferase (figure 4-15B). Our observations suggest that the regulation of astral microtubules during mitosis by RhoA and ROCK is not mediated via the cofilin pathway.

To further test for possible regulation of astral microtubules by the cofilin, we examined the effects of slingshot1 on metaphase microtubules. Slingshot1 is known to regulate the actin structures by de-phosphorylating and thereby activating cofilin (Niwa et al., 2002). Our data shows that metaphase cells over-expressing slingshot1 appeared to have normal mitotic spindle with no apparent increase in astral microtubules (figure 4-16A). Similarly, over-expression of slingshot1 was unable to rescue the increased astral microtubules phenotype caused by Y27632 and C3 transferase (figure 4-16B). This data further supports our findings that cofilin is not involved in the regulation of metaphase astral microtubules.

Figure 4-15. Cofilin does not function downstream of RhoA-ROCK pathway in the regulation of metaphase astral microtubules. **(A)** HeLa cells were transfected with vector control, cofilin WT, cofilin S3A or cofilin S3E constructs for 24 hours. Cells were stained with phalloidin or anti- α -tubulin. * represents transfected cell. All metaphase cells shown are transfected. Bars, 10 μ m. **(B)** HeLa cells were transfected with vector control, cofilin WT or cofilin S3A for 24 hours and co-treated with Y27632 or C3 transferase for 3 hours. Cells were stained with anti- α -tubulin (green) and DAPI (blue). Merged images on right panel. Bar, 10 μ m.

Figure 4-16. Slingshot1 does not play a role in regulation of metaphase astral microtubules. **(A)** HeLa cells were transfected with vector control or Slingshot1 and stained with anti- α -tubulin (green) and DAPI (blue). Merged images on right panel. Bar, 10 μ m. **(B)** HeLa cells were transfected with vector control or Slingshot1 for 24 hours and co-treated with Y27632 or C3 transferase for 3 hours. Cells were stained with anti- α -tubulin (green) and DAPI (blue). Merged images on right panel. Bar, 10 μ m.

4.1.7 *TPPP is a novel substrate for LIMK2*

As cofilin does not mediate astral microtubules formation, we sought to explore the substrate downstream of LIMK2 in mediating astral microtubules formation. As TPPP/P25 has previously been reported to be phosphorylated by LIMK1 (Acevedo et al., 2007), we examined if it can also be a substrate of LIMK2. TPPP/P25, described as TPPP here after, is a microtubule polymerisation promoting protein previously thought to be expressed solely in the brain (Hlavanda et al., 2002), but subsequent found to be ubiquitous (Acevedo et al., 2007). We were able to amplify the cDNA of TPPP from a total cDNA preparation from HeLa cells, suggesting that the transcript of TPPP is present in a non-neuronal cell type.

To examine if LIMK2 can phosphorylate TPPP, we performed an *in vitro* kinase assay. Active and inactive C-terminal GST-tagged LIMK1 and LIMK2 were transiently expressed in COS-7 cells and incubated with bacterially expressed TPPP in the presence of [32 P] γ -ATP. COS-7 cells were used for production of large amount of recombinant Flag-tagged LIMK1 and LIMK2 proteins used for kinase assay. COS-7 cells were preferred over HeLa cells due to its high transfection efficiency and ability to be grown at high densities, hence providing a higher protein yield. In addition, COS-7 cells are capable for correctly folding and processing recombinant proteins (Andersen and Krummen, 2002; Butler, 2005; Wurm, 2004). Cofilin was used as a positive control while GST as a negative control. Our results show that both LIMK1 and LIMK2 kinase-active constructs were able to auto-phosphorylate and phosphorylate cofilin and TPPP (figure 4-17B). The kinase-dead mutants of LIMK1 and LIMK2 did not show any phosphorylation on cofilin and TPPP. This suggests that TPPP is a novel substrate for LIMK2. (This work in section 4.1.7 was done in collaboration with Dr Lim Hong Hwa, Institute of Molecular and Cellular Biology, A*STAR, Singapore).

Figure 4-17. TPPP is a substrate of LIMK1 and LIMK2. *In vitro* kinase assays were performed on mammalian expressed active and inactive LIMK1 and LIMK2 constructs and bacterially expressed cofilin and TPPP. Kinase constructs loading: lanes 1-3: LIMK1-T505E-GST; lanes 4-6: LIMK1-D460A-GST; lanes 7-8: LIMK2-T484E-GST; lanes 10-12: LIMK2-D430A-GST. Substrates loading: lanes 1,3,7,8: GST; lanes 2,5,8,11: GST-Cofilin; lanes 3,6,9,12: GST-TPPP. (A) Coomassie stained gel. (B) Autoradiograph. Red arrows pointing to phosphorylated GST-TPPP bands.

4.1.8 *RhoA-ROCK-LIMK2 pathway controls TPPP localisation to the mitotic spindle which function to regulate astral microtubule formation*

To visualise localisation pattern of TPPP in HeLa cells, full-length cDNA encoding TPPP was cloned with an eGFP reporter tag under a weak promoter (pXJΔ2) expression vector. Weak expression of TPPP coupled with cytoskeleton buffer fixation (see methods) allows visualisation of localisation pattern of TPPP within the cells. Here, we show that TPPP co-localise to tubulin network at all stages of the cell cycle (figure 4-18A). During interphase, TPPP localised to form a meshwork of interconnecting fibres around the cell cytoplasmic region, with strong concentration to the microtubule organising centres (MTOC). The staining pattern of TPPP strongly co-localised with α -tubulin staining pattern. During metaphase, TPPP localised to the mitotic spindle. As the cells progress through anaphase, TPPP localised to the separating spindle and finally during anaphase it concentrates to the

central spindle at the midzone (figure 4-18A). Immunofluorescence staining revealed that at mitosis, TPPP co-localised with LIMK2 at the mitotic spindle (figure 4-18B), suggesting possible physical interactions between LIMK2 and TPPP at the mitotic spindle. To confirm this, we performed a pull-down assay. Cells were co-transfected with flag tagged-LIMK1 or LIMK2 and GST tagged-TPPP and subjected to pull-down analysis. Our data showed that GST-TPPP was capable of establishing physical interaction with both Flag-LIMK1 and Flag-LIMK2 (figure 4-18C).

Figure 4-18. TPPP co-localises with LIMK2 at the mitotic spindle. (A) TPPP localises with tubulin network at most cell cycle stages. Asynchronised HeLa cells were transfected with pXJΔ2-eGFP-TPPP (green) for 24 hours, fixed with cytoskeleton buffer and co-stained with anti- α -tubulin (red) and DAPI (blue). Cells at interphase and different stages of mitosis were imaged. Merged images on right panel. Bar, 10 μ m. (B) TPPP co-localizes with LIMK2 at the mitotic spindle. HeLa cells were transfected with pXJΔ2-eGFP-TPPP (green), fixed with cytoskeleton buffer and co-stained with anti-LIMK2 (red) and DAPI (blue). Merged images on right panel. Bar, 10 μ m. (C) TPPP interacts with LIMK1 and LIMK2. HeLa cells were transfected with GST control or GST-tagged TPPP and co-transfected with flag-LIMK1 or flag-LIMK2 for 24 hours. Lysates were subjected to glutathione beads pull down and western blotted with anti-GST and anti-Flag. Lane 1: Flag-LIMK1 and GST-TPPP; lane 2: Flag-LIMK2 and GST-TPPP; lane 3: Flag-LIMK1 and GST; lane 4: Flag-LIMK2 and GST.

Since TPPP can interact with LIMK2 and localises to the mitotic spindle during metaphase, we examined if this localisation pattern can be affected by treatment with Y27632, C3 transferase or knockdown of LIM kinases. In control metaphase cells or cells transfected with luciferase siRNA, $89.4 \% \pm 3.1 \text{ s.d.}$ and $85.8 \% \pm 8.0 \text{ s.d.}$ of metaphase cells displayed spindle localisation of TPPP respectively (figure 4-19A and B). Y27632 and C3 transferase treatment resulted in the abrogation of this localisation to $55.1 \% \pm 6.5 \text{ s.d.}$ and $45.8 \% \pm 9.1 \text{ s.d.}$ respectively (figure 4-19A and B). In cells transfected with LIMK1 siRNA, TPPP was still able to localise to the mitotic spindle of $77.83 \% \pm 9.3 \text{ s.d.}$ metaphase cells, although these spindles now became oddly-shaped (figure 4-19A). In contrast, cells that were transfected with either LIMK2 siRNA alone or double-transfected with LIMK1 and LIMK2 siRNA, such spindle localisation of TPPP was greatly diminished, $38.6 \% \pm 12.3 \text{ s.d.}$ and $23.7 \% \pm 7.9 \text{ s.d.}$ respectively (figure 4-19A and B). The percentages of metaphase cells

displaying TPPP spindle localisation was counted and represented in figure 4-19B. Our data show that TPPP localisation to the mitotic spindle is largely dependent on the activity of RhoA and ROCK, as well as the presence of LIMK2.

Figure 4-19. TPPP localization to the mitotic spindle is dependent on RhoA, ROCK and LIMK2, but independent of LIMK1. HeLa cells were transfected with pXJA2-eGFP-TPPP (green), and with Y27632, C3 transferase for 3 hours or co-transfected with LIMK1 siRNA, LIMK2 siRNA or both LIMK1 and LIMK2 siRNAs. Cells were fixed with cytoskeleton buffer and stained with DAPI (blue). Merged images on bottom panel. Bar, 10 μ m. (B) The mean percentages of metaphase cells displaying spindle localisation of LIMK2 were scored. Error bars represent standard deviation. * $p < 0.0001$. Experiment was performed in triplicate; $n \geq 122$.

As TPPP localises to the mitotic spindle in a RhoA-ROCK-LIMK2-dependent fashion, we set out to further investigate this localisation. First, we knockdown TPPP in HeLa cells and examined its effect. Two duplexes of siRNA targeting different regions of the TPPP mRNA were designed. The siRNAs provided 70 to 90 percent down-regulation of TPPP transcript levels as assayed by real-time PCR (figure 4-120). At the protein level, both siRNAs resulted in more that 90 % knock-down (figure 4-20B). Next, we examined the effect of TPPP knockdown in HeLa cells during interphase and mitosis. In both situations, the microtubule network does not seem to be greatly affected by the siRNA (figure 4-21). Thus TPPP does not appear to be the major protein involved in regulating microtubules formation in HeLa cells.

Figure 4-20. Knock-down of TPPP using siRNA. HeLa cells were transfected with luciferase siRNA or TPPP siRNAs for 48 hours. **(A)** RNA were extracted and subjected to RT-PCR and quantitative real-time PCR and relative quantity of TPPP transcripts were plotted. Error bars represent standard deviation. **(B)** Cell lysate were harvested and subjected to SDS-PAGE and western blotted with anti-TPPP and anti- α -tubulin.

Figure 4-21. Knock-down of TPPP does not affect interphase and metaphase microtubule network. HeLa cells were transfected with luciferase siRNA, TPPP siRNA (1) or TPPP siRNA (2) and stained with anti- α -tubulin (green) and DAPI (blue). Merged images on bottom panel. Bars, 10 μ m.

It has been reported that phosphorylation of TPPP inhibits its tubulin polymerising activity (Acevedo et al., 2007; Hlavanda et al., 2002), while microinjection of the protein into the *Drosophila* embryos has been found to inhibit mitotic spindle formation (Tirian et al., 2003). Thus, we examined if reduction of TPPP will have any effects on the astral microtubules induced by the drugs and LIMK2 siRNA. We found that in control cells transfected with luciferase siRNA, treatment with Y27632, C3 transferase or LIMK2 siRNA were able to cause excessive astral microtubule formation during metaphase (figure 4-22A, top panel). However, in cells transfected with either of the TPPP siRNAs, there was a reduction in the amount of astral microtubule formation after treatment with Y27632, C3 transferase or LIMK2 siRNA (figure 4-22A, middle and bottom panels). The percentage of metaphase cells with excessive astral microtubules were counted and scored in figure 4-22B. Our data suggest that that under normal circumstances, LIMK2 present at the mitotic spindle serves to phosphorylate TPPP and prevent excessive tubulin polymerisation and formation of excessive metaphase astral microtubules. In the event of RhoA or ROCK inhibition, the activity of

LIMK2 at the spindle is decreased; hence the level of TPPP phosphorylation is decreased. This renders TPPP active and resulted in excessive microtubule polymerisation, leading to increased astral microtubules observation.

Figure 4-22. TPPP functions downstream of RhoA-ROCK-LIMK2 in the regulation of metaphase astral microtubules. **(A)** HeLa cells were transfected with luciferase siRNA, TPPP siRNA (1) or TPPP siRNA (2) for 48 hours and co-treated with Y27632 or C3 transferase for 3 hours or co-transfected with LIMK2 siRNA for 48 hours. Cells were stained with anti- α -tubulin (green) and DAPI (blue). Merged images on right panel. Bar, 10 μ m. **(B)** The mean percentages of metaphase cells displaying increased astral microtubules were scored. Error bars represent standard deviation. Experiment was performed in triplicate; $n \geq 44$.

The phosphorylation of TPPP by LIMK1 has been reported to be essential for the control of its tubulin polymerising activity (Acevedo et al., 2007), and that mass spectrometry has mapped threonine-14 and serine-18 as the two major sites of phosphorylation on TPPP that is responsible for regulation of its activity (Hlavanda et al., 2007), we set to examine the effects of phosphorylation at these two sites on the formation of metaphase astral microtubules. TPPP phosphorylation at threonine-14 and serine-18 perturbs the structural alterations induced by the binding of TPPP to tubulin, thus rendering TPPP less active when phosphorylated (Kleinnijenhuis et al., 2008). We constructed phospho-mimetic mCherry tagged-TPPP T14D/S18D and TPPP T14E/S18E mutant constructs (inactive), and non-phosphorylatable form mutant TPPP T14A/S18A (active), and examined their effects on the metaphase spindles of HeLa cells. Immunostaining results showed that although interphase cells transfected with wildtype TPPP displayed increased microtubule filaments (figure 4-23A), metaphase cells transfected with either the wildtype or mutant constructs (figure 4-23B) did not showed significant increase in astral microtubules. Thus, our data proved inconclusive on the TPPP phosphorylation status of threonine-14 and serine-18 in the regulation of astral microtubules during mitosis.

Figure 4-23. Mutations of TPPP at threonine 14 and serine 18 does not affect metaphase astral microtubules formation. **(A)** HeLa cells were transfected with vector control or mCherry-tagged TPPP (WT) (red) and stained with anti- α -tubulin (green) and DAPI (blue). Merged images on right panel. Bar, 10 μ m. **(B)** HeLa cells were transfected with vector control, mCherry-tagged TPPP WT, TPPP T14D/S18D, TPPP T14E/S18E or TPPP T14A/S18A (red) and stained with anti- α -tubulin (green) and DAPI (blue). Metaphase cells were imaged. Merged images on right panel. Bar, 10 μ m.

4.1.9 *RhoA-ROCK-LIMK2-TPPP pathway controls metaphase spindle orientation*

Astral microtubules have long been implicated to control physical interaction between the spindle and the cell cortex, and hence controlling spindle orientation and positioning within the mitotic cell (Palmer et al., 1992). We examined the effects of Y27632-, C3 transferase- and LIMK knockdown-induced astral microtubule on spindle orientation, and the correlation of astral microtubule rescue by active LIMK2 and TPPP siRNA with the spindle orientation.

HeLa cells were transfected with LIMK1 and LIMK2 active and inactive constructs and assayed for metaphase spindle orientation, i.e. the angle of spindle in relation to the substratum (figure 4-24A). We found that in cells treated with Y27632 or C3 transferase, the spindle angle in relation to the substratum was increased (figure 4-24B and C), and only active LIMK2 were able to rescue this mis-orientation to some degree (figure 4-25A and B). This further reinforced our findings that LIMK2 functions downstream of RhoA and ROCK in the regulation of metaphase astral microtubules and hence, spindle orientation.

Next we examined if knock-down of TPPP has any effect on the mis-orientation of mitotic spindle orientation induced by the suppression of RhoA-ROCK-LIMK pathway. In cells treated with Y27632 or C3 transferase, knockdown of TPPP was able to lower the spindle orientation angle to near wildtype (figure 4-26A). However, TPPP knockdown was only able to rescue spindle mis-orientation caused by LIMK2 siRNA, but not LIMK1 siRNA nor LIMK1 and LIMK2 dual siRNA transfection (figure 4-26B). This suggests that RhoA, ROCK, LIMK1 and LIMK2 contribute to the control of spindle angle during mitosis, but TPPP can only regulate spindle orientation downstream of LIMK2, but not LIMK1. Spindle orientation regulation by LIMK1 could be regulated by cofilin instead (Kaji et al., 2008). This further supports our hypothesis that TPPP functions downstream of LIMK2 in regulating astral microtubules formation and hence controlling the spindle orientation.

Figure 4-24. RhoA and ROCK participate in the regulation of metaphase spindle orientation. **(A)** Schematic representation of spindle orientation measurement. **(B)** HeLa cells were treated with Y27632 or C3 transferase for 3 hours and stained with anti-pericentrin and DAPI. Metaphase cells were imaged for z-stack at 1 μ m distance apart. **(C)** Mean spindle angle from **(B)** were calculated and plotted. Experiment was performed in triplicate; $n \geq 50$. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent maximum and minimum; black dots represent mean; red circles represent outliers.

Figure 4-25. LIMK2 functions downstream of RhoA-ROCK in regulating metaphase spindle orientation. **(A),(B)** HeLa cells were cultured on fibronectin-coated coverslips and transfected with vector control, LIMK1 active, LIMK1 kinase dead, LIMK2 active or LIMK2 kinase dead constructs for 24 hours. Cells were treated with **(A)** Y27632 or **(B)** C3 transferase for 3 hours and stained with anti-pericentrin and DAPI. Metaphase cells were imaged for z-stack and spindle angle with respect to the substratum were calculated. Experiment was performed in triplicate; $n \geq 50$. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent maximum and minimum; black dots represent mean; red circles represent outliers.

Figure 4-26. TPPP functions downstream of RhoA-ROCK-LIMK2 in regulating metaphase spindle orientation. **(A)**, **(B)** HeLa cells were cultured on fibronectin-coated coverslips and transfected with luciferase siRNA, TPPP siRNA (1) or TPPP siRNA (2) for 48 hours. Cells were treated with **(A)** Y27632 or C3 transferase, or **(B)** co-transfected with LIMK1 siRNA, LIMK2 siRNA or both LIMK1 and LIMK2 siRNAs. Cells were stained with anti-pericentrin and DAPI. Metaphase cells were imaged for z-stack and spindle angle with respect to the substratum were calculated. Experiment was performed in triplicate; $n \geq 50$. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent maximum and minimum; black dots represent mean; red circles represent outliers.

Discussion

In this study we have explored the regulatory links between actin and the mitotic spindles, in particular the regulation of astral microtubules formation. We have found that perturbation of actin prior to mitosis onset can cause an increase in astral microtubules. This increase was regardless if actin polymerisation was enhanced or diminished. In addition, we have delineated the pathway of RhoA regulation of the mitotic astral microtubules formation via the RhoA/ROCK/LIMK2/TPPP pathway, which subsequently ensures proper spindle orientation. We hypothesise two possible model of RhoA pathway regulating spindle orientation; one involving the intrinsic influence of RhoA/ROCK/LIMK2 on microtubule regulating enzyme, TPPP, thereby affecting microtubules dynamics, and a second involving the regulation of cortical acto-myosin network and force translation from the cell cortex to the spindle microtubules. On the other hand, LIMK1 was found to regulate mainly centrosome integrity.

The RhoGTPase has previously been described in the control of spindle orientation; in particular the function of Cdc42 has been widely linked to the control of cell polarity and spindle alignment and orientation. The understanding of spindle orientation control was made possible with the study of cells that display asymmetric cell division. Par6 was initially identified in *C. elegans* to be responsible for asymmetric positioning of the mitotic spindle during zygotic cell division (Hung and Kemphues, 1999). Cdc42 was subsequently identified to also play a role in spindle orientation and act via Par6 in controlling cell polarity, migration and morphogenesis (Gotta et al., 2001; Na and Zernicka-Goetz, 2006). Additional studies have shown that Cdc42 contribute to the regulation of Caco-2 cells apical surface positioning by controlling the orientation of the spindle during cell division (Jaffe et al., 2008). Studies have shown that Cdc42 regulate spindle bi-orientation by stabilisation of kinetochore-microtubule attachment via the association and activation of mDia3 (Yasuda et al., 2004) and knockdown of Cdc42 was found to increase the number of mitotic cells with mis-oriented spindle (Yasuda et al., 2006). Later, it was found that Cdc42 regulates the spindle orientation via two independent pathway; Cdc42-PAK2-βPIX and the phosphatidyl 3,4,5 triphosphate (PIP3) (Mitsushima et al., 2009).

On the other hand, RhoA has been widely documented to regulate important mitotic events such as the positioning of the cleavage furrow (reviewed in (Piekny et al., 2005). Few studies

have previously linked RhoA to the regulation of early mitotic events such as the control of spindle orientation. One report has shown that the presence of dominant-negative RhoA in chick neuroepithelium cells caused an increase in the spindle orientation angle in mitotic cells, and the phenotype was dependent on the dosage of dominant negative RhoA present in the cell (Roszko et al., 2006). However, the exact mechanism of RhoA-regulated spindle orientation remains unclear. Here, we have shown that inhibition of Rho proteins using C3 transferase were able to cause similar spindle orientation defect (figure 4-23C), a phenotype which is accompanied by a decrease in cortical rigidity and increase of astral microtubules. These data show that RhoA can regulate early mitotic events.

In our current study, we have shown that RhoA and ROCK activities can affect microtubule dynamics within the cell, and inhibition of either protein leads to increase in microtubule polymerisation rate (figure 4-6C). Other studies have also shown that RhoA can both regulate and be regulated by microtubules dynamics; a function distinct from its more well known actin polymerisation-regulating activities. The depolymerisation of microtubules by nocodazole has been shown to release microtubule-associated RhoGEF, GEF-H1, leading to the activation of RhoA (Chang et al., 2008; Krendel et al., 2002). On the other hand, RhoA regulates lysophosphatidic acid-induced microtubule stabilisation at the edge of scratch wound in fibroblasts via mDia (Bartolini et al., 2008; Cook et al., 1998; Palazzo et al., 2001; Wen et al., 2004), and activation of RhoA appears to inhibit microtubule polymerisation (Grigoriev et al., 2006). In addition, the inhibition of ROCK was also found to decrease nocodazole induced membrane blebbings and serves to stabilise microtubules (Takesono et al., 2010), while inhibition of downstream myosinII by blebbistatin also stabilised microtubules (Takesono et al., 2010). It appears that RhoA and ROCK may regulate microtubule stability by activating histone deacetylase 6 (HDAC6) (Ling and Lobie, 2004), which deacetylates microtubules and decreases the amount of stable acetylated microtubules within the cell (Matsuyama et al., 2002). Thus, RhoA activity serves to prevent and regulate excessive microtubule polymerisation, coinciding with our data that the loss of RhoA signalling pathway can lead to increased net elongation of microtubules (figure 4-6C) and eventually lead to increased astral microtubules observed during metaphase (figure 4-1A).

LIMK1 and LIMK2 are both downstream of RhoA/ROCK activation and can act on the same substrate, cofilin, in regulating actin cytoskeleton network (Maekawa et al., 1999). Studies have shown LIMK1 and LIMK2 may play different roles during mitosis. LIMK1 and LIMK2

were found to be located to different regions of the mitotic apparatus; with LIMK1 mainly at the centrosomes and LIMK2 mainly at the spindle (Sumi et al., 2006). Similarly, we have demonstrated clear spindle localisation of LIMK2 during metaphase, which is disrupted when RhoA/ROCK activities are inhibited (figure 4-11). Our studies implicated LIMK2 as an important regulator in proper spindle formation as knockdown of LIMK2 caused increased in astral microtubules at the spindles (figure 4-8A) while active LIMK2 construct was able to restore the excessive astral microtubules induced by Y27632 and C3 transferase treatment (figure 4-10A). Indeed, LIMK2 has previously been linked to microtubule stability. A microarray study have identified LIMK2 expression as a predictive marker of anti-cancer drug resistance, many of which targets the microtubules (Dan et al., 2002). The reported data have shown negative correlation between LIMK2 expression and drug resistance; where low expression of LIMK2 in human cancer cell lines were able to confer resistance to anticancer drugs. Conversely in another study, it was found that vincristine (a microtubule targeting drug)-induced mitotic arrest was inhibited in LIMK2 knockdown cells, while LIMK2 expression increased sensitivity to the microtubule-depolymerising drug (Po'uha et al., 2009). It remains unclear whether LIMK2 activity elicits positive or negative effects on microtubule stability. Nevertheless, down-regulation of LIMK2 was similarly found to induce formation of abnormal mitotic spindles (Po'uha et al., 2009). Coupled to our findings, the data provide evidences that LIMK2 plays an important role in the regulation of microtubule dynamics, and in current observation, the regulation of astral microtubules and spindle orientation.

Apart from regulating microtubules dynamics, a number of studies have also linked LIMK2 functions to cell survival. A microarray study have showed that LIMK2 is up-regulated in CHK1 knockdown cells, suggesting that LIMK2 plays a role in DNA damage checkpoint regulation (Ou et al., 2007). In addition, the isoform LIMK2b, but not LIMK1 or LIMK2a, was identified as a direct transactivation target of p53 upon DNA damage; and serves to promote checkpoint regulation by promoting G₂/M arrest (Hsu et al., 2010). Similarly in a separate study, LIMK2 was also identified as a direct p53 target gene induced by genotoxic stress and down-regulation of LIMK2 by siRNA knockdown resulted in abrogation of mitotic arrest induced by chemotherapy and radiotherapy (Croft et al., 2010). The functional relevance of LIMK2-regulated astral microtubules formation and its checkpoint control function may still seem vague at this moment. Nevertheless, LIMK2 appears to play a more distinctive role than LIMK1 in cell cycle regulation during stress induction. It is unclear if

LIMK2-associated modulation of astral microtubule formation functions as a channel to dictate mitotic progression in the event of genotoxic stress.

Although we found that the RhoA/ROCK/LIMK2 pathway can regulate astral microtubule and hence affect the spindle orientation, it remains unclear if such effect is exerted directly on the microtubules alone or as a secondary effect via the altered actin network within the cell. The RhoA/ROCK/LIMK pathway has been widely known to regulate the actin cytoskeleton network within the cell (Etienne-Manneville and Hall, 2002) and controls mitotic rigidity (Maddox and Burridge, 2003). On the other hand, moesin-dependent cortical rigidity has been reported to be essential for the proper formation and positioning of the meiotic and mitotic spindles (Kunda et al., 2008; Larson et al., 2010). It is possible that the regulation of spindle orientation observed in our current study may also be attributed to the change in actin dynamics and cortical rigidity that is mediated through the RhoA/ROCK/LIMK2 pathway rather than the intrinsic activity of RhoA pathway acting directly on microtubules. In accordance, we have also shown that perturbation of the cortical rigidity via various RhoA-independent means such as cytochalasin D, jasplakinolide, concanavalin A treatment and α -actinin 1 over-expression were able to perturb mitotic cortical rigidity and induce the formation of excessive astral microtubules, suggesting a tight regulatory relationship between the actin network and the mitotic spindle (figures 4-1, 4-2 and 4-5). The importance of actomyosin-microtubules interaction has sparked recent interest, since a series of early fluorescence and electron microscopy studies revealed the presence of actin within the mitotic spindles (Forer et al., 1979; Schloss et al., 1977). More recent studies have demonstrated that various actin poisons and myosin inhibitors have varying effects on spindle structure and functions (Fabian and Forer, 2007; Forer et al., 2007), and that F-actin present in the spindle, together with myosin-10, function to provide mechanical strength to control spindle length (Woolner et al., 2008).

Moesin phosphorylation has previously been reported to play a role in cell rounding and cortical stability in the *Drosophila* mitotic cells (Carreno et al., 2008; Kunda et al., 2008). In current knowledge, moesin is phosphorylated by Slik (Hipfner et al., 2004; Hughes and Fehon, 2006) and is essential for spindle organisation and positioning during mitosis (Carreno et al., 2008; Kunda et al., 2008), although it is not clear if Slik directly phosphorylates moesin or through an intermediate protein. Similarly, we have shown that active and dominant negative moesin mutants were able to increase and decrease mitotic

cortical rigidity respectively (figure 4-13A), which subsequently leads to increased astral microtubules (figure 4-13B and C). In addition, we have shown decreased phospho-ERM levels in cells treated with C3 transferase (figure 4-14A), but such phosphorylation was not downstream of ROCK and mDia1 (figure 4-14C). In one study, it was found that the product of RhoA effector phosphatidylinositol 4-phosphate 5-kinase (PIP5K), phosphatidylinositol 4,5-bisphosphate (PIP₂), could lead to phosphorylation of moesin *in vivo* (Matsui et al., 1999), although the kinase has not been identified. Thus it seems that RhoA-mediated mitotic cortical rigidity could be regulated by PIP₂-regulated moesin phosphorylation. On the other hand, we have shown that although mitotic cells treated with Y27632 also lose its cortical rigidity (figure 4-2B), such phenotype could be rescued by over-expression of active LIMK2 kinase construct (figure 4-12), suggesting that ROCK and LIMK2 together mediate mitotic cortical rigidity. It appears unequivocal that mitotic cortical rigidity influences proper spindle orientation. Our data and published work suggest that there are at least two separate but functionally-related pathways regulating the rigid state of the mitotic cortex; one involving RhoA-mediated moesin phosphorylation and the other involving ROCK and its downstream effector LIMK2. Both pathways are known to involve the actin; moesin acting as a bridging protein which cross-links membrane proteins to the underlying actin filaments and the LIMK pathway responsible for maintenance of actin polymerisation within the cells. Both pathways transmit the physical tension strength at the cell cortex to the cell interior, possibly via the interconnecting astral microtubules; thereby regulate the positioning of the mitotic spindle.

Using different experimental cell types and models, the acto-myosin structures and the astral microtubules have been shown to be important in the anchoring of the spindle microtubules to the cell cortex. In budding yeast, actin structures were found to be crucial for guiding astral microtubules into the buds for proper alignment of spindle to the mother bud axis and subsequent segregation of the sister chromatid into the daughter bud (Yin et al., 2000), and selective actin disruption using conditional tropomyosin double mutant leads to defects in spindle orientation (Theesfeld et al., 1999). In another study, cultured fibroblasts were found to display spindles that orient parallel to the substratum surface and disruption of actin filaments by cytochalasin D or latrunculin A resulted in mis-localisation of the spindle that could not be properly anchored via the astral microtubules (Toyoshima and Nishida, 2007). Thus our proposed model is based on the information that changes in astral microtubules formation may be attributed to the changes in cortical state of the cells when treated with

various drugs (see chapter 3, figure 3-17). This phenomenon of cortical actin exerting a pulling force on the spindle has already been documented in the budding yeast and *C. elegans* embryo (Grill and Hyman, 2005; Hildebrandt and Hoyt, 2000). It is postulated that the cortical acto-myosin structures provide a relatively stiff platform at the plasma membrane which serves to anchor the astral microtubules for maintenance of force distribution acting on the spindle (Carreno et al., 2008; Goulding et al., 2007; Kunda et al., 2008). This is evident from studies which have shown that depletion of the *C. elegans* non-muscle myosin, NMY-2, interferes with anterior-posterior polarity and defects in asymmetrical pulling forces which are normally present within the embryo (Munro et al., 2004; Shelton et al., 1999). Indeed, a recent study has provided direct visualisation of such membrane force acting on the mitotic spindle positioning, where a weakened cell cortex resulted in membrane invaginations arising from the spindle microtubule pulling force (Redemann et al., 2010). Although in all the above studies, the relationship between the cell cortex and astral microtubules were not investigated, it is apparent that the cell cortex provides directional cues for positioning of the mitotic spindle, possibly exerting its effect via the astral microtubules.

In present study, we found that TPPP knockdown can rescue the excessive microtubule formation (figure 4-21) and aberrant spindle orientation (figure 4-25) induced by the inhibition of RhoA/ROCK/LIMK2 pathway. We have also shown that like LIMK2, TPPP localises to the spindle microtubules during metaphase and anaphase (figure 4-17A and B). In addition, we have demonstrated that TPPP is a novel substrate of LIMK2 (figure 4-17). The weaker binding efficiencies detected between LIMK2 and TPPP compared to LIMK1 and TPPP could be due to several reasons. The amount of LIMK2 expressed in this experiment was lower than the amount of LIMK1 expressed (see lane 2 is weaker than lane 1 for IB: anti-flag). In addition, the difference in binding efficiencies could be due to differences between LIMK1 and LIMK2. Although both LIMK1 and LIMK2 share high homology of 70% at the carboxyl-terminus kinase domain, they share roughly 50% and 46% sequence identities at the LIM and PDZ domains, the two regions commonly know to participate in protein-DNA or protein-protein interactions (Mizuno et al., 1994; Nunoue et al., 1995). These differences can therefore affect the binding properties between the kinases and TPPP.

To this end, our works suggest that TPPP may function downstream of RhoA signalling in the regulation of spindle astral microtubules. Analogous to our findings, several studies have also suggested possible function of TPPP in regulation of mitotic spindle formation. TPPP

expression at low levels was similarly found to localise to the mitotic spindle (Lehotzky et al., 2004), and that microinjection of TPPP into *Drosophila* embryos were found to inhibit mitotic spindle assembly (Tirian et al., 2003). As studies have found that phosphorylation of TPPP resulted in loss of its tubulin polymerisation activity (Acevedo et al., 2007; Hlavanda et al., 2002), we proposed that under normal mitotic circumstances RhoA, ROCK and LIMK2 activity serves to maintain TPPP in its phosphorylated form, thereby prohibiting excessive microtubule formation and thus controlling the number of astral microtubules present. Although we were unable to demonstrate the function of two previously known TPPP phosphorylation site, threonine 14 and serine 18 (Hlavanda et al., 2007), in the regulation of astral microtubules formation (figure 4-22), it has been reported that TPPP phosphorylation on a single serine site is responsible for inhibiting its tubulin bundling activity (Acevedo et al., 2007). Based on our reported data and other studies, we propose that TPPP provides the missing link between LIMK2 activity and astral microtubule regulation.

CHAPTER 5 ACTIN AND THE MITOTIC CENTROSOMES

In our previous chapter, we have shown that perturbation of actin and cortical rigidity can affect astral microtubules formation and spindle orientation. In this chapter, we will look briefly at the implications of LIM kinases at the mitotic centrosomes.

5.1 Results

5.1.1 *RhoA and ROCK play a role in mitotic centrosome integrity*

To examine the functions of RhoA and ROCK on the regulation of mitotic centrosomes, we treated HeLa cells with Y27632 and C3 transferase to inhibit ROCK and RhoA respectively, and examined the metaphase staining pattern of centrosomal pericentrin. Control metaphase centrosome usually appears focused as two distinct dots at either half of the spindle pole (figure 5-1A, left panel). Upon treatment with either Y27632 or C3 transferase, the centrosome appears de-focused and extends vertically beyond the usual dots (figure 5-1A, middle and right panels). Quantification of the centrosome staining intensity was calculated by measuring the background-correct intensity of a 50 by 50 pixel region surrounding each centrosome. Our data show that inhibition of RhoA and ROCK increased the integrated metaphase pericentrin intensity (intensity = 1130 ± 148 *s.d.* and 1001 ± 125 *s.d.* respectively) compared to control cells (intensity = 901 ± 64 *s.d.*) (figure 5-1B).

Figure 5-1. RhoA and ROCK activity are required for centrosome integrity. **(A)** HeLa cells were stained with pericentrin (green) and DAPI (blue). White bar, 10 μ m. Bottom panel represents enlarged image of boxed region. Black bar, 2 μ m. **(B)** Quantitation of integrated pericentrin intensity for control, Y27632 and C3 transferase-treated cells. Error bars represent standard deviation. * $p=0.0006$; ** $p<0.0001$. Experiment was performed in triplicate; $n\geq 16$.

5.1.2 LIMK1 and LIMK2 may be involved in regulation of mitotic centrosome integrity

Since RhoA and ROCK may play a role in the regulation of metaphase centrosome integrity, we set to examine if LIMK, the downstream target of RhoA/ROCK, may also play a role in the regulation of the centrosome. To examine the role of LIMK on metaphase centrosome, HeLa cells were transfected with LIMK siRNA and examined for centrosomal pericentrin staining (see chapter 4.1.3, figure 4-8A and B). Our results have shown that a large proportion of LIMK1 knockdown cells displayed multi-polar spindles while LIMK2 knockdown cells present with diffused centrosomal material. In addition, cells co-transfected with LIMK1 and LIMK2 siRNA displayed serious centrosomal defocusing and multi-asters formation (figure 4-8A). These data suggest that both LIMK1 and LIMK2 may participate in the regulation of metaphase centrosomal integrity.

5.1.3 *LIMK1 and LIMK2 knockdown does not affect cell's proliferation and division ability*

Because the activity of LIMK1 and LIMK2 are shown to play a role in the formation of the acto-myosin contractile ring that is essential for completion of cytokinesis (Sumi et al., 2006; Yang et al., 2004), we set to examine if the centrosomal defects caused by LIMK siRNAs that we observed were due to defective cytokinesis; hence, multi-polar spindles are observed in subsequent cell cycle. To check for defective cytokinesis, HeLa cells were transfected with LIMK siRNAs and its DNA content were tracked for a period of 72 hours post-transfection. FACS data shows that in control cells treated with cytochalasin D, an actin depolymerising drug known to induce cytokinesis defect, cells showed polyploidy at 24 hours post-drug treatment (4N) and duplicated chromosomal material (8N) at 48 and 72 hours post-drug treatment, indicating that these cells have defective cytokinesis. On the other hand, cells transfected with either luciferase, LIMK1 or LIMK2 siRNA has consistent G₁ population of diploid cells (2N) and cells with duplicated genetic material at G₂ stage (4N) even at 72 hours post-siRNA transfection, suggesting that cells with LIMK1 or LIMK2 knockdown were still able to undergo effective cell division (figure 5-2A).

To ensure that LIMK1 and LIMK2 knockdown cells are proliferating as usual, and that the consistent G₁ and G₂ population are not a result of cell cycle arrest, we performed a growth curve analysis of cells with LIMK knock down. MTT assay was performed over a period of 5 days post-transfection. MTT is a yellow tetrazole that is reduced to purple formazan by mitochondrial reductase in living cells. The resultant colorimetric absorbance measurement is proportional to the number of viable cells present. Growth curve analysis shows that cells transfected with LIMK1 siRNA, LIMK2 siRNA or double-transfect with LIMK1 and LIMK2 siRNAs were able to display exponential growth over the five days tracking period, similar to control cells transfected with luciferase siRNA (figure 5-2B). These data shows that knockdown of LIMK1 and LIMK2 does not affect cells ability to proliferate and divide its genetic material between the daughter cells.

Figure 5-2. LIMK1 and LIMK2 knock-down does not affect cell proliferation and division. **(A)** HeLa cells were treated with cytochalasin D or transfected with luciferase, LIMK1 or LIMK2 siRNA. Cells were harvested at 0, 24, 48 and 72 hours post-treatment and DNA content assessed by fluorescence-activated cell sorting with propidium iodide staining. **(B)** HeLa cells were transfected with luciferase siRNA, LIMK1 siRNA, LIMK2 siRNA, or double transfected with LIMK1 and LIMK2 siRNAs. 48 hours post-transfected, cells were trypsinised and equal numbers of cells were seeded into five 24-well culture plates. Plates were harvested 24 hours later and daily thereafter for 5 days and assayed for MTT growth curve. Error bar represents standard deviation. Experiment was performed in triplicate.

5.1.4 LIMK1 and LIMK2 co-fractionate with centrosomal proteins

To further identify the localisation pattern of LIMK1 and LIMK2 during interphase and mitosis, we isolated centrosome fractions via sucrose gradient ultracentrifugation and examined for their presence. Western immunoblot data shows that at interphase, both LIMK1 and LIMK2 co-fractionate with sucrose fractions containing centrosomal marker proteins γ -tubulin and centrin-2 (figure 5-3A). Similar results are shown for mitotic population cells (figure 5-3B), although a higher level of LIMK1 was detected at the centrosomal fractions than LIMK2. Our data thus show that LIMK1 and LIMK2 can localise and bind to the centrosome during interphase and mitosis.

Figure 5-3. LIMK1 and LIMK2 co-fractionate with centrosomal proteins. HeLa cells were cultured in large flasks. (A) Interphase cells and (B) mitotic cells were separated via shake-off. Cells were lysed while preserving organelles and lysates were loaded onto discontinuous sucrose gradient for ultracentrifugation. Ten fractions were collected and western blotted with anti-LIMK1, anti-LIMK2, anti- γ -tubulin and anti-centrin2.

5.1.5 *Specific localisation pattern of LIMK1 and/or LIMK2 phosphorylation during mitosis*

LIMK1 and LIMK2 have been previously reported to localise differentially during mitosis, with LIMK1 concentrating at the centrosome and LIMK2 at the mitotic spindle (Sumi et al., 2006). In accordance with reported data, we have also shown that LIMK2 localise to the mitotic spindle during metaphase (figure 4-11), and mediates the regulation of metaphase spindle astral microtubule formation by RhoA-ROCK and TPPP (see chapter 4). In addition we have shown that knock-down of LIMK1 posed a more serious phenotypic effect on the centrosome as silencing of LIMK1 resulted in both diffused centrosomes and multi-centrosome phenotypes. Knockdown of LIMK2, on the other hand, shows more subtle effects of diffused centrosomes (figure 4-8A and B). Furthermore, centrosome isolation of mitotic cells also revealed a higher amount of LIMK1 localised with centrosomal marker proteins (figure 5-3B). Taken together, the data provide preliminary evidence that LIMK1 and LIMK2 may indeed localise and function differently during mitosis; with LIMK1 regulating the mitotic centrosome integrity and LIMK2 regulating the mitotic astral microtubules.

To study the localisation pattern of phosphorylated LIMK1 and/or LIMK2 during the cell cycle, we immunostained HeLa cells with phospho-LIMK1/2 and found that at interphase, phospho-LIMK1/2 is diffusely localised throughout the cytoplasm (figure 5-4, top panel). As the cell enters metaphase, phospho-LIMK1/2 was detected at the spindle pole, and very weakly co-localise with the mitotic spindle. As the cell progress through mitosis, spindle pole staining decreases and phospho-LIMK1/2 begins to localise to the spindle midzone and eventually to the midbody.

Figure 5-4. Phospho-LIMK1/2 localisation pattern during cell cycle progression. HeLa cells were fixed with cytoskeleton buffer and immunostained with anti-phospho-LIMK1/2 (green), anti- α -tubulin (red) and DAPI (blue). Merged images on right panel. Bars, 10 μ m.

Next we examine if such phosphorylation of LIMK1/2 at the centrosomes is dependent on the activity of RhoA and ROCK. We found that in metaphase cells treated with Y27632 or C3 transferase, the centrosome appears diffused and spread out with significantly weaker staining intensities (intensity = 342 ± 53 s. d. and 320 ± 61 s. d. respectively) of phospho-LIMK1/2 compared to control cells (intensity = 417 ± 57 s. d.) which have a more focused centrosome with stronger staining intensity (figure 5-5A and B). Taken together, the above data suggest that LIMK1 may play a more significant role in regulation of the centrosomal integrity and may mediate its effect via phosphorylation downstream of RhoA-ROCK.

Figure 5-5. Phospho-LIMK1/2 at the spindle pole is dependent on Rho and ROCK activity. **(A)** HeLa cells were treated with control, Y27632 or C3 transferase for 3 hours and stained with anti-phospho-LIMK1/2 (green) and DAPI (blue). White bar, 10 μ m. Right panel represents enlarged region of boxed region. Black bar, 2 μ m. **(B)** The mean background-corrected integrated fluorescence intensities were plotted. Error bars represent standard deviation. * $p < 0.0001$. Experiment was performed in triplicate; $n = 30$.

Discussion

LIMK are LIM domain containing serine/threonine kinase that regulates actin dynamics through phosphorylation of cofilin (Mizuno et al., 1994; Yang et al., 1998). LIMK1 and LIMK2 has been identified to be differentially localised during mitosis; LIMK1 at the spindle poles and LIMK2 at the mitotic spindle (Sumi et al., 2006). Both LIMK1 and LIMK2 may participate in the maintenance of mitotic centrosomal integrity as knockdown of either LIM-kinases resulted in defocusing of centrosome and multi-centrosome phenotypes (figure 4-8). Similar defocused phenotypes are seen in C3 transferase- and Y27632-treated cells (figure 5-1), and the levels of phosphorylated LIMK1/2 at the centrosome during metaphase is dependent on the activities of RhoA and ROCK (figure 5-5). We suggest that RhoA signals via ROCK and LIMKs to maintain focused state of spindle pole during mitosis, although more information is required to indicate direct phosphorylation of LIMK by RhoA/ROCK signalling at the centrosome. Future experiments will involve attempts to rescue the diffused centrosome phenotype induced by C3 transferase and Y27632 treatment using active LIMK1 and LIMK2 constructs.

Phosphorylated LIMK1 and LIMK2 have previously been identified to co-localise and interact with γ -tubulin (Chakrabarti et al., 2007). However, the functional significance was not established. Phosphorylated cofilin has also been identified to be present in the mitotic centrosome (Andersen et al., 2003). In addition, the phosphorylation status of cofilin was found to change periodically during mitosis progression; increasing during early mitosis and peaks at metaphase and subsequently declining as the cell enters anaphase, telophase and cytokinesis (Amano et al., 2002). Such periodic rise and fall of phosphorylated cofilin also coincide with similar phosphorylation pattern for LIMK1 and Slingshot1 (SSH1) (Amano et al., 2002; Kaji et al., 2003), highly suggestive that LIMK1 may be responsible for phosphorylation of centrosomal cofilin during early metaphase. A number of LIM domain containing proteins such as LMO4, paxillin and Ajuba have also been found to be co-localised with γ -tubulin (Abe et al., 2006; Herreros et al., 2000; Hirota et al., 2003; Montanez-Wiscovich et al., 2010), suggesting a possibility that the LIM domain may serve as a general centrosomal binding domain that is responsible for anchoring proteins that are essential for centrosomal integrity.

The localisation of pLIMK1/2 at the centrosome is of particular interest as over-expression of LIMK1 has been associated with abnormal spindle assembly with supernumerary centrosome and micronuclei (Davila et al., 2007). It was also observed that pLIMK1 co-localised to the multi-polar centrosomes in the more invasive MDA-MB-231 breast cancer cells, which expressed higher levels of LIMK1 compared to the less invasive MCF7 breast cancer cells (Chakrabarti et al., 2007; Yoshioka et al., 2003). Thus LIMK1 may be involved in the maintenance of mitotic spindle integrity and a correlation between altered LIMK1 expression and the acquirement of chromosomal instability in cancer progression.

Our results have shown that knockdown of LIMK1 and LIMK2 does not affect cytokinesis and cell growth (figure 5-2) which is contrary to the belief that altered actin dynamics will affect the formation of contractile ring. Several previous studies have shown that over-expression of LIMK1 in HeLa cells caused cytokinesis defects (Amano et al., 2002; Sumi et al., 2002), while others have shown that transient expression of LIMK1 could prolong mitosis timing but cells subsequently resume cell cycle without defective cytokinesis (Davila et al., 2007). Therefore, the precise level of LIMK1 or its activity is tightly regulated for the proper progression of cell cycle. LIMK1 has been shown to bind to a number of microtubule associated proteins (MAPs) thereby promoting microtubule assembly and disassembly (Gorovoy et al., 2005). However no phosphorylated LIMK1 was found to be associated with the spindle microtubules (Chakrabarti et al., 2007), thus it is speculated that LIMK1 may bind to MAPs in its unphosphorylated form. Upon activation phosphorylation, LIMK1 translocates to the spindle pole and associates with γ -tubulin. This is also supported by the evidence that ROCK2 phosphorylation of LIMK1 decreases its binding with α -tubulin, leading to microtubule destabilisation (Gorovoy et al., 2005).

The data presented in this study does not sufficiently demonstrate the function of LIMK1/2 in centrosome integrity. What is certain is that LIMK1 does play an important role in regulating the centrosome integrity and numbers in cells, a condition which is often associated with metastatic and invasive properties in advanced cancers. Nevertheless, it remains unclear if LIMK functions in controlling the mechanical cohesion of the centrosomes, or does it ensure the proper duplication of centrosomes during the cell cycle. Further investigations are essential in understanding the molecular functions of LIMK in cancer progression and thus possible therapeutic intervention.

References

- Abe, Y., Ohsugi, M., Haraguchi, K., Fujimoto, J. and Yamamoto, T. (2006). LATS2-Ajuba complex regulates gamma-tubulin recruitment to centrosomes and spindle organization during mitosis. *FEBS Lett* 580, 782-8.
- Acevedo, K., Li, R., Soo, P., Suryadinata, R., Sarcevic, B., Valova, V. A., Graham, M. E., Robinson, P. J. and Bernard, O. (2007). The phosphorylation of p25/TPPP by LIM kinase 1 inhibits its ability to assemble microtubules. *Exp Cell Res* 313, 4091-106.
- Allen, L. A. and Aderem, A. (1996). Mechanisms of phagocytosis. *Curr Opin Immunol* 8, 36-40.
- Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* 271, 20246-9.
- Amano, T., Kaji, N., Ohashi, K. and Mizuno, K. (2002). Mitosis-specific activation of LIM motif-containing protein kinase and roles of cofilin phosphorylation and dephosphorylation in mitosis. *J Biol Chem* 277, 22093-102.
- Amon, A., Surana, U., Muroff, I. and Nasmyth, K. (1992). Regulation of p34^{CDC28} tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. *Nature* 355, 368-71.
- Andersen, D. C. and Krummen, L. (2002). Recombinant protein expression for therapeutic applications. *Curr Opin Biotechnol* 13, 117-23.
- Andersen, J. S., Wilkinson, C. J., Mayor, T., Mortensen, P., Nigg, E. A. and Mann, M. (2003). Proteomic characterization of the human centrosome by protein correlation profiling. *Nature* 426, 570-4.
- Arber, S., Barbayannis, F. A., Hanser, H., Schneider, C., Stanyon, C. A., Bernard, O. and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* 393, 805-9.
- Asiedu, M., Wu, D., Matsumura, F. and Wei, Q. (2009). Centrosome/spindle pole-associated protein regulates cytokinesis via promoting the recruitment of MyoGEF to the central spindle. *Mol Biol Cell* 20, 1428-40.
- Asoian, R. K. and Zhu, X. (1997). Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression. *Curr Opin Cell Biol* 9, 93-8.

- Atkin, C. L., Thelander, L., Reichard, P. and Lang, G. (1973). Iron and free radical in ribonucleotide reductase. Exchange of iron and Mossbauer spectroscopy of the protein B2 subunit of the Escherichia coli enzyme. *J Biol Chem* 248, 7464-72.
- Bailly, E., Pines, J., Hunter, T. and Bornens, M. (1992). Cytoplasmic accumulation of cyclin B1 in human cells: association with a detergent-resistant compartment and with the centrosome. *J Cell Sci* 101 (Pt 3), 529-45.
- Bakal, C. J., Finan, D., LaRose, J., Wells, C. D., Gish, G., Kulkarni, S., DeSepulveda, P., Wilde, A. and Rottapel, R. (2005). The Rho GTP exchange factor Lfc promotes spindle assembly in early mitosis. *Proc Natl Acad Sci U S A* 102, 9529-34.
- Balasenthil, S., Sahin, A. A., Barnes, C. J., Wang, R. A., Pestell, R. G., Vadlamudi, R. K. and Kumar, R. (2004). p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells. *J Biol Chem* 279, 1422-8.
- Baldin, V., Lukas, J., Marcote, M. J., Pagano, M. and Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev* 7, 812-21.
- Barr, F. A. and Gruneberg, U. (2007). Cytokinesis: placing and making the final cut. *Cell* 131, 847-60.
- Bartolini, F., Moseley, J. B., Schmoranzer, J., Cassimeris, L., Goode, B. L. and Gundersen, G. G. (2008). The formin mDia2 stabilizes microtubules independently of its actin nucleation activity. *J Cell Biol* 181, 523-36.
- Basto, R., Lau, J., Vinogradova, T., Gardiol, A., Woods, C. G., Khodjakov, A. and Raff, J. W. (2006). Flies without centrioles. *Cell* 125, 1375-86.
- Bement, W. M., Benink, H. A. and von Dassow, G. (2005). A microtubule-dependent zone of active RhoA during cleavage plane specification. *J Cell Biol* 170, 91-101.
- Bernards, A. (2003). GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila. *Biochim Biophys Acta* 1603, 47-82.
- Bershadsky, A., Chausovsky, A., Becker, E., Lyubimova, A. and Geiger, B. (1996). Involvement of microtubules in the control of adhesion-dependent signal transduction. *Curr Biol* 6, 1279-89.
- Birkenfeld, J., Nalbant, P., Bohl, B. P., Pertz, O., Hahn, K. M. and Bokoch, G. M. (2007). GEF-H1 modulates localized RhoA activation during cytokinesis under the control of mitotic kinases. *Dev Cell* 12, 699-712.
- Blethrow, J. D., Glavy, J. S., Morgan, D. O. and Shokat, K. M. (2008). Covalent capture of kinase-specific phosphopeptides reveals Cdk1-cyclin B substrates. *Proc Natl Acad Sci U S A* 105, 1442-7.

- Bogenhagen, D. and Clayton, D. A. (1976). Thymidylate nucleotide supply for mitochondrial DNA synthesis in mouse L-cells. Effect of 5-fluorodeoxyuridine and methotrexate in thymidine kinase plus and thymidine kinase minus cells. *J Biol Chem* 251, 2938-44.
- Bohmer, R. M., Scharf, E. and Assoian, R. K. (1996). Cytoskeletal integrity is required throughout the mitogen stimulation phase of the cell cycle and mediates the anchorage-dependent expression of cyclin D1. *Mol Biol Cell* 7, 101-111.
- Booher, R. N., Holman, P. S. and Fattaey, A. (1997). Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. *J Biol Chem* 272, 22300-6.
- Bottazzi, M. E., Buzzai, M., Zhu, X., Desdouets, C., Brechot, C. and Assoian, R. K. (2001). Distinct effects of mitogens and the actin cytoskeleton on CREB and pocket protein phosphorylation control the extent and timing of cyclin A promoter activity. *Mol Cell Biol* 21, 7607-16.
- Brennan, I. M., Peters, U., Kapoor, T. M. and Straight, A. F. (2007). Polo-like kinase controls vertebrate spindle elongation and cytokinesis. *PLoS ONE* 2, e409.
- Brown, E. J. and Baltimore, D. (2003). Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes Dev* 17, 615-28.
- Bubb, M. R., Spector, I., Beyer, B. B. and Fosen, K. M. (2000). Effects of jasplakinolide on the kinetics of actin polymerization. An explanation for certain in vivo observations. *J Biol Chem* 275, 5163-70.
- Burkard, M. E., Randall, C. L., Larochelle, S., Zhang, C., Shokat, K. M., Fisher, R. P. and Jallepalli, P. V. (2007). Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. *Proc Natl Acad Sci U S A* 104, 4383-8.
- Butler, M. (2005). Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. *Appl Microbiol Biotechnol* 68, 283-91.
- Cairns, J. (1966). Autoradiography of HeLa cell DNA. *J Mol Biol* 15, 372-3.
- Canman, J. C., Lewellyn, L., Laband, K., Smerdon, S. J., Desai, A., Bowerman, B. and Oegema, K. (2008). Inhibition of Rac by the GAP activity of centralspindlin is essential for cytokinesis. *Science* 322, 1543-6.
- Carnero, A. and Hannon, G. J. (1998). The INK4 family of CDK inhibitors. *Curr Top Microbiol Immunol* 227, 43-55.
- Caron, E. and Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* 282, 1717-21.

- Carreno, S., Kouranti, I., Glusman, E. S., Fuller, M. T., Echard, A. and Payre, F. (2008). Moesin and its activating kinase Slik are required for cortical stability and microtubule organization in mitotic cells. *J Cell Biol* 180, 739-46.
- Castellano, F., Montcourrier, P. and Chavrier, P. (2000). Membrane recruitment of Rac1 triggers phagocytosis. *J Cell Sci* 113 (Pt 17), 2955-61.
- Castellano, F., Montcourrier, P., Guillemot, J. C., Gouin, E., Machesky, L., Cossart, P. and Chavrier, P. (1999). Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. *Curr Biol* 9, 351-60.
- Chakrabarti, R., Jones, J. L., Oelschlager, D. K., Tapia, T., Tousson, A. and Grizzle, W. E. (2007). Phosphorylated LIM kinases colocalize with gamma-tubulin in centrosomes during early stages of mitosis. *Cell Cycle* 6, 2944-52.
- Chang, Y. C., Nalbant, P., Birkenfeld, J., Chang, Z. F. and Bokoch, G. M. (2008). GEF-H1 couples nocodazole-induced microtubule disassembly to cell contractility via RhoA. *Mol Biol Cell* 19, 2147-53.
- Chen, B., Li, A., Wang, D., Wang, M., Zheng, L. and Bartles, J. R. (1999). Espin contains an additional actin-binding site in its N terminus and is a major actin-bundling protein of the Sertoli cell-spermatid ectoplasmic specialization junctional plaque. *Mol Biol Cell* 10, 4327-39.
- Chen, E. and Li, C. C. (1998). Association of Cdk2/cyclin E and NF-kappa B complexes at G1/S phase. *Biochem Biophys Res Commun* 249, 728-34.
- Chow, J. P., Siu, W. Y., Ho, H. T., Ma, K. H., Ho, C. C. and Poon, R. Y. (2003). Differential contribution of inhibitory phosphorylation of CDC2 and CDK2 for unperturbed cell cycle control and DNA integrity checkpoints. *J Biol Chem* 278, 40815-28.
- Christov, K., Ikui, A., Shilkaitis, A., Green, A., Yao, R., You, M., Grubbs, C., Steele, V., Lubet, R. and Weinstein, I. B. (2003). Cell proliferation, apoptosis, and expression of cyclin D1 and cyclin E as potential biomarkers in tamoxifen-treated mammary tumors. *Breast Cancer Res Treat* 77, 253-64.
- Clark, K., Langeslag, M., Figdor, C. G. and van Leeuwen, F. N. (2007). Myosin II and mechanotransduction: a balancing act. *Trends Cell Biol* 17, 178-86.
- Clute, P. and Pines, J. (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat Cell Biol* 1, 82-7.
- Coleman, M. L., Densham, R. M., Croft, D. R. and Olson, M. F. (2006). Stability of p21Waf1/Cip1 CDK inhibitor protein is responsive to RhoA-mediated regulation of the actin cytoskeleton. *Oncogene* 25, 2708-16.

- Conti, M. A. and Adelstein, R. S. (2008). Nonmuscle myosin II moves in new directions. *J Cell Sci* 121, 11-8.
- Cook, T. A., Nagasaki, T. and Gundersen, G. G. (1998). Rho guanosine triphosphatase mediates the selective stabilization of microtubules induced by lysophosphatidic acid. *J Cell Biol* 141, 175-85.
- Cooper, J. A. (1987). Effects of cytochalasin and phalloidin on actin. *J Cell Biol* 105, 1473-8.
- Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M. and Greenberg, S. (1997). Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. *J Exp Med* 186, 1487-94.
- Crawford, D. F. and Piwnica-Worms, H. (2001). The G(2) DNA damage checkpoint delays expression of genes encoding mitotic regulators. *J Biol Chem* 276, 37166-77.
- Croft, D. R., Crighton, D., Samuel, M. S., Lourenco, F. C., Munro, J., Wood, J., Bensaad, K., Vousden, K. H., Sansom, O. J., Ryan, K. M. et al. (2010). p53-mediated transcriptional regulation and activation of the actin cytoskeleton regulatory RhoC to LIMK2 signaling pathway promotes cell survival. *Cell Res*.
- Croft, D. R. and Olson, M. F. (2006). The Rho GTPase effector ROCK regulates cyclin A, cyclin D1, and p27Kip1 levels by distinct mechanisms. *Mol Cell Biol* 26, 4612-27.
- Dadke, D., Jarnik, M., Pugacheva, E. N., Singh, M. K. and Golemis, E. A. (2006). Deregulation of HEF1 impairs M-phase progression by disrupting the RhoA activation cycle. *Mol Biol Cell* 17, 1204-17.
- Dan, S., Tsunoda, T., Kitahara, O., Yanagawa, R., Zembutsu, H., Katagiri, T., Yamazaki, K., Nakamura, Y. and Yamori, T. (2002). An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines. *Cancer Res* 62, 1139-47.
- Davila, M., Jhala, D., Ghosh, D., Grizzle, W. E. and Chakrabarti, R. (2007). Expression of LIM kinase 1 is associated with reversible G1/S phase arrest, chromosomal instability and prostate cancer. *Mol Cancer* 6, 40.
- De Brabander, M., Geuens, G., Nuydens, R., Willebrords, R. and De Mey, J. (1981). Microtubule assembly in living cells after release from nocodazole block: the effects of metabolic inhibitors, taxol and PH. *Cell Biol Int Rep* 5, 913-20.
- Deng, M., Williams, C. J. and Schultz, R. M. (2005). Role of MAP kinase and myosin light chain kinase in chromosome-induced development of mouse egg polarity. *Dev Biol* 278, 358-66.

- Depue, R. H., Jr. and Rice, R. V. (1965). F-Actin Is a Right-Handed Helix. *J Mol Biol* 12, 302-3.
- Didsbury, J., Weber, R. F., Bokoch, G. M., Evans, T. and Snyderman, R. (1989). rac, a novel ras-related family of proteins that are botulinum toxin substrates. *J Biol Chem* 264, 16378-82.
- Draetta, G. and Beach, D. (1988). Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. *Cell* 54, 17-26.
- Draviam, V. M., Xie, S. and Sorger, P. K. (2004). Chromosome segregation and genomic stability. *Curr Opin Genet Dev* 14, 120-5.
- Ducommun, B., Brambilla, P., Felix, M. A., Franza, B. R., Jr., Karsenti, E. and Draetta, G. (1991). cdc2 phosphorylation is required for its interaction with cyclin. *EMBO J* 10, 3311-9.
- Dunphy, W. G., Brizuela, L., Beach, D. and Newport, J. (1988). The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* 54, 423-31.
- Dynlacht, B. D., Flores, O., Lees, J. A. and Harlow, E. (1994). Differential regulation of E2F transactivation by cyclin/cdk2 complexes. *Genes Dev* 8, 1772-86.
- Ekholm, S. V. and Reed, S. I. (2000). Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. *Curr Opin Cell Biol* 12, 676-84.
- el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* 75, 817-25.
- Elgin, S. C. and Weintraub, H. (1975). Chromosomal proteins and chromatin structure. *Annu Rev Biochem* 44, 725-74.
- Enomoto, T. (1996). Microtubule disruption induces the formation of actin stress fibers and focal adhesions in cultured cells: possible involvement of the rho signal cascade. *Cell Struct Funct* 21, 317-26.
- Espreafico, E. M., Coling, D. E., Tsakraklides, V., Krogh, K., Wolenski, J. S., Kalinec, G. and Kachar, B. (1998). Localization of myosin-V in the centrosome. *Proc Natl Acad Sci U S A* 95, 8636-41.
- Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* 420, 629-35.
- Fabian, L. and Forer, A. (2007). Possible roles of actin and myosin during anaphase chromosome movements in locust spermatocytes. *Protoplasma* 231, 201-13.

- Fasshauer, M., Iwig, M. and Glaesser, D. (1998). Synthesis of proto-oncogene proteins and cyclins depends on intact microfilaments. *Eur J Cell Biol* 77, 188-95.
- Field, C. M. and Alberts, B. M. (1995). Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex. *J Cell Biol* 131, 165-78.
- Fishkind, D. J., Cao, L. G. and Wang, Y. L. (1991). Microinjection of the catalytic fragment of myosin light chain kinase into dividing cells: effects on mitosis and cytokinesis. *J Cell Biol* 114, 967-75.
- Forer, A., Jackson, W. T. and Engberg, A. (1979). Actin in spindles of *Haemanthus katherinae* endosperm. II. Distribution of actin in chromosomal spindle fibres, determined by analysis of serial sections. *J Cell Sci* 37, 349-71.
- Forer, A. and Pickett-Heaps, J. D. (1998). Cytochalasin D and latrunculin affect chromosome behaviour during meiosis in crane-fly spermatocytes. *Chromosome Res* 6, 533-49.
- Forer, A., Spurck, T. and Pickett-Heaps, J. D. (2007). Actin and myosin inhibitors block elongation of kinetochore fibre stubs in metaphase crane-fly spermatocytes. *Protoplasma* 232, 79-85.
- Forer, A., Spurck, T., Pickett-Heaps, J. D. and Wilson, P. J. (2003). Structure of kinetochore fibres in crane-fly spermatocytes after irradiation with an ultraviolet microbeam: neither microtubules nor actin filaments remain in the irradiated region. *Cell Motil Cytoskeleton* 56, 173-92.
- Forsberg, M., Druid, P., Zheng, L., Stendahl, O. and Sarndahl, E. (2003). Activation of Rac2 and Cdc42 on Fc and complement receptor ligation in human neutrophils. *J Leukoc Biol* 74, 611-9.
- Gachet, Y., Tournier, S., Millar, J. B. and Hyams, J. S. (2001). A MAP kinase-dependent actin checkpoint ensures proper spindle orientation in fission yeast. *Nature* 412, 352-5.
- Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119, 493-501.
- Gibson, D. A., Marquardt, M. D. and Gordon, J. A. (1975). Cell rigidity: Effect on concanavalin A-mediated agglutinability of fibroblasts after fixation. *Science* 189, 45-6.
- Glotzer, M. (2004). Cleavage furrow positioning. *J Cell Biol* 164, 347-51.
- Glotzer, M. (2005). The molecular requirements for cytokinesis. *Science* 307, 1735-9.

- Gorovoy, M., Niu, J., Bernard, O., Profirovic, J., Minshall, R., Neamu, R. and Voynoyasenskaya, T. (2005). LIM kinase 1 coordinates microtubule stability and actin polymerization in human endothelial cells. *J Biol Chem* 280, 26533-42.
- Gossen, M. and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 89, 5547-51.
- Gotta, M., Abraham, M. C. and Ahringer, J. (2001). CDC-42 controls early cell polarity and spindle orientation in *C. elegans*. *Curr Biol* 11, 482-8.
- Goulding, M. B., Canman, J. C., Senning, E. N., Marcus, A. H. and Bowerman, B. (2007). Control of nuclear centration in the *C. elegans* zygote by receptor-independent Galpha signaling and myosin II. *J Cell Biol* 178, 1177-91.
- Gregory, S. L., Ebrahimi, S., Milverton, J., Jones, W. M., Bejsovec, A. and Saint, R. (2008). Cell division requires a direct link between microtubule-bound RacGAP and Anillin in the contractile ring. *Curr Biol* 18, 25-9.
- Grigoriev, I., Borisy, G. and Vorobjev, I. (2006). Regulation of microtubule dynamics in 3T3 fibroblasts by Rho family GTPases. *Cell Motil Cytoskeleton* 63, 29-40.
- Grill, S. W. and Hyman, A. A. (2005). Spindle positioning by cortical pulling forces. *Dev Cell* 8, 461-5.
- Guadagno, T. M. and Newport, J. W. (1996). Cdk2 kinase is required for entry into mitosis as a positive regulator of Cdc2-cyclin B kinase activity. *Cell* 84, 73-82.
- Gupton, S. L. and Gertler, F. B. (2007). Filopodia: the fingers that do the walking. *Sci STKE* 2007, re5.
- Hagting, A., Jackman, M., Simpson, K. and Pines, J. (1999). Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. *Curr Biol* 9, 680-9.
- Hagting, A., Karlsson, C., Clute, P., Jackman, M. and Pines, J. (1998). MPF localization is controlled by nuclear export. *EMBO J* 17, 4127-38.
- Hannon, G. J. and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 371, 257-61.
- Hara, T., Abe, M., Inoue, H., Yu, L. R., Veenstra, T. D., Kang, Y. H., Lee, K. S. and Miki, T. (2006). Cytokinesis regulator ECT2 changes its conformation through phosphorylation at Thr-341 in G2/M phase. *Oncogene* 25, 566-78.
- Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E. et al. (1995). Inhibition of cyclin-dependent kinases by p21. *Mol Biol Cell* 6, 387-400.

-
- Hartshorne, D. J., Ito, M. and Erdodi, F. (2004). Role of protein phosphatase type 1 in contractile functions: myosin phosphatase. *J Biol Chem* 279, 37211-4.
- Hayles, J., Fisher, D., Woollard, A. and Nurse, P. (1994). Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2-mitotic B cyclin complex. *Cell* 78, 813-22.
- Heald, R., McLoughlin, M. and McKeon, F. (1993). Human wee1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated Cdc2 kinase. *Cell* 74, 463-74.
- Heath, J. P. (1983). Behaviour and structure of the leading lamella in moving fibroblasts. I. Occurrence and centripetal movement of arc-shaped microfilament bundles beneath the dorsal cell surface. *J Cell Sci* 60, 331-54.
- Helfman, D. M., Levy, E. T., Berthier, C., Shtutman, M., Rivelino, D., Grosheva, I., Lachish-Zalait, A., Elbaum, M. and Bershadsky, A. D. (1999). Caldesmon inhibits nonmuscle cell contractility and interferes with the formation of focal adhesions. *Mol Biol Cell* 10, 3097-112.
- Heng, Y. W. and Koh, C. G. (2010). Actin cytoskeleton dynamics and the cell division cycle. *Int J Biochem Cell Biol* 42, 1622-33.
- Herreros, L., Rodriguez-Fernandez, J. L., Brown, M. C., Alonso-Lebrero, J. L., Cabanas, C., Sanchez-Madrid, F., Longo, N., Turner, C. E. and Sanchez-Mateos, P. (2000). Paxillin localizes to the lymphocyte microtubule organizing center and associates with the microtubule cytoskeleton. *J Biol Chem* 275, 26436-40.
- Hewish, D. R. (1976). DNA replication in eukaryotes: a model for the specific involvement of chromatin subunits. *Nucleic Acids Res* 3, 69-78.
- Hickson, G. R. and O'Farrell, P. H. (2008). Rho-dependent control of anillin behavior during cytokinesis. *J Cell Biol* 180, 285-94.
- Hildebrandt, E. R. and Hoyt, M. A. (2000). Mitotic motors in *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1496, 99-116.
- Hipfner, D. R., Keller, N. and Cohen, S. M. (2004). Slik Sterile-20 kinase regulates Moesin activity to promote epithelial integrity during tissue growth. *Genes Dev* 18, 2243-8.
- Hirota, T., Kunitoku, N., Sasayama, T., Marumoto, T., Zhang, D., Nitta, M., Hatakeyama, K. and Saya, H. (2003). Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. *Cell* 114, 585-98.
- Hirota, T., Morisaki, T., Nishiyama, Y., Marumoto, T., Tada, K., Hara, T., Masuko, N., Inagaki, M., Hatakeyama, K. and Saya, H. (2000). Zyxin, a regulator of actin filament
-

- assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumor suppressor. *J Cell Biol* 149, 1073-86.
- Hlavanda, E., Klement, E., Kokai, E., Kovacs, J., Vincze, O., Tokesi, N., Orosz, F., Medzihradszky, K. F., Dombradi, V. and Ovadi, J. (2007). Phosphorylation blocks the activity of tubulin polymerization-promoting protein (TPPP): identification of sites targeted by different kinases. *J Biol Chem* 282, 29531-9.
- Hlavanda, E., Kovacs, J., Olah, J., Orosz, F., Medzihradszky, K. F. and Ovadi, J. (2002). Brain-specific p25 protein binds to tubulin and microtubules and induces aberrant microtubule assemblies at substoichiometric concentrations. *Biochemistry* 41, 8657-64.
- Hotulainen, P. and Lappalainen, P. (2006). Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J Cell Biol* 173, 383-94.
- Hsu, F. F., Lin, T. Y., Chen, J. Y. and Shieh, S. Y. (2010). p53-Mediated transactivation of LIMK2b links actin dynamics to cell cycle checkpoint control. *Oncogene* 29, 2864-76.
- Hsu, S. L., Yin, S. C., Liu, M. C., Reichert, U. and Ho, W. L. (1999). Involvement of cyclin-dependent kinase activities in CD437-induced apoptosis. *Exp Cell Res* 252, 332-41.
- Huang, S., Chen, C. S. and Ingber, D. E. (1998). Control of cyclin D1, p27(Kip1), and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. *Mol Biol Cell* 9, 3179-93.
- Huang, S. and Ingber, D. E. (2002). A discrete cell cycle checkpoint in late G(1) that is cytoskeleton-dependent and MAP kinase (Erk)-independent. *Exp Cell Res* 275, 255-64.
- Huberman, J. A. and Riggs, A. D. (1968). On the mechanism of DNA replication in mammalian chromosomes. *J Mol Biol* 32, 327-41.
- Hughes, S. C. and Fehon, R. G. (2006). Phosphorylation and activity of the tumor suppressor Merlin and the ERM protein Moesin are coordinately regulated by the Slik kinase. *J Cell Biol* 175, 305-13.
- Hung, T. J. and Kemphues, K. J. (1999). PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in *Caenorhabditis elegans* embryos. *Development* 126, 127-35.
- Huyghe, P., Dassonneville, L., Fenaux, P. and Bailly, C. (2004). Hydroxyurea-induced apoptosis in an EBV-immortalized lymphoblastoid cell line. *Oncol Res* 14, 235-45.

- Ingber, D. E., Prusty, D., Sun, Z., Betensky, H. and Wang, N. (1995). Cell shape, cytoskeletal mechanics, and cell cycle control in angiogenesis. *J Biomech* 28, 1471-84.
- Iwig, M., Czeslick, E., Muller, A., Gruner, M., Spindler, M. and Glaesser, D. (1995). Growth regulation by cell shape alteration and organization of the cytoskeleton. *Eur J Cell Biol* 67, 145-57.
- Jackman, M., Firth, M. and Pines, J. (1995). Human cyclins B1 and B2 are localized to strikingly different structures: B1 to microtubules, B2 primarily to the Golgi apparatus. *EMBO J* 14, 1646-54.
- Jaffe, A. B. and Hall, A. (2005). Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol* 21, 247-69.
- Jaffe, A. B., Kaji, N., Durgan, J. and Hall, A. (2008). Cdc42 controls spindle orientation to position the apical surface during epithelial morphogenesis. *J Cell Biol* 183, 625-33.
- Jahn, R. and Scheller, R. H. (2006). SNAREs--engines for membrane fusion. *Nat Rev Mol Cell Biol* 7, 631-43.
- Jantsch-Plunger, V., Gonczy, P., Romano, A., Schnabel, H., Hamill, D., Schnabel, R., Hyman, A. A. and Glotzer, M. (2000). CYK-4: A Rho family gtpase activating protein (GAP) required for central spindle formation and cytokinesis. *J Cell Biol* 149, 1391-404.
- Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J. and Pavletich, N. P. (1995). Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* 376, 313-20.
- Jin, P., Gu, Y. and Morgan, D. O. (1996). Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. *J Cell Biol* 134, 963-70.
- Jin, P., Hardy, S. and Morgan, D. O. (1998). Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. *J Cell Biol* 141, 875-85.
- Joe, A. K., Liu, H., Suzui, M., Vural, M. E., Xiao, D. and Weinstein, I. B. (2002). Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin Cancer Res* 8, 893-903.
- Johnson, C. A., Forster, T. H., Winterford, C. M. and Allan, D. J. (1992). Hydroxyurea induces apoptosis and regular DNA fragmentation in a Burkitt's lymphoma cell line. *Biochim Biophys Acta* 1136, 1-4.
- Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F. and Holmes, K. C. (1990). Atomic structure of the actin:DNase I complex. *Nature* 347, 37-44.

- Kaji, N., Muramoto, A. and Mizuno, K. (2008). LIM kinase-mediated cofilin phosphorylation during mitosis is required for precise spindle positioning. *J Biol Chem* 283, 4983-92.
- Kaji, N., Ohashi, K., Shuin, M., Niwa, R., Uemura, T. and Mizuno, K. (2003). Cell cycle-associated changes in Slingshot phosphatase activity and roles in cytokinesis in animal cells. *J Biol Chem* 278, 33450-5.
- Kamm, K. E. and Stull, J. T. (1985). The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu Rev Pharmacol Toxicol* 25, 593-620.
- Kane, R. E. (1975). Preparation and purification of polymerized actin from sea urchin egg extracts. *J Cell Biol* 66, 305-15.
- Kang, J. S. and Krauss, R. S. (1996). Ras induces anchorage-independent growth by subverting multiple adhesion-regulated cell cycle events. *Mol Cell Biol* 16, 3370-80.
- Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K. et al. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 273, 245-8.
- King, R. W., Deshaies, R. J., Peters, J. M. and Kirschner, M. W. (1996). How proteolysis drives the cell cycle. *Science* 274, 1652-9.
- Kleinnijenhuis, A. J., Hedegaard, C., Lundvig, D., Sundbye, S., Issinger, O. G., Jensen, O. N. and Jensen, P. H. (2008). Identification of multiple post-translational modifications in the porcine brain specific p25alpha. *J Neurochem* 106, 925-33.
- Kondo, A., Hashimoto, S., Yano, H., Nagayama, K., Mazaki, Y. and Sabe, H. (2000). A new paxillin-binding protein, PAG3/Papalpha/KIAA0400, bearing an ADP-ribosylation factor GTPase-activating protein activity, is involved in paxillin recruitment to focal adhesions and cell migration. *Mol Biol Cell* 11, 1315-27.
- Konishi, Y., Lehtinen, M., Donovan, N. and Bonni, A. (2002). Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. *Mol Cell* 9, 1005-16.
- Kovacs, M., Toth, J., Hetenyi, C., Malnasi-Csizmadia, A. and Sellers, J. R. (2004). Mechanism of blebbistatin inhibition of myosin II. *J Biol Chem* 279, 35557-63.
- Kovar, D. R., Harris, E. S., Mahaffy, R., Higgs, H. N. and Pollard, T. D. (2006). Control of the assembly of ATP- and ADP-actin by formins and profilin. *Cell* 124, 423-35.
- Krendel, M. and Mooseker, M. S. (2005). Myosins: tails (and heads) of functional diversity. *Physiology (Bethesda)* 20, 239-51.
- Krendel, M., Zenke, F. T. and Bokoch, G. M. (2002). Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. *Nat Cell Biol* 4, 294-301.

-
- Kunda, P., Pelling, A. E., Liu, T. and Baum, B. (2008). Moesin controls cortical rigidity, cell rounding, and spindle morphogenesis during mitosis. *Curr Biol* 18, 91-101.
- Larson, S. M., Lee, H. J., Hung, P. H., Matthews, L. M., Robinson, D. N. and Evans, J. P. (2010). Cortical mechanics and meiosis II completion in mammalian oocytes are mediated by myosin-II and Ezrin-Radixin-Moesin (ERM) proteins. *Mol Biol Cell* 21, 3182-92.
- Lee, K. and Song, K. (2007). Actin dysfunction activates ERK1/2 and delays entry into mitosis in mammalian cells. *Cell Cycle* 6, 1487-95.
- Lee, M. H., Reynisdottir, I. and Massague, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* 9, 639-49.
- Lehotzky, A., Tirian, L., Tokesi, N., Lenart, P., Szabo, B., Kovacs, J. and Ovadi, J. (2004). Dynamic targeting of microtubules by TPPP/p25 affects cell survival. *J Cell Sci* 117, 6249-59.
- Leung, T., Chen, X. Q., Tan, I., Manser, E. and Lim, L. (1998). Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization. *Mol Cell Biol* 18, 130-40.
- Li, F. and Higgs, H. N. (2003). The mouse Formin mDia1 is a potent actin nucleation factor regulated by autoinhibition. *Curr Biol* 13, 1335-40.
- Ling, L. and Lobie, P. E. (2004). RhoA/ROCK activation by growth hormone abrogates p300/histone deacetylase 6 repression of Stat5-mediated transcription. *J Biol Chem* 279, 32737-50.
- Liu, B. P., Chrzanowska-Wodnicka, M. and Burridge, K. (1998). Microtubule depolymerization induces stress fibers, focal adhesions, and DNA synthesis via the GTP-binding protein Rho. *Cell Adhes Commun* 5, 249-55.
- Liu, F., Stanton, J. J., Wu, Z. and Piwnicka-Worms, H. (1997). The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex. *Mol Cell Biol* 17, 571-83.
- Lohez, O. D., Reynaud, C., Borel, F., Andreassen, P. R. and Margolis, R. L. (2003). Arrest of mammalian fibroblasts in G1 in response to actin inhibition is dependent on retinoblastoma pocket proteins but not on p53. *J Cell Biol* 161, 67-77.
- Lukas, J., Bartkova, J., Rohde, M., Strauss, M. and Bartek, J. (1995). Cyclin D1 is dispensable for G1 control in retinoblastoma gene-deficient cells independently of cdk4 activity. *Mol Cell Biol* 15, 2600-11.
-

- Lukas, J., Muller, H., Bartkova, J., Spitkovsky, D., Kjerulff, A. A., Jansen-Durr, P., Strauss, M. and Bartek, J. (1994). DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. *J Cell Biol* 125, 625-38.
- Mabuchi, I. and Okuno, M. (1977). The effect of myosin antibody on the division of starfish blastomeres. *J Cell Biol* 74, 251-63.
- Maddox, A. S. and Burridge, K. (2003). RhoA is required for cortical retraction and rigidity during mitotic cell rounding. *J Cell Biol* 160, 255-65.
- Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. and Narumiya, S. (1999). Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 285, 895-8.
- Maiato, H., Rieder, C. L. and Khodjakov, A. (2004). Kinetochore-driven formation of kinetochore fibers contributes to spindle assembly during animal mitosis. *J Cell Biol* 167, 831-40.
- Mailand, N., Podtelejnikov, A. V., Groth, A., Mann, M., Bartek, J. and Lukas, J. (2002). Regulation of G(2)/M events by Cdc25A through phosphorylation-dependent modulation of its stability. *EMBO J* 21, 5911-20.
- Mammoto, A., Huang, S., Moore, K., Oh, P. and Ingber, D. E. (2004). Role of RhoA, mDia, and ROCK in cell shape-dependent control of the Skp2-p27kip1 pathway and the G1/S transition. *J Biol Chem* 279, 26323-30.
- Maness, P. F. and Walsh, R. C., Jr. (1982). Dihydrocytochalasin B disorganizes actin cytoarchitecture and inhibits initiation of DNA synthesis in 3T3 cells. *Cell* 30, 253-62.
- Margadant, C., van Opstal, A. and Boonstra, J. (2007). Focal adhesion signaling and actin stress fibers are dispensable for progression through the ongoing cell cycle. *J Cell Sci* 120, 66-76.
- Maroto, B., Ye, M. B., von Lohneysen, K., Schnelzer, A. and Knaus, U. G. (2008). P21-activated kinase is required for mitotic progression and regulates Plk1. *Oncogene* 27, 4900-8.
- Massol, P., Montcourrier, P., Guillemot, J. C. and Chavrier, P. (1998). Fc receptor-mediated phagocytosis requires CDC42 and Rac1. *EMBO J* 17, 6219-29.
- Matsui, T., Yonemura, S. and Tsukita, S. (1999). Activation of ERM proteins in vivo by Rho involves phosphatidylinositol 4-phosphate 5-kinase and not ROCK kinases. *Curr Biol* 9, 1259-62.

- Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S. et al. (2002). In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J* 21, 6820-31.
- McKenna, N. M. and Wang, Y. L. (1986). Possible translocation of actin and alpha-actinin along stress fibers. *Exp Cell Res* 167, 95-105.
- McMillan, J. N., Sia, R. A. and Lew, D. J. (1998). A morphogenesis checkpoint monitors the actin cytoskeleton in yeast. *J Cell Biol* 142, 1487-99.
- Miki, H., Suetsugu, S. and Takenawa, T. (1998). WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J* 17, 6932-41.
- Miller, A. L. and Bement, W. M. (2009). Regulation of cytokinesis by Rho GTPase flux. *Nat Cell Biol* 11, 71-7.
- Minoshima, Y., Kawashima, T., Hirose, K., Tono-zuka, Y., Kawajiri, A., Bao, Y. C., Deng, X., Tatsuka, M., Narumiya, S., May, W. S., Jr. et al. (2003). Phosphorylation by aurora B converts MgcRacGAP to a RhoGAP during cytokinesis. *Dev Cell* 4, 549-60.
- Mitsushima, M., Toyoshima, F. and Nishida, E. (2009). Dual role of Cdc42 in spindle orientation control of adherent cells. *Mol Cell Biol* 29, 2816-27.
- Mizuno, K., Okano, I., Ohashi, K., Nunoue, K., Kuma, K., Miyata, T. and Nakamura, T. (1994). Identification of a human cDNA encoding a novel protein kinase with two repeats of the LIM/double zinc finger motif. *Oncogene* 9, 1605-12.
- Montanez-Wiscovich, M. E., Shelton, M. D., Seachrist, D. D., Lozada, K. L., Johnson, E., Miedler, J. D., Abdul-Karim, F. W., Visvader, J. E. and Keri, R. A. (2010). Aberrant expression of LMO4 induces centrosome amplification and mitotic spindle abnormalities in breast cancer cells. *J Pathol* 222, 271-81.
- Morgan, D. O. (1995). Principles of CDK regulation. *Nature* 374, 131-4.
- Moulding, D. A., Blundell, M. P., Spiller, D. G., White, M. R., Cory, G. O., Calle, Y., Kempski, H., Sinclair, J., Ancliff, P. J., Kinnon, C. et al. (2007). Unregulated actin polymerization by WASp causes defects of mitosis and cytokinesis in X-linked neutropenia. *J Exp Med* 204, 2213-24.
- Munro, E., Nance, J. and Priess, J. R. (2004). Cortical flows powered by asymmetrical contraction transport PAR proteins to establish and maintain anterior-posterior polarity in the early *C. elegans* embryo. *Dev Cell* 7, 413-24.

-
- Murata-Hori, M., Suizu, F., Iwasaki, T., Kikuchi, A. and Hosoya, H. (1999). ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. *FEBS Lett* 451, 81-4.
- Mykkanen, O. M., Gronholm, M., Ronty, M., Lalowski, M., Salmikangas, P., Suila, H. and Carpen, O. (2001). Characterization of human palladin, a microfilament-associated protein. *Mol Biol Cell* 12, 3060-73.
- Na, J. and Zernicka-Goetz, M. (2006). Asymmetric positioning and organization of the meiotic spindle of mouse oocytes requires CDC42 function. *Curr Biol* 16, 1249-54.
- Newman, S. L., Mikus, L. K. and Tucci, M. A. (1991). Differential requirements for cellular cytoskeleton in human macrophage complement receptor- and Fc receptor-mediated phagocytosis. *J Immunol* 146, 967-74.
- Nheu, T., He, H., Hirokawa, Y., Walker, F., Wood, J. and Maruta, H. (2004). PAK is essential for RAS-induced upregulation of cyclin D1 during the G1 to S transition. *Cell Cycle* 3, 71-4.
- Niiya, F., Tatsumoto, T., Lee, K. S. and Miki, T. (2006). Phosphorylation of the cytokinesis regulator ECT2 at G2/M phase stimulates association of the mitotic kinase Plk1 and accumulation of GTP-bound RhoA. *Oncogene* 25, 827-37.
- Nishimura, Y. and Yonemura, S. (2006). Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. *J Cell Sci* 119, 104-14.
- Niwa, R., Nagata-Ohashi, K., Takeichi, M., Mizuno, K. and Uemura, T. (2002). Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. *Cell* 108, 233-46.
- Noble, M. E., Endicott, J. A. and Johnson, L. N. (2004). Protein kinase inhibitors: insights into drug design from structure. *Science* 303, 1800-5.
- Norman, J. C., Jones, D., Barry, S. T., Holt, M. R., Cockcroft, S. and Critchley, D. R. (1998). ARF1 mediates paxillin recruitment to focal adhesions and potentiates Rho-stimulated stress fiber formation in intact and permeabilized Swiss 3T3 fibroblasts. *J Cell Biol* 143, 1981-95.
- Nunoue, K., Ohashi, K., Okano, I. and Mizuno, K. (1995). LIMK-1 and LIMK-2, two members of a LIM motif-containing protein kinase family. *Oncogene* 11, 701-10.
- Oceguera-Yanez, F. and Narumiya, S. (2006). Measurement of activity of rho GTPases during mitosis. *Methods Enzymol* 406, 332-45.
- O'Connell, C. B. and Khodjakov, A. L. (2007). Cooperative mechanisms of mitotic spindle formation. *J Cell Sci* 120, 1717-22.
-

- O'Connell, C. B., Wheatley, S. P., Ahmed, S. and Wang, Y. L. (1999). The small GTP-binding protein rho regulates cortical activities in cultured cells during division. *J Cell Biol* 144, 305-13.
- Oegema, K., Savoian, M. S., Mitchison, T. J. and Field, C. M. (2000). Functional analysis of a human homologue of the *Drosophila* actin binding protein anillin suggests a role in cytokinesis. *J Cell Biol* 150, 539-52.
- Ohta, T., Takasuka, T., Ishibashi, S. and Ide, T. (1985). Cytochalasin D inhibits the progression from the Go to S phase at the mid-prereplicative stage in GC-7 cells stimulated with serum. *Cell Struct Funct* 10, 37-46.
- Okamoto, A., Demetrick, D. J., Spillare, E. A., Hagiwara, K., Hussain, S. P., Bennett, W. P., Forrester, K., Gerwin, B., Serrano, M., Beach, D. H. et al. (1994). Mutations and altered expression of p16INK4 in human cancer. *Proc Natl Acad Sci U S A* 91, 11045-9.
- Olofsson, B. (1999). Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. *Cell Signal* 11, 545-54.
- Olson, M. F., Ashworth, A. and Hall, A. (1995). An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* 269, 1270-2.
- Ou, Y. H., Chung, P. H., Hsu, F. F., Sun, T. P., Chang, W. Y. and Shieh, S. Y. (2007). The candidate tumor suppressor BTG3 is a transcriptional target of p53 that inhibits E2F1. *EMBO J* 26, 3968-80.
- Palazzo, A. F., Cook, T. A., Alberts, A. S. and Gundersen, G. G. (2001). mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nat Cell Biol* 3, 723-9.
- Palmer, R. E., Sullivan, D. S., Huffaker, T. and Koshland, D. (1992). Role of astral microtubules and actin in spindle orientation and migration in the budding yeast, *Saccharomyces cerevisiae*. *J Cell Biol* 119, 583-93.
- Pan, W., Sun, T., Hoess, R. and Grafstrom, R. (1998). Defining the minimal portion of the retinoblastoma protein that serves as an efficient substrate for cdk4 kinase/cyclin D1 complex. *Carcinogenesis* 19, 765-9.
- Pan, Z. Q., Reardon, J. T., Li, L., Flores-Rozas, H., Legerski, R., Sancar, A. and Hurwitz, J. (1995). Inhibition of nucleotide excision repair by the cyclin-dependent kinase inhibitor p21. *J Biol Chem* 270, 22008-16.
- Parker, L. L. and Piwnicka-Worms, H. (1992). Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. *Science* 257, 1955-7.

- Paulovich, A. G. and Hartwell, L. H. (1995). A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. *Cell* 82, 841-7.
- Pavalko, F. M., Otey, C. A. and Burrridge, K. (1989). Identification of a filamin isoform enriched at the ends of stress fibers in chicken embryo fibroblasts. *J Cell Sci* 94 (Pt 1), 109-18.
- Pedoux, R., Al-Irani, N., Marteau, C., Pellicier, F., Branche, R., Ozturk, M., Franchi, J. and Dore, J. F. (1998). Thymidine dinucleotides induce S phase cell cycle arrest in addition to increased melanogenesis in human melanocytes. *J Invest Dermatol* 111, 472-7.
- Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S. and Piwnicka-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* 277, 1501-5.
- Peng, J., Marshall, N. F. and Price, D. H. (1998). Identification of a cyclin subunit required for the function of *Drosophila* P-TEFb. *J Biol Chem* 273, 13855-60.
- Peng, J., Wallar, B. J., Flanders, A., Swiatek, P. J. and Alberts, A. S. (2003). Disruption of the Diaphanous-related formin Drf1 gene encoding mDia1 reveals a role for Drf3 as an effector for Cdc42. *Curr Biol* 13, 534-45.
- Perez, F., Diamantopoulos, G. S., Stalder, R. and Kreis, T. E. (1999). CLIP-170 highlights growing microtubule ends in vivo. *Cell* 96, 517-27.
- Petronczki, M., Glotzer, M., Kraut, N. and Peters, J. M. (2007). Polo-like kinase 1 triggers the initiation of cytokinesis in human cells by promoting recruitment of the RhoGEF Ect2 to the central spindle. *Dev Cell* 12, 713-25.
- Piekny, A., Werner, M. and Glotzer, M. (2005). Cytokinesis: welcome to the Rho zone. *Trends Cell Biol* 15, 651-8.
- Piekny, A. J. and Glotzer, M. (2008). Anillin is a scaffold protein that links RhoA, actin, and myosin during cytokinesis. *Curr Biol* 18, 30-6.
- Pines, J. (1995). Cyclins and cyclin-dependent kinases: theme and variations. *Adv Cancer Res* 66, 181-212.
- Pines, J. and Hunter, T. (1991). Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J Cell Biol* 115, 1-17.
- Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P. and Massague, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 78, 59-66.

- Po'uha, S. T., Shum, M. S., Goebel, A., Bernard, O. and Kavallaris, M. (2009). LIM-kinase 2, a regulator of actin dynamics, is involved in mitotic spindle integrity and sensitivity to microtubule-destabilizing drugs. *Oncogene* 29, 597-607.
- Pugacheva, E. N. and Golemis, E. A. (2005). The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome. *Nat Cell Biol* 7, 937-46.
- Redemann, S., Pecreaux, J., Goehring, N. W., Khairy, K., Stelzer, E. H., Hyman, A. A. and Howard, J. (2010). Membrane invaginations reveal cortical sites that pull on mitotic spindles in one-cell *C. elegans* embryos. *PLoS ONE* 5, e12301.
- Ren, S. and Rollins, B. J. (2004). Cyclin C/cdk3 promotes Rb-dependent G0 exit. *Cell* 117, 239-51.
- Ren, X. D., Kiosses, W. B. and Schwartz, M. A. (1999). Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J* 18, 578-85.
- Reshetnikova, G., Barkan, R., Popov, B., Nikolsky, N. and Chang, L. S. (2000). Disruption of the actin cytoskeleton leads to inhibition of mitogen-induced cyclin E expression, Cdk2 phosphorylation, and nuclear accumulation of the retinoblastoma protein-related p107 protein. *Exp Cell Res* 259, 35-53.
- Reynisdottir, I., Polyak, K., Iavarone, A. and Massague, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. *Genes Dev* 9, 1831-45.
- Rhind, N., Furnari, B. and Russell, P. (1997). Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. *Genes Dev* 11, 504-11.
- Riabowol, K., Draetta, G., Brizuela, L., Vandre, D. and Beach, D. (1989). The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. *Cell* 57, 393-401.
- Rickert, P., Seghezzi, W., Shanahan, F., Cho, H. and Lees, E. (1996). Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. *Oncogene* 12, 2631-40.
- Rodriguez-Fraticelli, A. E., Vargarajauregui, S., Eastburn, D. J., Datta, A., Alonso, M. A., Mostov, K. and Martin-Belmonte, F. (2010). The Cdc42 GEF Intersectin 2 controls mitotic spindle orientation to form the lumen during epithelial morphogenesis. *J Cell Biol* 189, 725-38.
- Rogers, G. C., Rogers, S. L., Schwimmer, T. A., Ems-McClung, S. C., Walczak, C. E., Vale, R. D., Scholey, J. M. and Sharp, D. J. (2004). Two mitotic kinesins cooperate to drive sister chromatid separation during anaphase. *Nature* 427, 364-70.

-
- Ronty, M., Taivainen, A., Moza, M., Otey, C. A. and Carpen, O. (2004). Molecular analysis of the interaction between palladin and alpha-actinin. *FEBS Lett* 566, 30-4.
- Roovers, K. and Assoian, R. K. (2003). Effects of rho kinase and actin stress fibers on sustained extracellular signal-regulated kinase activity and activation of G(1) phase cyclin-dependent kinases. *Mol Cell Biol* 23, 4283-94.
- Rosenblatt, J., Cramer, L. P., Baum, B. and McGee, K. M. (2004). Myosin II-dependent cortical movement is required for centrosome separation and positioning during mitotic spindle assembly. *Cell* 117, 361-72.
- Roszko, I., Afonso, C., Henrique, D. and Mathis, L. (2006). Key role played by RhoA in the balance between planar and apico-basal cell divisions in the chick neuroepithelium. *Dev Biol* 298, 212-24.
- Royou, A., Sullivan, W. and Karess, R. (2002). Cortical recruitment of nonmuscle myosin II in early syncytial *Drosophila* embryos: its role in nuclear axial expansion and its regulation by Cdc2 activity. *J Cell Biol* 158, 127-37.
- Rubtsova, S. N., Kondratov, R. V., Kopnin, P. B., Chumakov, P. M., Kopnin, B. P. and Vasiliev, J. M. (1998). Disruption of actin microfilaments by cytochalasin D leads to activation of p53. *FEBS Lett* 430, 353-7.
- Sahai, E. and Marshall, C. J. (2002). ROCK and Dia have opposing effects on adherens junctions downstream of Rho. *Nat Cell Biol* 4, 408-15.
- Sampson, K. and Pickett-Heaps, J. D. (2001). Phalloidin stains the kinetochore region in the mitotic spindle of the green algae *Oedogonium* spp. *Protoplasma* 217, 166-76.
- Sanger, J. W. (1975). Changing patterns of actin localization during cell division. *Proc Natl Acad Sci U S A* 72, 1913-6.
- Schloss, J. A., Milsted, A. and Goldman, R. D. (1977). Myosin subfragment binding for the localization of actin-like microfilaments in cultured cells. A light and electron microscope study. *J Cell Biol* 74, 794-815.
- Schmidt, A., Durgan, J., Magalhaes, A. and Hall, A. (2007). Rho GTPases regulate PRK2/PKN2 to control entry into mitosis and exit from cytokinesis. *EMBO J* 26, 1624-36.
- Schmidt, A. and Hall, A. (2002). Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev* 16, 1587-609.
- Schulze, A., Zerfass-Thome, K., Berges, J., Middendorp, S., Jansen-Durr, P. and Henglein, B. (1996). Anchorage-dependent transcription of the cyclin A gene. *Mol Cell Biol* 16, 4632-8.
-

- Schuyler, S. C. and Pellman, D. (2001). Microtubule "plus-end-tracking proteins": The end is just the beginning. *Cell* 105, 421-4.
- Senderowicz, A. M., Kaur, G., Sainz, E., Laing, C., Inman, W. D., Rodriguez, J., Crews, P., Malspeis, L., Grever, M. R., Sausville, E. A. et al. (1995). Jasplakinolide's inhibition of the growth of prostate carcinoma cells in vitro with disruption of the actin cytoskeleton. *J Natl Cancer Inst* 87, 46-51.
- Shelton, C. A., Carter, J. C., Ellis, G. C. and Bowerman, B. (1999). The nonmuscle myosin regulatory light chain gene *mlc-4* is required for cytokinesis, anterior-posterior polarity, and body morphology during *Caenorhabditis elegans* embryogenesis. *J Cell Biol* 146, 439-51.
- Sherr, C. J. and Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* 9, 1149-63.
- Shi, L., Chen, G., He, D., Bosc, D. G., Litchfield, D. W. and Greenberg, A. H. (1996). Granzyme B induces apoptosis and cyclin A-associated cyclin-dependent kinase activity in all stages of the cell cycle. *J Immunol* 157, 2381-5.
- Shi, L., Nishioka, W. K., Th'ng, J., Bradbury, E. M., Litchfield, D. W. and Greenberg, A. H. (1994). Premature p34cdc2 activation required for apoptosis. *Science* 263, 1143-5.
- Shimizu, T., O'Connor, P. M., Kohn, K. W. and Pommier, Y. (1995). Unscheduled activation of cyclin B1/Cdc2 kinase in human promyelocytic leukemia cell line HL60 cells undergoing apoptosis induced by DNA damage. *Cancer Res* 55, 228-31.
- Silverman-Gavrila, R. V. and Forer, A. (2000). Evidence that actin and myosin are involved in the poleward flux of tubulin in metaphase kinetochore microtubules of crane-fly spermatocytes. *J Cell Sci* 113 (Pt 4), 597-609.
- Silverman-Gavrila, R. V. and Forer, A. (2003). Myosin localization during meiosis I of crane-fly spermatocytes gives indications about its role in division. *Cell Motil Cytoskeleton* 55, 97-113.
- Simerly, C., Nowak, G., de Lanerolle, P. and Schatten, G. (1998). Differential expression and functions of cortical myosin IIA and IIB isotypes during meiotic maturation, fertilization, and mitosis in mouse oocytes and embryos. *Mol Biol Cell* 9, 2509-25.
- Small, J. V., Rottner, K., Kaverina, I. and Anderson, K. I. (1998). Assembling an actin cytoskeleton for cell attachment and movement. *Biochim Biophys Acta* 1404, 271-81.
- Smeets, M. F., Mooren, E. H. and Begg, A. C. (1994). The effect of radiation on G2 blocks, cyclin B expression and cdc2 expression in human squamous carcinoma cell lines with different radiosensitivities. *Radiother Oncol* 33, 217-27.

- Snapper, S. B., Takeshima, F., Anton, I., Liu, C. H., Thomas, S. M., Nguyen, D., Dudley, D., Fraser, H., Purich, D., Lopez-Illasaca, M. et al. (2001). N-WASP deficiency reveals distinct pathways for cell surface projections and microbial actin-based motility. *Nat Cell Biol* 3, 897-904.
- Sneeden, J. L. and Loeb, L. A. (2004). Mutations in the R2 subunit of ribonucleotide reductase that confer resistance to hydroxyurea. *J Biol Chem* 279, 40723-8.
- Snyder, J. A., Ha, Y., Olsofka, C. and Wahdan, R. (2010). Both actin and myosin inhibitors affect spindle architecture in PtK1 cells: does an actomyosin system contribute to mitotic spindle forces by regulating attachment and movements of chromosomes in mammalian cells? *Protoplasma* 240, 57-68.
- Somlyo, A. P. and Somlyo, A. V. (2003). Ca²⁺ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 83, 1325-58.
- Sorger, P. K. and Murray, A. W. (1992). S-phase feedback control in budding yeast independent of tyrosine phosphorylation of p34cdc28. *Nature* 355, 365-8.
- Straight, A. F., Field, C. M. and Mitchison, T. J. (2005). Anillin binds nonmuscle myosin II and regulates the contractile ring. *Mol Biol Cell* 16, 193-201.
- Strausfeld, U., Fernandez, A., Capony, J. P., Girard, F., Lautredou, N., Derancourt, J., Labbe, J. C. and Lamb, N. J. (1994). Activation of p34cdc2 protein kinase by microinjection of human cdc25C into mammalian cells. Requirement for prior phosphorylation of cdc25C by p34cdc2 on sites phosphorylated at mitosis. *J Biol Chem* 269, 5989-6000.
- Sumi, T., Hashigasako, A., Matsumoto, K. and Nakamura, T. (2006). Different activity regulation and subcellular localization of LIMK1 and LIMK2 during cell cycle transition. *Exp Cell Res* 312, 1021-30.
- Sumi, T., Matsumoto, K. and Nakamura, T. (2001). Specific activation of LIM kinase 2 via phosphorylation of threonine 505 by ROCK, a Rho-dependent protein kinase. *J Biol Chem* 276, 670-6.
- Sumi, T., Matsumoto, K. and Nakamura, T. (2002). Mitosis-dependent phosphorylation and activation of LIM-kinase 1. *Biochem Biophys Res Commun* 290, 1315-20.
- Sumi, T., Matsumoto, K., Takai, Y. and Nakamura, T. (1999). Cofilin phosphorylation and actin cytoskeletal dynamics regulated by rho- and Cdc42-activated LIM-kinase 2. *J Cell Biol* 147, 1519-32.

- Takasuka, T., Ishibashi, S. and Ide, T. (1987). Expression of cell-cycle-dependent genes in serum stimulated cells whose entry into S phase is blocked by cytochalasin D. *Biochim Biophys Acta* 909, 161-4.
- Takesono, A., Heasman, S. J., Wojciak-Stothard, B., Garg, R. and Ridley, A. J. (2010). Microtubules regulate migratory polarity through Rho/ROCK signaling in T cells. *PLoS ONE* 5, e8774.
- Tapia, C., Kutzner, H., Mentzel, T., Savic, S., Baumhoer, D. and Glatz, K. (2006). Two mitosis-specific antibodies, MPM-2 and phospho-histone H3 (Ser28), allow rapid and precise determination of mitotic activity. *Am J Surg Pathol* 30, 83-9.
- Taylor, W. R., DePrimo, S. E., Agarwal, A., Agarwal, M. L., Schonthal, A. H., Katula, K. S. and Stark, G. R. (1999). Mechanisms of G2 arrest in response to overexpression of p53. *Mol Biol Cell* 10, 3607-22.
- Theesfeld, C. L., Irazoqui, J. E., Bloom, K. and Lew, D. J. (1999). The role of actin in spindle orientation changes during the *Saccharomyces cerevisiae* cell cycle. *J Cell Biol* 146, 1019-32.
- Thery, M. and Bornens, M. (2006). Cell shape and cell division. *Curr Opin Cell Biol* 18, 648-57.
- Tirian, L., Hlavanda, E., Olah, J., Horvath, I., Orosz, F., Szabo, B., Kovacs, J., Szabad, J. and Ovadi, J. (2003). TPPP/p25 promotes tubulin assemblies and blocks mitotic spindle formation. *Proc Natl Acad Sci U S A* 100, 13976-81.
- Toyoshima, F., Matsumura, S., Morimoto, H., Mitsushima, M. and Nishida, E. (2007). PtdIns(3,4,5)P3 regulates spindle orientation in adherent cells. *Dev Cell* 13, 796-811.
- Toyoshima, F., Moriguchi, T., Wada, A., Fukuda, M. and Nishida, E. (1998). Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G2 checkpoint. *EMBO J* 17, 2728-35.
- Toyoshima, F. and Nishida, E. (2007). Integrin-mediated adhesion orients the spindle parallel to the substratum in an EB1- and myosin X-dependent manner. *EMBO J* 26, 1487-98.
- Tsakiridis, T., Bergman, A., Somwar, R., Taha, C., Aktories, K., Cruz, T. F., Klip, A. and Downey, G. P. (1998). Actin filaments facilitate insulin activation of the src and collagen homologous/mitogen-activated protein kinase pathway leading to DNA synthesis and c-fos expression. *J Biol Chem* 273, 28322-31.
- Vadlamudi, R. K., Adam, L., Wang, R. A., Mandal, M., Nguyen, D., Sahin, A., Chernoff, J., Hung, M. C. and Kumar, R. (2000). Regulatable expression of p21-activated kinase-1

- promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. *J Biol Chem* 275, 36238-44.
- Varmark, H. (2004). Functional role of centrosomes in spindle assembly and organization. *J Cell Biochem* 91, 904-14.
- Vicente-Manzanares, M., Ma, X., Adelstein, R. S. and Horwitz, A. R. (2009). Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nat Rev Mol Cell Biol* 10, 778-90.
- Waga, S., Li, R. and Stillman, B. (1997). p53-induced p21 controls DNA replication. *Leukemia* 11 Suppl 3, 321-3.
- Wang, W., Eddy, R. and Condeelis, J. (2007). The cofilin pathway in breast cancer invasion and metastasis. *Nat Rev Cancer* 7, 429-40.
- Wang, Y. L. (1991). Dynamics of the cytoskeleton in live cells. *Curr Opin Cell Biol* 3, 27-32.
- Watanabe, N., Kato, T., Fujita, A., Ishizaki, T. and Narumiya, S. (1999). Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nat Cell Biol* 1, 136-43.
- Watanabe, S., Ando, Y., Yasuda, S., Hosoya, H., Watanabe, N., Ishizaki, T. and Narumiya, S. (2008). mDia2 induces the actin scaffold for the contractile ring and stabilizes its position during cytokinesis in NIH 3T3 cells. *Mol Biol Cell* 19, 2328-38.
- Welsh, C. F., Roovers, K., Villanueva, J., Liu, Y., Schwartz, M. A. and Assoian, R. K. (2001). Timing of cyclin D1 expression within G1 phase is controlled by Rho. *Nat Cell Biol* 3, 950-7.
- Wen, Y., Eng, C. H., Schmoranzner, J., Cabrera-Poch, N., Morris, E. J., Chen, M., Wallar, B. J., Alberts, A. S. and Gundersen, G. G. (2004). EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. *Nat Cell Biol* 6, 820-30.
- Werner, M., Munro, E. and Glotzer, M. (2007). Astral signals spatially bias cortical myosin recruitment to break symmetry and promote cytokinesis. *Curr Biol* 17, 1286-97.
- Wheeler, A. P. and Ridley, A. J. (2004). Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility. *Exp Cell Res* 301, 43-9.
- Woolner, S., O'Brien, L. L., Wiese, C. and Bement, W. M. (2008). Myosin-10 and actin filaments are essential for mitotic spindle function. *J Cell Biol* 182, 77-88.
- Wurm, F. M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol* 22, 1393-8.

- Xie, L. and Forer, A. (2008). Jasplakinolide, an actin stabilizing agent, alters anaphase chromosome movements in crane-fly spermatocytes. *Cell Motil Cytoskeleton* 65, 876-89.
- Yamamoto, M., Marui, N., Sakai, T., Morii, N., Kozaki, S., Ikai, K., Imamura, S. and Narumiya, S. (1993). ADP-ribosylation of the rhoA gene product by botulinum C3 exoenzyme causes Swiss 3T3 cells to accumulate in the G1 phase of the cell cycle. *Oncogene* 8, 1449-55.
- Yamashiro, S. and Matsumura, F. (1991). Mitosis-specific phosphorylation of caldesmon: possible molecular mechanism of cell rounding during mitosis. *Bioessays* 13, 563-8.
- Yamashiro, S., Totsukawa, G., Yamakita, Y., Sasaki, Y., Madaule, P., Ishizaki, T., Narumiya, S. and Matsumura, F. (2003). Citron kinase, a Rho-dependent kinase, induces di-phosphorylation of regulatory light chain of myosin II. *Mol Biol Cell* 14, 1745-56.
- Yamashiro, S., Yamakita, Y., Hosoya, H. and Matsumura, F. (1991). Phosphorylation of non-muscle caldesmon by p34cdc2 kinase during mitosis. *Nature* 349, 169-72.
- Yang, G., Houghtaling, B. R., Gaetz, J., Liu, J. Z., Danuser, G. and Kapoor, T. M. (2007). Architectural dynamics of the meiotic spindle revealed by single-fluorophore imaging. *Nat Cell Biol* 9, 1233-42.
- Yang, J., Bardes, E. S., Moore, J. D., Brennan, J., Powers, M. A. and Kornbluth, S. (1998). Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. *Genes Dev* 12, 2131-43.
- Yang, J., Winkler, K., Yoshida, M. and Kornbluth, S. (1999). Maintenance of G2 arrest in the *Xenopus* oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. *EMBO J* 18, 2174-83.
- Yang, N., Higuchi, O., Ohashi, K., Nagata, K., Wada, A., Kangawa, K., Nishida, E. and Mizuno, K. (1998). Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. *Nature* 393, 809-12.
- Yang, X., Yu, K., Hao, Y., Li, D. M., Stewart, R., Insogna, K. L. and Xu, T. (2004). LATS1 tumour suppressor affects cytokinesis by inhibiting LIMK1. *Nat Cell Biol* 6, 609-17.
- Yasuda, H., Kanda, K., Koiwa, H., Suenaga, K., Kidou, S. and Ejiri, S. (2005). Localization of actin filaments on mitotic apparatus in tobacco BY-2 cells. *Planta* 222, 118-29.
- Yasuda, S., Ocegüera-Yanez, F., Kato, T., Okamoto, M., Yonemura, S., Terada, Y., Ishizaki, T. and Narumiya, S. (2004). Cdc42 and mDia3 regulate microtubule attachment to kinetochores. *Nature* 428, 767-71.

- Yasuda, S., Taniguchi, H., Ocegüera-Yanez, F., Ando, Y., Watanabe, S., Monypenny, J. and Narumiya, S. (2006). An essential role of Cdc42-like GTPases in mitosis of HeLa cells. *FEBS Lett* 580, 3375-80.
- Yin, H., Pruyne, D., Huffaker, T. C. and Bretscher, A. (2000). Myosin V orientates the mitotic spindle in yeast. *Nature* 406, 1013-5.
- Yoshioka, K., Foletta, V., Bernard, O. and Itoh, K. (2003). A role for LIM kinase in cancer invasion. *Proc Natl Acad Sci U S A* 100, 7247-52.
- Yuce, O., Piekny, A. and Glotzer, M. (2005). An ECT2-centralspindlin complex regulates the localization and function of RhoA. *J Cell Biol* 170, 571-82.
- Zha, J., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S. J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* 87, 619-28.
- Zhang, Q., Magnusson, M. K. and Mosher, D. F. (1997). Lysophosphatidic acid and microtubule-destabilizing agents stimulate fibronectin matrix assembly through Rho-dependent actin stress fiber formation and cell contraction. *Mol Biol Cell* 8, 1415-25.
- Zhao, W. M. and Fang, G. (2005). MgcRacGAP controls the assembly of the contractile ring and the initiation of cytokinesis. *Proc Natl Acad Sci U S A* 102, 13158-63.
- Zhao, Z. S., Lim, J. P., Ng, Y. W., Lim, L. and Manser, E. (2005). The GIT-associated kinase PAK targets to the centrosome and regulates Aurora-A. *Mol Cell* 20, 237-49.
- Zhou, J., Yao, J. and Joshi, H. C. (2002). Attachment and tension in the spindle assembly checkpoint. *J Cell Sci* 115, 3547-55.
- Zhu, X., Ohtsubo, M., Bohmer, R. M., Roberts, J. M. and Assoian, R. K. (1996). Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2, and phosphorylation of the retinoblastoma protein. *J Cell Biol* 133, 391-403.
- Zieve, G. W., Turnbull, D., Mullins, J. M. and McIntosh, J. R. (1980). Production of large numbers of mitotic mammalian cells by use of the reversible microtubule inhibitor nocodazole. Nocodazole accumulated mitotic cells. *Exp Cell Res* 126, 397-405.

Related publications

1. Heng, Y.W. and Koh, C.G. 2010. Actin cytoskeleton dynamics and the cell division cycle. *Int J Biochem Cell Biol* 42(10): 1622-1633.
2. Heng, Y.W., Mina, T., Zhong, S., Lim, C.T. and Koh, C.G. 2011. Actin cytoskeleton signaling regulates astral microtubule organization and spindle orientation. *Manuscript in preparation.*