LIMK1 regulates centrosome integrity and mitotic spindle positioning

OU SIRONG
SCHOOL OF BIOLOGICAL SCIENCES
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### Abbreviations

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<td>AurkA</td>
<td>Aurora kinase A</td>
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<td>AurkB</td>
<td>Aurora kinase B</td>
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<tr>
<td>CAK</td>
<td>Cyclin dependent kinase activating kinase</td>
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<tr>
<td>CDC25</td>
<td>M-phase inducer phosphatase</td>
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<tr>
<td>Cdc42</td>
<td>Cell division control protein 42 homolog</td>
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<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
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<td>CEP192</td>
<td>Centrosomal protein 192kDa</td>
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<tr>
<td>Cip/Kip</td>
<td>CDK interacting protein/Kinase inhibitory protein</td>
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<tr>
<td>CKI</td>
<td>Cyclin dependent kinase inhibitor</td>
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<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
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<td>DMSO</td>
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<td>DYNC1I</td>
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<td>DYNC1LIs</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<td>LATS1</td>
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<td>Protein diaphanous homolog</td>
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<td>MTOC</td>
<td>Microtubule organizing center</td>
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<td>NEBD</td>
<td>Nuclear envelop breakdown</td>
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<td>Nuclear mitotic apparatus protein</td>
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<td>ROCK</td>
<td>Rho-associated coiled coil containing kinase</td>
</tr>
<tr>
<td>RZZ</td>
<td>Rod/Zw10/Zwilch complex</td>
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<tr>
<td>S-phase</td>
<td>Synthesis phase</td>
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<tr>
<td>Ser</td>
<td>Serine</td>
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<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
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<tr>
<td>Slik</td>
<td>Sterile 20 like kinase</td>
</tr>
<tr>
<td>SSH1</td>
<td>Slingshot1</td>
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<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>Thr</td>
<td>Threonine</td>
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<tr>
<td>TPPP/p25</td>
<td>Tubulin polymerization-promoting protein</td>
</tr>
<tr>
<td>TubGCP</td>
<td>Gamma complex associated tubulin protein</td>
</tr>
<tr>
<td>γ-Tubulin</td>
<td>Tubulin gamma-1 chain</td>
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</table>
Abstract

Centrosome is the primary microtubule organizing center (MTOC) in mammalian cells. Several different types of cancer are observed to contain abnormal centrosome number, suggesting that abnormal centrosome function and number may contribute to tumor development (Chan, 2011; D’Assoro et al., 2002). Therefore, it is of interest to study and understand the proteins and factors that are involved in regulating the function and number of centrosome.

The actin cytoskeleton is involved in several cellular functions, such as intracellular vesicular trafficking, cell migration, cell-cell/extracellular matrix adhesion and cell division (Heng and Koh, 2010; Sit and Manser, 2011). Several actin associated proteins are observed to localize at the mitotic spindle apparatus, suggesting that these proteins are involved in regulating the functions of spindle apparatus (Heng and Koh, 2010). LIM-kinase 1 (LIMK1) belongs to the LIM motif containing protein kinase (LIMK) family and regulates actin cytoskeleton dynamics through phosphorylation of Cofilin (Agnew et al., 1995; Scott and Olson, 2007). Cyclin dependent kinase 1 (CDK1) is reported to phosphorylate and activate LIMK1 during mitosis (Amano et al., 2002; Sumi et al., 2002). In addition, phosphorylated form of LIMK1 is observed to localize at mitotic spindle apparatus during M-phase, suggesting that LIMK1 is involved in regulating the function of mitotic spindle apparatus (Chakrabarti et al., 2007). Although earlier findings have identified the role of LIMK1 in regulating spindle orientation and cytokinesis, the exact role of the kinase at the mitotic centrosome is not fully explored (Kaji et al., 2008; Kaji et al., 2003; Yang et al., 2004b).

The primary aim of this study is to investigate the role of LIMK1 in mitosis, with an emphasis on its possible role in regulating centrosome integrity. We also set out to investigate and confirm the role of LIMK1 in regulating mitotic spindle orientation and positioning.
We observed that LIMK1-depleted metaphase cells formed multi-polar spindle and displayed defects in mitotic centrosome focusing. These observations suggest that the kinase is important for regulating mitotic centrosome integrity. In addition, LIMK1 depletion resulted in decrease accumulation of centrosomal proteins at spindle poles, suggesting that LIMK1 is important in regulating centrosomal protein localization at the spindle pole. From the rescue experiments that we had conducted, we discovered that both Cofilin and TPPP/p25, two well-studied substrates of LIMK1, did not function downstream of LIMK1 in regulating centrosome integrity. However, cytoplasmic dynein light intermediate chains 1 and 2 (DYNC1LI1 and DYNC1LI2) were able to rescue the defects observed in LIMK1-depleted cells, suggesting that both proteins function downstream of LIMK1 in regulating centrosome integrity. In addition, our data also suggests that DYNC1LI1 and DYNC1LI2 could potentially be novel substrates of LIMK1 in regulating centrosome integrity and centrosomal protein transportation. Based on the findings in the current study, we propose that LIMK1 could potentially phosphorylate DYNC1LI1 and DYNC1LI2. LIMK1-mediated phosphorylation of DYNC1LIs could potentially regulate the function(s) of cytoplasmic dynein and influence the transportation of centrosomal proteins to the mitotic centrosome for mitotic spindle pole integrity maintenance.

Lastly, we validated the role of LIMK1 in spindle orientation and positioning during mitosis. LIMK1 depletion increased the spindle angle and off-center positioning of the mitotic spindle apparatus, suggesting that LIMK1 is involved in regulating spindle orientation and positioning. The Rho-ROCK pathway functions upstream of LIMK1 in controlling spindle orientation and positioning as a phospho-mimic form of LIMK1 was able to rescue the defects in Rho- and ROCK-inhibited cells. LIMK1 regulates spindle orientation and positioning possibly via its activity on Cofilin and actin cytoskeleton. The findings in our current study suggest that LIMK1 is involved in maintaining spindle orientation and positioning by regulating the cortical actin network for stable astral microtubule attachment.
Chapter 1   Introduction

The actin cytoskeleton is one of the three cytoskeletal structures found in mammalian cell and is involved in several cellular functions. Several proteins, which are involved in regulating actin cytoskeleton dynamics, are reported to play a role in regulating cell cycle progression and associate with the mitotic spindle apparatus during M-phase (Chircop, 2014; Heng and Koh, 2010). Therefore, we are interested to decipher the functional links between the regulation of the actin cytoskeleton and cell cycle progression.

LIM motif containing protein kinase 1 (LIMK1), a kinase involved in regulating actin cytoskeleton, has been reported to regulate cell cycle progression (Arber et al., 1998; Davila et al., 2007; Higuchi et al., 1996). In addition, phosphorylated LIMK1 is observed to associate with mitotic centrosome, suggesting that the kinase plays a role in regulating the function of mitotic centrosome during M-phase (Sumi et al., 2006). Although several studies have showed that LIMK1 is localized at the mitotic centrosome, the function of the kinase on spindle poles is not fully understood (Chakrabarti et al., 2007; Sumi et al., 2006). Therefore, the primary aim of this study is to investigate the role of LIMK1 on the mitotic centrosome. In the following sections, we will introduce the key elements and proteins that are relevant to our study.

1.1   The cell cycle

The mammalian cell cycle consists of several highly regulated events where a mother cell gives rise to two daughter cells. Several external and internal stimuli trigger a series of signaling cascades to regulate the cell’s entry into and progression through the cell cycle. Defects in cell cycle regulation or progression have been linked to many cancer and developmental defects, highlighting the importance of
proper cell cycle regulation (Budirahardja and Gonczy, 2009; Hanahan and Weinberg, 2011).

1.1.1 Different stages of the cell cycle.

The cell cycle can be divided into interphase and mitotic phase (M-phase) (Figure 1-1). Interphase comprises Gap 1 (G1), Synthesis (S) and Gap 2 (G2) phases. At G1-phase, the cells integrate various extra-cellular and intra-cellular signals to determine if the environment is optimum for commitment into cell division. After making the decision, the cell begins to synthesize proteins that are needed for S-phase entry and completion. Chromosomes are replicated during S-phase and surveillance systems are present to ensure that each chromosome is replicated with high fidelity. The centrosome is also duplicated during S-phase and begins to mature for the preparation of M-phase. When chromosome replication is completed without error, cells enter into G2-phase. At this phase of the cell cycle, the cell synthesizes factors that prepare them for entering M-phase.

M-phase can be sub-divided into prophase, pro-metaphase, metaphase, anaphase, telophase and cytokinesis. During M-phase, the replicated genomic material is equally divided between the two daughter cells. At prophase, chromosomes condense and the nuclear envelope begins to breakdown. The centrosomes begin to migrate away from each other and initiate the formation of mitotic spindle apparatus. During pro-metaphase, microtubules radiate from the spindle poles and attach to specialized structure, known as kinetochores, on the chromosome (Rieder and Salmon, 1998). Once attached to the spindle microtubules radiating from both spindle poles, the chromosomes move towards the metaphase plate with the aid of microtubule motor proteins (Rieder and Salmon, 1994). At metaphase, the chromosomes congregate at the equatorial plane and spindle assembly checkpoint ensures the integrity of kinetochores attachment to spindle microtubules (Zhou et al., 2002). When all the criteria of spindle assembly checkpoint are met, the chromosomes begin to segregate and migrate towards the spindle poles. This
segregation process begins at anaphase and continues till telophase. At late
telophase, the nuclear envelope begins to assemble around the segregated
chromosomes to form two daughter nuclei. The two daughter nuclei are then
physically separated from each other during cytokinesis, thus giving rise to two cells.

![Figure 1-1. A typical mammalian cell cycle. A typical cell cycle consists of Gap 1 (G₁-phase), Synthesis (S-phase) and Gap 2 phases (G₂-phase). During these phases, the cell synthesizes proteins that are needed for progression through the different stages of cell cycle and entry into Mitosis (M-phase). In addition, the cell replicates its genomic material and centrosome during S-phase before progressing into M-phase. During M-phase, the replicated genomic material is segregated and the two daughter cells are physically separated during cytokinesis.](image)

1.1.2 Regulation of the cell cycle

Proper progression of the cell cycle is mainly regulated by cyclin-dependent kinases (CDKs) and their interacting partner, cyclins (Malumbres and Barbacid, 2005). The different combinations of cyclin/CDK complexes induce the phosphorylation of their respective substrates, thus triggering the necessary downstream signaling cascades for cell cycle progression. For G₁-phase progression, cyclin-D interacts
with CDK4 and CDK6, leading to the activation of the kinases. Active CDK4/6 triggers the expression of proteins that are required for G₁/S-phase transition and successful completion of S-phase. For example, active CDK4/6 phosphorylates the retinoblastoma protein (pRb) to prevent Rb from binding and inactivating E2F transcription factors (Harbour et al., 1999). E2F will then activate the transcription of cyclin-E and cyclin-A, which interact with CDK2 to form cyclin-E/CDK2 and cyclin-A/CDK2 complexes (Botz et al., 1996; Schulze et al., 1995). Cyclin-E/CDK2 complex regulates the successful transition into S-phase, and initiates DNA and centrosome duplications during S-phase. Once the DNA replication is initiated, cyclin-E is rapidly degraded by ubiquitin-mediated proteolysis (Singer et al., 1999; Strohmaier et al., 2001). Cyclin-A/CDK2 activity is crucial for regulating S-phase progression and S/G₂-phase transition. As the cells progress through G₂-phase, cyclin-A is degraded and cyclin-B begins to accumulate. Cyclin-B interacts with CDK1 to form the mitosis promoting factor (MPF). The activity of MPF is crucial for several events in G₂/M-phase transitions and during M-phase.

The activity of cyclin/CDK complexes is regulated by their phosphorylation status and its interaction with CDK inhibitors (CKIs). CDK7, Cyclin-H and menage a trois-1 (MAT1) assembly protein form a complex known as CDK activating kinase (CAK) (Kaldis, 1999). CAK phosphorylates and activates CDK1, CDK2, CDK4 and CDK6 (Lolli and Johnson, 2005). Activated CDKs phosphorylates CAK on residue serine 168 (Ser168) and threonine 170 (Thr170) to enhance CAK kinase activity (Garrett et al., 2001). CDKs-mediated phosphorylation of CAK is proposed to allow both kinases to form a positive feedback loop signaling to ensure continuous CDKs activation (Garrett et al., 2001).

Phosphorylation of CDKs does not always lead to its activation. For example, Wee1-like protein kinase (WEE1) and membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase (MYT1) phosphorylate CDK1 on threonine 14 (Thr14) and tyrosine 15 (Tyr15) residues ( Booher et al., 1997; Parker et al., 1992). Phosphorylation on Thr14 and Tyr15 maintains the cyclin-B/CDK1 complex in an inactive state. CDC25 family of phosphatase catalyzes the removal of Thr14 and
Tyr15 phosphorylation, leading to the activation of CDK1 (Strausfeld et al., 1991). Active CDK1 in turn phosphorylates CDC25 phosphatase and increase the phosphatase’s activity (Strausfeld et al., 1994). CDK1-mediated phosphorylation of CDC25 phosphatase allows both proteins to form a positive feedback loop signaling to ensure progression into M-phase.

Besides phosphorylation and dephosphorylation, CDK activity is negatively regulated by interacting with CDK inhibitors (CKIs). There are two families of CKI; INK4 and Cip/Kip families (Sherr and Roberts, 1999). The INK4 family of CKI consists of p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d}. They bind exclusively with CDK4 and CDK6 (Carnero and Hannon, 1998). Interaction with INK4 proteins prevents CDK4 and CDK6 from associating with cyclin-D, resulting in G1-phase arrest. The Cip/Kip family of CKI consists of p21\textsuperscript{Waf/Cip1}, p27\textsuperscript{Cip2} and p57\textsuperscript{Kip2}. They interact with a wider spectrum of cyclin/CDK complexes and inhibit their catalytic activities (Gu et al., 1993; Harper et al., 1993; Lee et al., 1995; Polyak et al., 1994). The protein levels and activities of CKIs are regulated by proteins responding to anti-mitogenic stimuli, cell-cell contact inhibition and DNA damages (Polyak et al., 1994; Sherr and Roberts, 1999).

### 1.2 The mammalian centrosome

The centrosome is the primary microtubule organizing center (MTOC) in mammalian cells. It helps to initiate microtubule polymerization and provides an anchorage point for microtubule filaments to radiate towards the cell periphery. These highly organized microtubules provide a platform for regulatory and motor proteins to regulate organelle positioning, vesicular transportation, and microtubule cytoskeleton dynamics. The centrosome is located close to the nucleus during interphase and its position in relation to the nucleus is tightly regulated during cell migration and polarity establishment (Etienne-Manneville, 2013; Hoon et al., 2014). The organization and function of the centrosome undergo dramatic changes as the cell progresses through the cell cycle. Several complex and interlinked signaling pathways regulate the replication and maturation of the centrosome as the cells progresses through cell
cycle. After maturation, the centrosomes arrange the microtubules into a highly complex and organized mitotic spindle apparatus for chromosome segregation during mitosis.

1.2.1 Centrosome structural organization

Interphase centrosome comprises of a mature and immature centriole known as mother and daughter centrioles, respectively (Bornens, 2002). Each centriole is composed of 9 microtubule triplets arranged in a 9-fold radial symmetry. The daughter centriole is the newly assembled centriole from the previous cell cycle. Sub-distal and distal appendages, such as Ninein and Centriolin, are attached to the mother centrioles (Bornens, 2002; Piel et al., 2000). These appendages function to nucleate microtubules to the centrosome. The mother and daughter centrioles are held together by several proteins, such as centrosomal NEK2-associated protein 1 (C-Nap1), Rootletin and CDK5RAP2 (Bahe et al., 2005; Barrera et al., 2010; Fry et al., 1998; Mayor et al., 2000). These proteins prevent pre-mature separation of the centrioles, which can lead to centrosome over-duplication. A proteinaceous matrix, known as pericentriolar material (PCM), surrounds the pair of centrioles. PCM contains numerous proteins, such as Pericentrin, and γ-Tubulin, that are important for the centrosome to perform its functions (Dictenberg et al., 1998; Doxsey et al., 1994; Takahashi et al., 2002; Zimmerman et al., 2004). γ-Tubulin, together with other proteins such as gamma complex associated tubulin protein (TubGCP) 2 and 3, are organized into a structure known as the γ-tubulin ring complex (γ-TuRC) (Murphy et al., 1998; Zheng et al., 1995). γ-TuRC and the appendages on mother centrioles contribute towards the centrosome’s ability to anchor the minus ends of microtubules to the centrosome.

1.2.2 The centrosome cycle

The centrosome cycle (Figure 1-2) describes the process in which the centrosome duplicates, matures and separates during the cell cycle (Bettencourt-Dias and Glover,
2007). In order for the daughter cells to inherit a centrosome each after cell division, the centrosome must duplicate only once per cell cycle. Abnormal centrosome numbers and mitotic spindle apparatus have been associated with genomic instability, which could potentially lead to the development of tumor (Jiang et al., 2003). Therefore, centrosome duplication and maturation are tightly synchronized with the cell cycle.

Although the details of centrosome duplication remains poorly understood, data from neonates and human cells have provided some insights into the key events involve in centrosome duplication (Bettencourt-Dias and Glover, 2007). Centrosome duplication begins with disengagement of the mother and daughter centrioles at the transition between mitotic exit and early G₁-phase. At this stage, both the mother and daughter centrioles are still held together in close proximity by interlinking proteins. At G₁/S-phase transition, the existing centrioles serve as a template for pro-centrioles assembly and elongation. The elongation process begins at S phase and is completed at late G₂-phase. The two centrosomes begin to mature and separate from each other to form the mitotic apparatus during G₂/M-phase transition. During centrosome maturation, numerous proteins are recruited to the centrosome to regulate the function and structural integrity of the centrosomes during mitosis (Lee and Rhee, 2011; Oshimori et al., 2006). Several cell cycle regulated kinases, such as CDK1 and NIMA-related kinase 2 (NEK2A), also regulate the separation of the two pairs of centrioles as the cell enters into M-phase (Wang et al., 2014).

CDK2 and Polo-like kinase (PLK) 4 are some of the key kinases identified to initiate the signaling cascade for centriole formation (Habedanck et al., 2005; Hinchcliffe and Sluder, 2002; Kleylein-Sohn et al., 2007). Cyclin-E/CDK2 phosphorylates nucleophosmin (NPM/B23), leading to the release of NPM/B23 from the centriole pair during G₁/S-phase (Okuda et al., 2000). Over-expression of a NPM/B23 mutant that is not phosphorylatable by CDK2 prevents disengagement of the mother and daughter centrioles, suggesting that NPM/B23 prevents pre-mature disengagement of centrioles (Okuda et al., 2000). It is believed that CDK2-mediated phosphorylation of NPM/B23 releases the protein from centrioles to allow other factors to be recruited
for centrosome duplication (Hinchcliffe and Sluder, 2001). In addition, CDK2 links the centrosome cycle with the cell cycle and limits the duplication of centrioles to once per cell cycle (Lange, 2002; Nigg, 2007).

**Figure 1-2. The centrosome cycle.** Centrosome duplication begins with partial disengagement of centrioles during G1-phase. As the cell enters into S-phase, centriole duplication is initiated and elongation follows soon after. The initiation of centriole duplication is probably triggered by cyclin-E/CDK2 or cyclin-A/CDK2 complex. PLK4 and its downstream effectors (boxed) are thought to regulate the centriole elongation process. Centriole elongation is completed by the end of G2-phase and is followed by centrosome maturation. During M-phase, the two centrosomes migrate away from each other and are segregated equally into two daughter cells.

PLK4 depletion has a negative impact on centrosome duplication, suggesting that PLK4 is crucial for this process (Habedanck et al., 2005). Centrosomal protein 192kDa (CEP192) and centrosomal protein 152kDa (CEP152) recruit PLK4 to the
site of pro-centriole formation (Kim et al., 2013; Sonnen et al., 2013). PLK4 then phosphorylates and recruits spindle assembly 6 homolog (hSAS6) to the site of pro-centriole formation (Kitagawa et al., 2009). Together with CEP135, centrosomal P4.1-associated protein (CPAP), POC1 centriolar protein A and B (POC1A and POC1B) and SCL/TAL1 interrupting locus (STIL), hSAS6 regulates the elongation of pro-centriole (Castiel et al., 2011; Kohlmaier et al., 2009; Lin et al., 2013; Schmidt et al., 2009; Venoux et al., 2013). During the elongation process, centriolar coiled-coil protein 110kDa (CP110) and its interacting partners act as a cap to regulate the length of the newly synthesized centrioles (Delgehyr et al., 2012; Kobayashi et al., 2011; Spektor et al., 2007; Tsang et al., 2008).

At late G₂ phase and G₂/M-phase transition, many proteins are recruited to the centrosome to increase its microtubule nucleation activity. This process is regulated by several kinases that are involved in regulating G₂/M-phase transition. For example, Polo-like kinase (PLK) 1 is important in regulating the accumulation of CEP192, CEP215, Neural precursor cell expressed developmentally down-regulated gene 1 (NEDD1), and Pericentrin (PCTN) at the centrosome (Haren et al., 2009; Lee and Rhee, 2011). These proteins act as a scaffold for the formation of additional γ-TuRC at the centrosome to increase the microtubule nucleation activity of the mitotic centrosome in M-phase (Fong et al., 2008; Gomez-Ferreria et al., 2007; Lee and Rhee, 2010; Zimmerman et al., 2004). PLK1 and CDK11 recruit Aurora kinase A (AurkA) to the centrosome at late G₂-phase. (Hanisch et al., 2006; Petretti et al., 2006). AurkA is important for recruiting proteins for centrosome maturation and spindle formation (Barr and Gergely, 2007; Lens et al., 2010; Terada et al., 2003). In addition, AurkA mediates the phosphorylation of Eg5; a kinesin related motor protein that is crucial for centrosome separation (Blangy et al., 1995; Giet et al., 1999).

### 1.3 Actin cytoskeleton

Actin microfilament is one of the three types of cytoskeleton found in mammalian cells. Actin microfilament and its associated proteins form the actin cytoskeleton
network. The actin cytoskeleton network is involved in many cellular processes, such as vesicle transport, cell migration, cell-cell/extracellular matrix adhesion and cell division (Chircop, 2014; Heng and Koh, 2010; Sit and Manser, 2011). Actin microfilament (F-actin) is composed of monomeric globular actin (G-actin) units. Each G-actin protein consists of a large and a small domain. A nucleotide binding region, which binds to either ATP or ADP, is located in the cleft between the large and small domains (Kabsch et al., 1990). During F-actin formation, G-actins orientate their cleft in the same direction and the ATP bound to G-actin is hydrolyzed by its ATPase domain to ADP. The end with the cleft is known as the “pointed end” and contains ADP bound form of G-actin (ADP-actin). In contrast, the opposite end is known as the “barbed end” and contains the ATP bound form of G-actin (ATP-actin). Although polymerization and de-polymerization can occur at both ends, there is a net elongation at the barbed end of F-actin.

1.3.1 Actin cytoskeleton and Rho-GTPase.

Actin polymerization and de-polymerization are regulated by Rho-GTPase proteins. These proteins help to translate extracellular signals into intra-cellular signaling cascades to regulate the actin cytoskeleton dynamics. Rho-GTPases regulate actin cytoskeleton dynamics by cycling between active (GTP bound form) and inactive (GDP bound form) states. The cycling between active and inactive states is regulated by three groups of proteins (Cherfils and Zeghouf, 2013). The guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP to GTP, resulting in the activation of Rho-GTPases. GTPase activating proteins (GAPs) help to activate the intrinsic GTPase activity of Rho-GTPase, leading to GTP hydrolysis. This hydrolysis event returns the activities of Rho-GTPases back to their basal levels. Guanine nucleotide dissociation inhibitors (GDIs) associate with GDP bound form of Rho-GTPases and prevent them from entering into the Rho-GTPase cycle.

RhoA, Cdc42 and Rac1 are the three most well characterized Rho-GTPases known to regulate actin cytoskeleton dynamics and remodeling (Sit and Manser, 2011).
These Rho-GTPases activate their respective downstream effectors to regulate actin cytoskeleton dynamics. For example, active RhoA activates mammalian diaphanous homologue (mDia) to induce actin polymerization and form long strands of actin microfilament (Watanabe et al., 1999). RhoA also activates Rho-associated coiled-coil containing protein kinase (ROCK), which phosphorylates and activates myosin (Amano et al., 1996; Kimura et al., 1996; Watanabe et al., 1999). ROCK-mediated activation of myosin will regulate actin microfilament cross-linking and acto-myosin contractility (Amano et al., 1996; Kimura et al., 1996). Rac1 activation leads to the formation of lamellipodia, a membrane protrusion needed for cell migration. Rac1 stimulates lamellipodia formation by activating Wiskott-Aldrich syndrome protein-family Verprolin-homologous (WAVE) family of proteins (Miki et al., 1998). Cdc42 activation leads to the formation of filopodia via its activity on Wiskott-Aldrich syndrome family of proteins (WASP) (Miki et al., 1996). Both WAVE and WASP proteins act as a scaffold to recruit proteins that are needed for actin polymerization and the formation of a dense meshwork of actin filaments in membrane extension (Takenawa and Suetsugu, 2007).

1.3.2 Actin cytoskeleton and Rho-GTPases in cell cycle

Previous studies on actin cytoskeleton and Rho-GTPases have focused on their roles in maintaining cell morphology, cell migration and cell-cell/ECM adhesion. However, recent findings have suggested that actin cytoskeleton and Rho-GTPases are involved in regulating various stages of cell cycle (Chircop, 2014; Heng and Koh, 2010). The state of actin organization within the cell has been shown to influence G1 phase progression and G1/S-phase transition. Treating cells with actin depolymerization toxin disrupts cyclin production, decreases Rb protein phosphorylation and up-regulates p27Kip1 (Huang and Ingber, 2002; Reshetnikova et al., 2000). The combined outcome of all these effects is G1-phase arrest and/or delay in G1/S-phase transition. In addition, disrupting actin polymerization or myosin activity prevents the activation of CDC25 phosphatase, thus resulting in a delay in G2/M transition (Lee and Song, 2007). Although more experimental data are needed, the above findings point to the possibility that a check-point exist to “sense” the
integrity of actin cytoskeleton before committing to the subsequent stages of the cell cycle.

In addition to its function in regulating actin cytoskeleton dynamics, Rho-GTPase signaling is shown to regulate cell cycle progression (Heng and Koh, 2010). For example, inhibition of RhoA slows down $p21^{\text{Waf/Cip1}}$ mRNA degradation and results in increase $p21^{\text{Waf/Cip1}}$ protein level (Coleman et al., 2006). The c-Jun N-terminal kinase (JNK) functions downstream of RhoA as inhibiting JNK abolishes the stabilization of $p21^{\text{Waf/Cip1}}$ mRNA induced by inhibition of RhoA (Coleman et al., 2006). The effect of RhoA on cell cycle progression is also mediated by some of its well-known downstream effectors (Croft and Olson, 2006; Mammoto et al., 2004). Ubiquitin dependent degradation of $p27^{\text{Kip1}}$ protein is modulated by the balance of mDia and ROCK activities (Mammoto et al., 2004). In addition, ROCK positively influences the activity of Ras/MAPK signaling to regulate cyclin-D expression (Croft and Olson, 2006). In contrast, ROCK-mediated phosphorylation and activation of LIM motif containing protein kinase 2 (LIMK2) is shown to influence the expression of cyclin-A (Croft and Olson, 2006). These observations highlight the importance of RhoA in regulating cell cycle progression.

The role of actin cytoskeleton and Rho-GTPases in mitosis was initially thought to be limited to cytokinesis (Piekny et al., 2005). However, increasing evidences from recent studies suggest that actin cytoskeleton and Rho-GTPases play a wider role in mitosis (Chircop, 2014; Narumiya and Yasuda, 2006). Interphase cells undergo dramatic actin cytoskeleton re-organization to achieve the characteristic rounded shape during mitosis. This remodeling process is mediated by RhoA-ROCK signaling pathway, as treatment with Rho or ROCK inhibitors disrupt the rounding process (Maddox and Burridge, 2003). RhoA-ROCK mediates cell rounding through its activity on myosin II motor protein (Sandquist et al., 2006). Myosin II slides along the cortical actin cytoskeleton, causing the cell periphery to retract and round up (Guha et al., 2005).
In addition to cell rounding, RhoA-ROCK is shown to be important for maintaining cortical rigidity. (Heng et al., 2012; Kunda et al., 2008; Maddox and Burridge, 2003). Proper cortical rigidity maintenance is thought to be important for proper mitotic spindle formation (Heng et al., 2012). ROCK has been shown to positively regulate Moesin function, suggesting that RhoA-ROCK signaling cooperates with Moesin to maintain cortical rigidity during mitosis (Hebert et al., 2008). Moesin and its activating kinase, Sterile20-like kinase (Slik), have been implicated in maintaining cortical rigidity (Carreno et al., 2008; Kunda et al., 2008). Inhibiting the function of Moesin or Slik by siRNA reduces cortical rigidity and leads to the formation of abnormal spindle apparatus (Carreno et al., 2008; Kunda et al., 2008). These observations highlight the importance of both proteins in regulating rigidity and spindle formation. Moesin is proposed to organize the cortical actin network during mitosis as RNAi-mediated depletion of Moesin and Slik have negative effects on cortical acto-myosin cytoskeleton organization (Carreno et al., 2008). An organized cortical acto-myosin network helps to maintain a rigid cell cortex and provide a stable platform for spindle positioning (Carreno et al., 2008; Kaji et al., 2008). In addition, Moesin also regulates the activity of myosin II to prevent excessive membrane blebbing, which results in abnormal mitotic spindle morphology (Carreno et al., 2008; Speck et al., 2003).

Centrosomes migrate apart from each other during the formation of mitotic spindle apparatus at the beginning of prophase. Cortical acto-myosin network and cortical actin dynamics are important for centrosome migration. Blocking myosin activity by blebbistatin or RNA interference of myosin disrupts centrosome separation, suggesting that myosin activity is important for centrosome migration (Rosenblatt et al., 2004). When cortical actin flow or dynamics is disrupted by actin cytoskeleton toxin or concanavalin A, centrosome separation is impaired or delayed (Cao et al., 2010; Rosenblatt et al., 2004). Impaired or delayed centrosome separation during M-phase leads to abnormal spindle morphology and results in abnormal chromosome segregation.

The activity of Cdc42 peaks during metaphase and declines back to basal level during telophase (Oceguera-Yanez and Narumiya, 2006). This observation suggests
that Cdc42 could be involved in regulating certain process during metaphase. RNAi-mediated knock-down of Cdc42 results in chromosome mis-alignment and activation of spindle assembly check-point, suggesting that Cdc42 is involved in regulating proper microtubule-kinetochore attachment (Yasuda et al., 2006). In agreement with the above observation, active Cdc42 positively regulates mDia3, a protein that is found to form a complex with centromere protein A (CENP-A) (Yasuda et al., 2004). The presence of Cdc42/mDia3/CENP-A complex at the kinetochore appears to be important for proper bi-oriented microtubule attachment of chromosome during chromosome alignment.

RhoA is crucial for successful cytokinesis completion, as disrupting RhoA signaling leads to defects in acto-myosin formation and cytokinesis failure (Chircop, 2014; Kamijo et al., 2006; Narumiya and Yasuda, 2006). The activity of RhoA is limited to the metaphase plate, the site where the formation of contractile ring for furrow ingression is initiated (Yuce et al., 2005). Interestingly, the activities of RhoA GEF (Ect2, GEF-H1, Myo-GEF) and GAP (MgcRacGAP, and p190RhoGAP) are regulated both spatially and temporally by CDK1, PLK1 and AurkB during cytokinesis (Chircop, 2014). Therefore, it is possible that RhoA GEF and GAP function synergistically in regulating the activity of RhoA for proper formation and contractility of the contractile ring. Besides regulating contractile ring formation, RhoA also regulates the contractility of acto-myosin network via Citron kinase- and ROCK-mediated phosphorylation of myosin light chain. Both kinases phosphorylate myosin light chain to control the constriction of membrane between the segregated chromosomes during cytokinesis (Piekny et al., 2005).

1.3.3 Rho-GTPases and centrosome cycle.

Rho-GTPase and its downstream effectors are implicated in centrosome duplication and maturation processes. RhoD, which is involved in regulating the endocytic pathway and actin cytoskeleton dynamics, is found to be involved in PLK4-induced centrosome duplication (Kyrkou et al., 2013). Over-expression of constitutively active
RhoD results in centrosome over-duplication, while dominant negative RhoD suppresses PLK4-induced centrosome over-duplication (Kyrkou et al., 2013). In addition, siRNA-mediated depletion of RhoA and RhoC disrupts centrosome duplication, suggesting that the Rho sub-family plays a role in regulating centrosome duplication (Kanai et al., 2010). RhoA and RhoC regulate centrosome duplication through ROCKII, which in turn positively activates nucleophosmin for centrosome duplication (Ma et al., 2006). Besides centrosome duplication, Rho GTPase signaling is proposed to be involved in regulating centrosome maturation. Treating cells with Clostridium difficile toxin B, a toxin known to inactivate all members of Rho GTPases, impairs AurkA activation (Ando et al., 2007). Impaired AurkA activation has a negative impact on centrosome maturation and function (Carmena and Earnshaw, 2003). PAK1 could be functioning downstream of Rho-GTPases in mediating AurkA activation as it is found to catalyze the phosphorylation of Serine 288 and Serine 342 residues on AurkA (Zhao et al., 2005). PAK1-mediated phosphorylation positively regulates the activity of AurkA at the centrosome and ensures proper mitotic spindle formation (Zhao et al., 2005).

1.4 LIM motif containing protein kinases (LIM-kinases)

LIM-kinase 1 (LIMK1) and LIM-kinase 2 (LIMK2) are two highly related kinases belonging to the LIM motif containing protein kinase (LIMK) family. Human LIMK1 and LIMK2 genes are located on chromosome 7 in the region of q11.23 and chromosome 22 in the region of q12.2, respectively (Martindale et al., 2000; Okano et al., 1995). The LIMK1 gene consists of 16 exons and alternate splicing generates two LIMK1 mRNAs species. One of the splice variants encodes a 647 amino acid long full length protein kinase. The second splice variant encodes for a catalytically inactive truncated protein. The LIMK2 gene also contains 16 exons and alternate splicing generates three LIMK2 mRNAs species. Different LIMK2 splice variants are reported to localize at different sub-cellular compartments and tissues, suggesting that there might be some regulatory or functional differences among these different splice variants (Ikebe et al., 1997; Ikebe et al., 1998; Osada et al., 1996).
1.4.1 Protein organization of LIM-kinases

Both LIMK1 and LIMK2 share similar protein domain organization and have a 50% overall amino acid sequence homology (Okano et al., 1995). Both LIM kinases contain two tandemly arranged amino-terminal LIM domains, followed by a PDZ domain and a protein kinase domain at the carboxyl-terminal (Figure 1-3). LIMK1 and 2 share about 50%, 46% and 70% amino acid sequence similarity at the LIM domain, PDZ domain, and protein kinase domain, respectively (Okano et al., 1995).

The LIM domains of LIMKs consist of conserved zinc-binding Cysteine and Histidine residues and are involved in regulating LIMK kinase activity (Nagata et al., 1999). In addition, the LIM domains are involved in regulating inter- and intra-molecular interactions between LIMKs and their interacting partners (Hiraoka et al., 1996; Yang et al., 2004b; Yokoo et al., 2003). The PDZ domain, together with the LIM domains, are shown to negatively regulate the kinase activity of LIMKs (Nagata et al., 1999). The PDZ domain of LIMK also contains Leucine rich nuclear export signal (NES) and nuclear localization signal (NLS) (Goyal et al., 2006; Yang et al., 1998; Yang and Mizuno, 1999). In addition to the NLS in the PDZ domain, LIMK2 contains an additional NLS in its kinase domain (Goyal et al., 2006). Although their functions in the nucleus are not fully understood, nuclear localization of LIMKs has been shown to regulate actin cytoskeleton dynamics and cell cycle progression (Goyal et al., 2005; Yokoo et al., 2003).

The protein kinase domains of LIMK1 and LIMK2 share the highest amino acid sequence homology. The kinase domains of both LIMKs are predicted to be dual specificity kinases that phosphorylate both Serine/Threonine and Tyrosine residues (Scott and Olson, 2007). Although several studies have suggested that LIMK is strictly a serine/threonine kinase, studies on LIMK auto-phosphorylation illustrate that it could also phosphorylate tyrosine residues (Nunoue et al., 1995; Okano et al., 1995; Proschel et al., 1995).
LIMK1 and 2 share many similarities in terms of structural organization, regulation and functions. For the purpose of this dissertation, we will be focusing our discussion on LIMK1.

Figure 1-3. Domain organization of LIMK1 and 2. Both LIMKs consist of two LIM domains, one PDZ domain, and a protein kinase domain. LIMK1 and 2 share an overall 50% amino acid homology (indicated by the black arrow). Both LIMKs share 50%, 46%, and 70% amino acid sequence similarity at their LIM, PDZ and protein kinase domain, respectively.

1.4.2 Activation and regulation of LIM-kinase 1 (LIMK1)

The kinase activity of LIMK1 is positively regulated by phosphorylation on its Threonine 508 (Thr508) residue, which is located in the activation loop (Ohashi et al., 2000). LIMK1 is phosphorylated and activated by Rho kinase (ROCK), a downstream effector of RhoA, on Thr508 (Ohashi et al., 2000). Besides ROCK, other protein kinases also positively regulate LIMK1 activity by phosphorylating Thr508. p21 activated kinase (PAK) 1, 2 and 4, downstream effectors of Cdc42 and Rac1, activate LIMK1 activity by phosphorylating Thr508 (Dan et al., 2001; Edwards et al., 1999; Misra et al., 2005; Wu et al., 2003). Myotonic dystrophy kinase-related Cdc42 binding kinase (MRCKα), a downstream effector of Cdc42, also activates LIMK1 by phosphorylating Thr508 (Sumi et al., 2001b). As LIMK1 is phosphorylated by many upstream kinases, many researchers have hypothesized that LIMK1 might serve to
integrate signals from various upstream pathways to regulate actin cytoskeleton dynamics (Figure 1-4) (Manetti, 2012; Scott and Olson, 2007).

In addition to Thr508 phosphorylation, phosphorylation on other residues on LIMK1 also modulates LIMK1 activity. VEGF stimulation leads to the activation of MAPKAPK2 and activated MAPKAPK2 phosphorylate LIMK1 at Serine 323 (Ser323) (Kobayashi et al., 2006). MAPKAPK2-mediated Ser323 phosphorylation increases LIMK1 activity and is important for VEGF-induced stress fiber formation and cell migration (Kobayashi et al., 2006). p38 mitogen-activated protein kinase (MAPK), an upstream kinase of MAPKAPK2, also phosphorylates LIMK1 at serine 310 (Ser310) during VEGF stimulations (Kobayashi et al., 2006). However, the functional significance of Ser310 phosphorylation is still unknown as it does not affect the kinase activity of LIMK1.

LIMK1 also undergoes auto-phosphorylation and trans-phosphorylation to upregulate its kinase activity. Auto-phosphorylation occurs exclusively on serine and tyrosine residues of LIMK1, leading many to speculate that LIMK1 may phosphorylates some still unknown substrates on tyrosine residues (Proschel et al., 1995). Surprisingly, T508V mutant undergoes auto-phosphorylation to similar extend as wild-type LIMK1, suggesting that Thr508 phosphorylation is not needed prior to any auto-phosphorylation (Kobayashi et al., 2006). However, the function of these auto-phosphorylation sites and how they regulate LIMK1 functions are still unknown. Trans-phosphorylation regulates LIMK1 by affecting its protein turnover rate and elevating its kinase activity. Heat shock protein 90 (Hsp90) is a molecular chaperone that protects protein from degradation and is involved in signal transduction (Nollen and Morimoto, 2002). Hsp90 interacts with LIMK1 and promotes the kinase to form homo-dimers for trans-phosphorylation (Li et al., 2006). Trans-phosphorylated LIMK1 protein has longer protein half-life and higher kinase activity (Li et al., 2006).
Figure 1-4. Summary of pathways regulating LIMK1. The activity of LIMK1 is positively regulated by phosphorylation and is catalyzed by the downstream effectors of Rho-GTPases. Slingshot 1 (SSH1) catalyzes the de-phosphorylation of LIMK1 and return the kinase activity of LIMK1 back to basal level.

The activity of LIMK1 can be regulated by intra-molecular interactions. LIM and PDZ domains are known to regulate protein-protein interactions and sub-cellular localization of LIMK1 (Lee-Hoeflich et al., 2004; Yang and Mizuno, 1999; Yokoo et al., 2003). In addition, LIM and PDZ domains are involved in regulating the activity of LIMK1 (Nagata et al., 1999). Deleting LIM and/or PDZ domains of LIMK1 resulted in increased kinase activity, suggesting that LIM and PDZ domains negatively regulate LIMK1 activity (Nagata et al., 1999; Tomiyoshi et al., 2004). The LIM and PDZ domains negatively regulates LIMK1 activity by binding to the kinase domain and hinder the kinase’s ability to phosphorylate its substrate(s) (Nagata et al., 1999).

Phosphorylation on LIMK1 is reversed by phosphatases; a family of proteins that removes phosphate group from their target substrates. Slingshot 1 (SSH1), initially shown to dephosphorylate Cofilin, interacts with LIMK1 and dephosphorylates Thr508 residue on the kinase (Soosairajah et al., 2005). In addition, several auto-phosphorylated serine residues on LIMK1 are shown to be dephosphorylated by...
SSH1 (Niwa et al., 2002; Soosairajah et al., 2005). SSH1-mediated dephosphorylation reduces LIMK1 kinase activity, resulting in a decrease in LIMK1-induced stress fiber formation. The phosphatase activity of SSH1 is inhibited when cells are treated with actin depolymerization drug, while the presence of F-actin enhances SSH1 phosphatase activity (Nagata-Ohashi et al., 2004; Soosairajah et al., 2005). These observations suggest that the activity of SSH1 and LIMK1 is directly regulated by actin cytoskeleton dynamics. Interestingly, SSH1 activity is also negatively regulated by PAK4 phosphorylation, suggesting that PAK4 activates LIMK1 through direct phosphorylation and regulation of SSH1 activity (Soosairajah et al., 2005).

Besides phosphorylation and dephosphorylation, the activity of LIMK1 can be regulated through sub-cellular localization. p57\textsuperscript{KIP2}, a cyclin dependent kinase inhibitor (CKI), binds to LIMK1 and promotes its translocation into the nucleus (Yokoo et al., 2003). Ectopic expression of p57\textsuperscript{KIP2}, but not a related CKI p21\textsuperscript{KIP1}, results in a reduction of LIMK1-induced stress fiber formation (Yokoo et al., 2003). As p57\textsuperscript{KIP2} binding to LIMK1 did not alter its kinase activity, the finding suggests that LIMK1 activity can be regulated by changing its sub-cellular localization.

LIMK1 activity is also regulated by modulating its protein level. Rnf6, a RING finger E3 ligases, catalyzes the poly-ubiquitination of LIMK1 and promotes the kinase’s degradation by proteasome during axon development (Tursun et al., 2005). miR-134, a brain specific microRNA, blocks LIMK1 mRNA translation during synapse development (Schratt et al., 2006). Although these mechanisms of regulating LIMK1 are not yet identified in other cell types, they suggest that additional means are involved in regulating LIMK1 activity.
1.4.3 Biological function of LIMK1

LIMK1 regulates actin dynamics that is important in many cellular processes such as cell migration, differentiation, apoptosis and membrane vesicle transportation. LIMK1 activation leads to the phosphorylation and inactivation of Cofilin at Serine 3 (Ser3) (Agnew et al., 1995; Arber et al., 1998). Cofilin depolymerizes actin filaments to maintain the G-actin pool for actin polymerization at other sites (Bravo-Cordero et al., 2013; Pavlov et al., 2007). Cofilin also severs F-actin to generate a de novo actin nucleation site for actin filament branching needed for leading edge protrusion (Bravo-Cordero et al., 2013; Pavlov et al., 2007). Phosphorylation of Cofilin inhibits its ability to sever F-actin, leading to increase stress fiber formation and decrease leading edge protrusion (Arber et al., 1998; Bravo-Cordero et al., 2013).

Cell migration is a complex process and requires rapid changes to the actin cytoskeleton network. When stimulated with chemotactic factors, cells form actin rich structures, known as lamellipodia, in the direction of movement. The formation of lamellipodia requires a balance between actin polymerization and depolymerization. LIMK1 is implicated in the initial stages of lamellipodia formation (Nishita et al., 2005). LIMK1 is important for actin re-organization during chemotaxis as knocking down LIMK1 results in a loss of lamellipodia formation upon chemotactic stimulation (Nishita et al., 2005). The authors of the study propose that the loss of LIMK1 results in higher Cofilin activity and this increase in activity shifts the balance towards actin depolymerization. Over-expression of constitutively active LIMK1 results in multiple lamellipodia formation, leading to non-directional migration (Dawe et al., 2003). This defect could be due to over-stabilization of actin filament, resulting in persistent presence of lamellipodia. SSH1, a phosphatase of LIMK1 and Cofilin, is found to localize at the lamellipodia and is important in maintaining directional cell migration (Nagata-Ohashi et al., 2004; Nishita et al., 2005). RNAi-mediated SSH1 knock-down results in the loss of directional lamellipodia formation, a phenotype similar to over-expression of constitutively active LIMK1 in migrating cells (Nishita et al., 2005). These findings suggest that the activity of LIMK1 is highly regulated spatially to generate directional migration. This finding also highlights the importance of
regulating the activity of both SSH1 and LIMK1 spatially and temporally for directional cell migration.

Neuronal differentiation requires actin cytoskeleton architecture remodeling for neurite outgrowth, axon extension and synapse maturation (Song and Poo, 2001). LIMK1 is shown to be involved in neuronal differentiation via its ability to regulate actin cytoskeleton. limk1 knock-out mice have abnormal dendrite and growth cone morphology and these defects are more pronounced in limk1limk2 double knock-out mouse (Meng et al., 2004; Meng et al., 2002). These knock-out mouse models indicate that LIMK1 is important for brain development. Furthermore, LIMK1 and Cofilin are highly expressed in the central nervous system during embryo development, highlighting their importance in neuronal differentiation (Bamburg and Bray, 1987; Bernard et al., 1994; Mori et al., 1997). The first stage of neurite outgrowth involves the formation of actin rich lamellipodia and filopodia, which are membrane protrusions crucial for sensing attractive and repulsive cues (Kalil and Dent, 2005). LIMK1-Cofilin pathway regulates actin polymerization for the formation of filopodia and lamellipodia during neurite outgrowth and neurite extension (Sarmiere and Bamburg, 2004; Tojima et al., 2003). In addition, over-expression of either LIM or PDZ domain of LIMK1 suppresses neurite extension (Birkenfeld et al., 2001). This finding further highlights the importance of LIMK1 activity in axon growth. At later stages of neuronal differentiation, LIMK1 negatively regulates growth cone and neurite extension for fine tuning the synaptic connections (Aizawa et al., 2001; Endo et al., 2003). The positive and negative effects of LIMK1 on neurite outgrowth are regulated by factors, such as bone morphogenetic protein 7 (BMP7) and Semaphorin3A (Aizawa et al., 2001; Lee-Hoeftich et al., 2004). LIMK1 is also found to be involved in synaptic maturation and regulation of neurotransmitter release, as LIMK1 deficient mice have smaller synapse and enhanced neurotransmitter release (Meng et al., 2004; Meng et al., 2002; Sarmiere and Bamburg, 2002).

In addition to its role in cell migration and neurite development, LIMK1 has been shown to be involved in endocytosis and apoptosis. The movement of endosomes is sensitive to both actin polymerizing and depolymerizing drugs, suggesting that actin
cytoskeleton dynamics is important for endosome trafficking (Cordonnier et al., 2001). Over-expression of LIMK1 results in reduced internalization of epidermal growth factor (EGF) bound EGF receptors (EGFR) and their transportation to the late endosomes (Nishimura et al., 2004). These defects are rescued by introducing kinase dead LIMK1 mutant or non-phosphorylatable form of Cofilin (Nishimura et al., 2006). This finding suggests that LIMK1-Cofilin pathway mediated regulation of actin cytoskeleton dynamics is involved in endosomes trafficking.

Apoptotic cells form membrane blebs and this process requires actin cytoskeleton remodeling. Knocking down LIMK1 prevents the formation of membrane blebs, suggesting that LIMK1 activity is important during the process of apoptosis (Tomiyoshi et al., 2004). Caspase 3, a key enzyme for initiating apoptosis, is reported to cleave LIMK1 during apoptosis (Porter and Janicke, 1999; Tomiyoshi et al., 2004). Caspase 3-mediated cleavage creates a constitutively active LIMK1 fragment that is involved in membrane bleb formation (Tomiyoshi et al., 2004).

1.4.4 Microtubule cytoskeleton and LIMK1

Microtubules are involved in numerous processes, such as cell migration, mitosis, organelle positioning, and membrane vesicles transportation. Microtubules are assembled from proto-filaments, which are composed of repeating units of α- and β-Tubulin (Nogales et al., 1999). Similar to the actin filament, the microtubule cytoskeleton has a rapidly growing “plus end” and a slower growing “minus end”. The faster growing ends contain β-Tubulin, while the slower growing ends contain α-Tubulin. Microtubule constantly switches between phases of growth and shrinkage; a process known as dynamic instability (Matov et al., 2010). The transition from growth to shrinkage is known as catastrophe, while the transition from shrinkage to growth is known as rescue (Mitchison and Kirschner, 1984; Walker et al., 1988).
The signaling pathways regulating actin and microtubule cytoskeleton dynamics are highly inter-connected. Treating cells with microtubule destabilizing drugs leads to actin stress fiber formation (Danowski, 1989). In contrast, Taxol, a microtubule stabilizing drug, induces actin stress fiber disassembly (Danowski, 1989). In addition, the RhoA-ROCK pathway plays a role in linking actin and microtubule dynamics. Disrupting either RhoA or ROCK signaling cascades leads to the formation of thick microtubule bundles, but at the same time results in actin depolymerization (Gao et al., 2004). This observation suggests that RhoA-ROCK activation causes actin polymerization and microtubule depolymerization. LIMK1, a downstream effector of RhoA-ROCK signaling pathway, aids in coordinating actin polymerization and microtubule depolymerization. LIMK1 is reported to localize to the microtubules and interacts with tubulin through its PDZ domain (Gorovoy et al., 2005). RNAi-mediated LIMK1 knockdown inhibits thrombin-induced microtubule destabilization, suggesting that LIMK1 is involved in regulating microtubule dynamics (Gorovoy et al., 2005). Over-expression of wild-type LIMK1 leads to microtubule destabilization and this effect is reversed when cells are pre-treated with Y-27632, a ROCK inhibitor (Gorovoy et al., 2005). This observation suggests that LIMK1 functions downstream of ROCK to regulate microtubule dynamics.

Over-expressing kinase dead LIMK1 mutant (LIMK1-D460A) does not induce microtubule destabilization, suggesting the presence of a downstream substrate in regulating microtubule (Gorovoy et al., 2005). Tubulin polymerization promoting protein (TPPP/p25) is found to be a substrate of LIMK1 in regulating microtubule (Acevedo et al., 2007). TPPP/p25 is first isolated and characterized in brain extracts and is discovered to interact with microtubules (Hlavanda et al., 2002; Takahashi et al., 1991). TPPP/p25 induces microtubule polymerization in-vitro and promotes microtubule bundling (Hlavanda et al., 2002). In addition, TPPP/p25 helps to stabilize microtubule structures by regulating the acetylation status of tubulin (Hlavanda et al., 2002; Tokesi et al., 2010). The activity of TPPP/p25 is regulated by phosphorylation and LIMK1 is one of the many kinases identified to phosphorylate TPPP/p25 (Acevedo et al., 2007; Hlavanda et al., 2007). LIMK1-mediated phosphorylation of TPPP/p25 inactivates its ability to promote microtubule polymerization (Acevedo et al., 2007). Given that LIMK1 also regulates actin cytoskeleton dynamics, the findings
suggest that LIMK1 may coordinate both actin and microtubule dynamics in processes, such as cell migration, that involve both actin and microtubule cytoskeleton (Figure 1-5).

**Figure 1-5. LIMK1 regulates actin and microtubule cytoskeleton dynamics.** LIMK1 regulates actin and microtubule dynamics via Cofilin and TPPP/p25, respectively. LIMK1-mediated phosphorylation of Cofilin and TPPP/p25 results in the inactivation of both proteins. Inactivation of Cofilin and TPPP/p25 result in actin polymerization and microtubule de-polymerization, respectively.

### 1.4.5 The role of LIMK1 in cell cycle and mitosis

LIMK1 is implicated in regulating cell cycle progression and many events during mitosis. LIMK1 protein is highly expressed in actively dividing cells, but not in serum starved and growth arrested cells (Higuchi et al., 1996). These data suggest that LIMK1 expression is growth phase dependent and could be involved in cell cycle regulation. Although partial depletion of LIMK1 results in cell cycle arrest at G2/M phase, other studies have shown that over-expression of wild-type LIMK1 results in growth inhibition (Davila et al., 2003; Higuchi et al., 1996). These findings point to the
possibility that the activity of LIMK1 needs to be tightly regulated for proper cell cycle progression. LIMK1 could regulate cell cycle progression through its activity on actin cytoskeleton. LIMK1 translocates to the nucleus and this translocation is regulated by p57KIP2, a member of the CKI (Vlachos and Joseph, 2009; Yokoo et al., 2003). Once in the nucleus, it is possible that LIMK1 then regulate the expression of genes important for cell cycle progression. Recent data seem to support this hypothesis as cAMP response element-binding protein (CREB) and Nuclear receptor subfamily 4 group A member (NURR1) transcription factors are shown to be substrates of LIMK1 (Sacchetti et al., 2006; Yang et al., 2004a).

LIMK1 is hyper-phosphorylated and thus hyper-activated during mitosis (Sumi et al., 2002). The phosphorylation is reported to be dependent on CDK1, suggesting that LIMK1 activity is likely to be linked to mitosis (Amano et al., 2002; Sumi et al., 2002). This hypothesis is supported by findings, which illustrate the involvement of LIMK1 in mitotic spindle formation and cytokinesis (Amano et al., 2002; Kaji et al., 2008; Yang et al., 2004b). Stable astral microtubule interaction with the cortical actin is important for accurate mitotic spindle positioning and orientation. The astral microtubule-cortical actin interaction requires a stable cortical actin network (Kaji et al., 2008). LIMK1 has been implicated in regulating cortical actin stability during mitosis.

Depletion of LIMK1 causes higher Cofilin activity, which disrupts cortical actin and astral microtubule organization (Kaji et al., 2008). This disruption in turn leads to mitotic spindle mis-orientation and off-center spindle positioning.

LIMK1 localizes to the cleavage furrow site (Sumi et al., 2006; Yang et al., 2004b). Over-expression of LIMK1 causes the formation of multinucleated cells suggesting a defective cytokinesis (Amano et al., 2002). This observation also suggests that LIMK1 activity needs to be tightly modulated for the completion of cytokinesis. This conclusion is supported by the discovery of a LIMK1 inhibitor, LATS1, at the cleavage furrow. Co-expression of LIMK1 and LATS1 reversed the cytokinesis defects observed in LIMK1 over-expressing cells (Yang et al., 2004b). In addition, a balance between LIMK1-mediated inactivation and SSH1-mediated activation of Cofilin at the cleavage furrow is important for cytokinesis (Kaji et al., 2003). LIMK1-
mediated inactivation of Cofilin could be required for the formation acto-myosin contractile ring. At the end of cytokinesis, LIMK1 needs to be inactivated for Cofilin to depolymerize and disassemble the acto-myosin ring.

Recently, LIMK1 and AurkA are reported to function together for proper spindle formation (Ritchey et al., 2012). RNAi-mediated depletion of LIMK1 results in abnormal spindle morphology, suggesting that LIMK1 could be involved in spindle formation (Ritchey et al., 2012). AurkA phosphorylates LIMK1 on residue Serine 307 (Ser307) and this phosphorylation event is important for LIMK1 localization to the mitotic centrosome (Ritchey et al., 2012). Interestingly, AurkA is reported to be a substrate of LIMK1 and RNAi-mediated depletion of LIMK1 reduces phospho-AurkA levels (Ritchey et al., 2012). Although further experimental evidences are needed, it is reasonable to speculate that LIMK1 and AurkA form a positive feedback loop during mitosis for proper mitotic spindle formation.

1.5 Cytoplasmic dynein

Dynein is a multi-subunit motor protein and is responsible for minus-end directed intra-cellular transport. There are three major forms of dynein in the mammalian cells: axonemal dynein, cytoplasmic dynein 1 and cytoplasmic dynein 2. Axonemal dynein is found exclusively in cilia and flagella, and are mainly involved in coordinating the “beating motion” of both cilia and flagella. Cytoplasmic dynein 2 is also found in cilia and flagella, and is involved in cargo/protein transportation within cilia and flagella structure. Cytoplasmic dynein 1 (commonly known as cytoplasmic dynein) is the major form of cytoplasmic dynein found in mammalian cells and is ubiquitously expressed. Cytoplasmic dynein 1 is involved in minus-end directed transportation of various cargoes/proteins, organelle positioning, cell migration and mitotic spindle assembly (Allan, 2011; Kardon and Vale, 2009). In this study, we will be focusing our discussion on the role of cytoplasmic dynein 1 in M-phase and it is referred to as “cytoplasmic dynein” or “dynein” throughout this manuscript.
1.5.1 Cytoplasmic dynein 1 subunits

Cytoplasmic dynein heavy chain (DYNC1H) is the largest subunit of the dynein complex and comprises a tail and a motor domain. The motor domain is responsible for hydrolyzing ATP to generate energy for dynein movement along the microtubules. The motor domain also mediates dynein interaction with the microtubule as it contains a microtubule-binding domain. Dynein heavy chain forms homo-dimers through its tail domain to serve as a scaffold for other subunits to assemble around it. Dynein intermediate chains (DYNC1I) and light intermediate chains (DYNC1LI) interact directly with the heavy chain tail domain, while dynein light chains assemble onto intermediate chains. Like the dynein heavy chains, these subunits also form dimers before assembling onto the dynein heavy chains.

The cytoplasmic dynein intermediate chain 1 and 2 (DYNC1I1 and DYNC1I2, respectively) are the two types of dynein intermediate chains identified in mammalian cells to date. Each of these intermediate chains have several isoforms due to alternate splicing of their respective genes (Vaughan and Vallee, 1995). Some DYNC1I1 and 2 isoforms displays tissue specific expression, leading some to hypothesize that these isoforms target the dynein to specific cargoes (Kuta et al., 2010). Indeed, DYNC1I1B isoform containing dynein complex is shown to specifically transport tropomyosin receptor kinase (TrkB) positive endosome in neuronal cells (Ha et al., 2008). In addition to binding to specific cargo, DYNC1I1 is shown to interact with p150Glued, a component of the dynactin complex (Vaughan and Vallee, 1995). The dynactin complex in turn helps to stabilize dynein interaction with the microtubules and enhance dynein processivity (King and Schroer, 2000).

The dynein light intermediate chains consist of three members, DYNC1LI1, DYNC1LI2 and DYNC2LI1. The DYNC2LI1 is the main light intermediate chain found in cytoplasmic dynein 2, which is involved in Golgi organization and intra-flagella transports (Grissom et al., 2002; Pazour et al., 1999; Porter et al., 1999). DYNC1LI1 and DYNC1LI2 forms homo-dimers and interact with cytoplasmic dynein 1 motor
complex in a mutually exclusive manner (Tynan et al., 2000). This mutually exclusive association with the dynein motor complex has an impact on the cargo transported by the microtubule motor complex. For instance, dynein containing DYNC1LI1 interacts with and mediates Pericentrin transportation to the centrosome, a process that is important for spindle formation (Purohit et al., 1999; Tynan et al., 2000). In contrast, only DYNC1LI2 isoform containing dynein interacts with Partitioning defective protein 3 (Par-3) to control centrosome positioning during cell migration (Schmoranzer et al., 2009). In addition, RNAi-mediated depletion of DYNC1LI1, but not DYNC1LI2, affects endoplasmic reticulum-to-Golgi complex transportation (Palmer et al., 2009). In contrast, depletion of DYNC1LI2, but not DYNC1LI1, affects endosomal vesicular transportation (Palmer et al., 2009). These observations further confirm the possibility that dynein containing different members or isoforms of its constituent subunits could be involved in specific dynein-dependent cellular processes.

There are three distinct type of dynein light chains, DYNLT (commonly known as TeTex/RP3), DYNLRB (commonly known as Roadblock/LC7), and DYNLL (commonly known as LC8). Each of these dynein light chains consists of several members and the expression levels of some of these members are found to be tissue specific (King et al., 1998). Dynein light chains are able to mediate cargo interactions and define the proteins/molecules transported by dynein. For instance, dynein containing DYNLT1 (commonly known as TeTex1) specifically interacts with and transports rhodopsin, while dynein containing DYNLT3 (commonly known as RP3) binds to mitotic checkpoint protein Bub3 (Lo et al., 2007a; Tai et al., 1999). These observations suggest that dynein light chains are also important for determining cargo specificity. Unlike DYNC1I and DYNC1LI, some members of the dynein light chains are capable of forming heterodimers (Lo et al., 2007b). Interestingly, not all combination of dynein light chain dimers are capable of being incorporated into the dynein motor complex. For instance, DYNLT type of light chain can form homo-dimers and heterodimers but only the homo-dimers are able to interact with dynein intermediate chains (Lo et al., 2007b). Therefore, further studies are needed to identify the role of light chains in dynein motor complex assembly and regulation of dynein function.
1.5.2 Cytoplasmic dynein 1 function in Mitosis

Cytoplasmic dynein is released from its interphase cargoes and recruited to various locations during the onset of mitosis (Roberts et al., 2013). This localization pattern of cytoplasmic dynein 1 suggests that it can perform multiple roles during mitosis. For instance, dynein-dynactin complexes are shown to localize at the nuclear envelope immediately before the onset of nuclear envelope break-down (NEBD), suggesting that dynein is involved in this process (Busson et al., 1998). Indeed, over-expression of p65 dynactin subunit, which disrupts dynein interaction with the nuclear envelope, prolongs the time taken for NEBD to complete (Salina et al., 2002). RNAi-mediated depletion of Bicaudal-D2 and nudE neurodevelopment protein 1-like 1 (NDEL1) reduce dynein-dynactin recruitment to the nuclear envelope, resulting in prolong NEBD (Hebbar et al., 2008; Splinter et al., 2010). These observations highlight the importance of dynein for proper NEBD.

Duplicated centrosomes migrate apart for the formation of mitotic spindle apparatus and cytoplasmic dynein is implicated in this process. The mitotic centrosomes of Drosophila embryo carrying a dysfunctional dynein heavy chain mutant do not move apart from each other (Robinson et al., 1999). In addition, disrupting dynein function either through RNAi-mediated depletion or microinjection of inhibiting antibodies abolishes centrosome separation, leading to failure in bipolar spindle formation (Gonczy et al., 1999; Vaisberg et al., 1993). These observations suggest that cytoplasmic dynein is involved in centrosome separation during the onset of mitosis. It is possible that cytoplasmic dynein, that are localized to the front of the migrating centrosome and anchored on the nuclear envelop, pulls the centrosome towards itself through its minus-end directed movement. This pulling force will help in the initial step of centrosome separation at the onset of mitosis.

Rod/Zw10/Zwilch (RZZ) and Gαi-LGN-NuMA complexes mediate the recruitment of cytoplasmic dynein to the kinetochores and cell cortex, respectively (Gassmann et al., 2008; Woodard et al., 2010). Dynein at the kinetochores participate in regulating
microtubule-kinetochore attachment, mitotic chromosome motion, and spindle assembly checkpoint signaling (Howell et al., 2001; Varma et al., 2008; Yang et al., 2007). Ran-GTP gradient from the mitotic chromosome and PLK1 at the centrosomes regulate the cortical localization of Gαi-LGN-NuMA complex (Kiyomitsu and Cheeseman, 2012; Kotak et al., 2012). This will in turn lead to the recruitment of dynein-dynactin complex to the cell cortex. Through its minus-end directed microtubule movement, cortical dynein pulls the mitotic spindle apparatus and helps to position it at the center of the cells (McNally, 2013). Besides the kinetochores and cell cortex, cytoplasmic dynein is also found to localize at the mitotic spindle poles, suggesting a possible role at the mitotic centrosome. Indeed, cytoplasmic dynein have been implicated in transporting factors/proteins, such as Pericentrin, γ-Tubulin, and Nuclear mitotic apparatus protein (NuMA), to the centrosome (Merdes et al., 2000; Young et al., 2000). These proteins will in turn regulate centrosome maturation and mitotic spindle formation. In addition, dynein-dynactin complex helps to focus and anchor the mitotic microtubule at the spindle poles (Quintyne et al., 1999).

1.6 Aim of the project

Centrosome is the primary microtubule organizing center (MTOC) in mammalian cells and is involved in regulating organelle positioning, vesicular transportation, and mitotic spindle formation. Abnormal centrosome function and numbers have been found to be contributing factors for tumor development (D’Assoro et al., 2002). This hypothesis is supported by clinical studies which reported abnormal centrosome numbers in numerous cancer types (Chan, 2011). Therefore, it is of interest to study and understand the proteins and factors that regulate the function and number of centrosome in the cells.

LIM-kinase 1 (LIMK1) is one of the many proteins identified to be involved in regulating actin cytoskeleton dynamics (Agnew et al., 1995; Scott and Olson, 2007). Although previous studies have focused on the role of LIMK1 in interphase actin cytoskeleton regulation, recent studies have suggested that LIMK1 is involved in
regulating M-phase events. CDK1 is shown to phosphorylate and activate LIMK1 during mitosis (Amano et al., 2002; Sumi et al., 2002). In addition, phosphorylated form of LIMK1 localizes to the mitotic spindle apparatus during M-phase (Chakrabarti et al., 2007). These findings suggest that LIMK1 could be involved in regulating the function of mitotic spindle apparatus. We are keen to explore the functional links between the regulation of the actin cytoskeleton and the control of the cell cycle. Since LIMK1 is actively involved in regulating actin dynamics in the cells and is implicated in cell cycle related events, it is a potential molecule that can link these two processes.

Although earlier studies have explored the role of LIMK1 in cytokinesis, the function of LIMK1 on the spindle apparatus is not fully understood. Based on previous observations that silencing LIMK1 led to multi-polar spindle and defocused centrosomes, we hypothesize that LIMK1 could regulate centrosome integrity and proper spindle formation (Heng et al., 2012). In this study, we set out to investigate the role(s) of LIMK1 in regulating the function of M-phase spindle apparatus.

The specific aims of this project are summarized below:

1. To explore the role of LIMK1 in regulating the function of mitotic centrosome.

2. To identify potential downstream effectors of LIMK1 involved in regulating the function of M-phase spindle apparatus.

3. To uncover additional function of LIMK1 during mitosis, with an emphasis on its role in regulating mitotic spindle apparatus orientation and positioning. We focused on these two aspects as earlier studies suggest that cortical actin cytoskeleton dynamics is important for spindle orientation and positioning (Kaji et al., 2008).

4. To discover novel substrates of LIMK1 in M-phase using SILAC based mass spectrometry analysis.
Chapter 2    Material and Methods

2.1     Materials

2.1.1     Chemicals and Reagents

<table>
<thead>
<tr>
<th>Name of chemical and reagents</th>
<th>Company</th>
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<tbody>
<tr>
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<td>Bio-Rad</td>
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<tr>
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<td>Bisacrylamide</td>
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<td>Hydrochloric acid</td>
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<td>Invitrogen</td>
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<td>Tween 20</td>
<td>Bio-Rad</td>
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<tr>
<td>Vectashield® with DAPI</td>
<td>Vector Labs</td>
</tr>
<tr>
<td>Y27632 (ROCK inhibitor)</td>
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2.1.2 Primary Antibodies

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<tr>
<th>Antibody</th>
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<th>Company</th>
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<tr>
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<tr>
<td>Anti-Actin</td>
<td>mouse monoclonal</td>
<td>Chemicon International</td>
</tr>
<tr>
<td>Antibody Name</td>
<td>Type</td>
<td>Vendor</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
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<td>rabbit polyclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-Centrin 3</td>
<td>mouse monoclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Anti-Cofilin</td>
<td>mouse monoclonal</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-phospho-Cofilin (Ser3)</td>
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<td>Abcam</td>
</tr>
<tr>
<td>Anti-Cyclin A</td>
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</tr>
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<td>Anti-Cyclin D1</td>
<td>mouse polyclonal</td>
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</tr>
<tr>
<td>Anti-Cyclin E</td>
<td>mouse polyclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
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<td>rabbit polyclonal</td>
<td>Abcam</td>
</tr>
<tr>
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<td>Abcam</td>
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<td>mouse monoclonal</td>
<td>Sigma-Aldrich</td>
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<td>rabbit polyclonal</td>
<td>Sigma-Aldrich</td>
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<td>Anti-GAPDH</td>
<td>mouse monoclonal</td>
<td>Ambion</td>
</tr>
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<td>mouse polyclonal</td>
<td>Santa Cruz Biotechnology</td>
</tr>
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<td>Anti-GST</td>
<td>rabbit polyclonal</td>
<td>Bethyl Laboratories</td>
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<td>Anti-LIMK1</td>
<td>rabbit polyclonal</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-LIMK2</td>
<td>rabbit polyclonal</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-NuMA</td>
<td>rabbit polyclonal</td>
<td>Cell Signaling Technology</td>
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<td>Anti-Pericentrin</td>
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<td>Abcam</td>
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<td>mouse monoclonal</td>
<td>Epitomics</td>
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<td>Sigma Aldrich</td>
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<td>Sigma Aldrich</td>
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</table>
2.1.3 Secondary Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse IgG, Alexa Fluor 488</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Goat anti-mouse IgG, Alexa Fluor 546</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Goat anti-mouse IgG, HRP conjugated</td>
<td>Dako Cytomation</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG, Alexa Fluor 488</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG, Alexa Fluor 546</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG, HRP conjugated</td>
<td>Dako Cytomation</td>
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</table>

2.1.4 Mammalian cell culturing materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Company</th>
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<tr>
<td>Dulbecco's modified Eagle's medium (DMEM)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>PAA</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>Gibco</td>
</tr>
<tr>
<td>Minimum Essential Medium Eagle (MEM)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Sigma Aldrich</td>
</tr>
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</table>

2.1.5 Commercial Kits

<table>
<thead>
<tr>
<th>Name of commercial kit</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>AxyPrep™ Plasmid Miniprep Kit</td>
<td>Axygen</td>
</tr>
<tr>
<td>Novex® ECL Chemiluminescent Substrate Reagent Kit</td>
<td>Invitrogen</td>
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</table>
### 2.1.6 Bacteria strains and mammalian cell lines

<table>
<thead>
<tr>
<th>Strain/Cell Line</th>
<th>Description</th>
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<tbody>
<tr>
<td>DH5α</td>
<td>Chemically competent <em>Escherichia coli</em> (Invitrogen)</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human embryonic kidney epithelial (ATCC-CRL-1573)</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervix adenocarcinoma epithelial (ATCC-CL-2)</td>
</tr>
<tr>
<td>U2-OS</td>
<td>Human osteosarcoma epithelial (ATCC-HTB-96)</td>
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### 2.1.7 Buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
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</thead>
<tbody>
<tr>
<td>Lysogeny Broth (LB)</td>
<td>10 g bacto-tryptone</td>
</tr>
<tr>
<td></td>
<td>5 g bacto-yeast extract</td>
</tr>
<tr>
<td></td>
<td>10 g NaCl</td>
</tr>
<tr>
<td></td>
<td>1000 mL MilliQ water</td>
</tr>
<tr>
<td>Lysogeny Broth (LB) Agar</td>
<td>10 g bacto-tryptone</td>
</tr>
<tr>
<td></td>
<td>5 g bacto-yeast extract</td>
</tr>
<tr>
<td></td>
<td>20 g bacto Agar</td>
</tr>
</tbody>
</table>
| **Mammalian cell lysis buffer** | 10 g NaCl  
1000 mL MilliQ water |
|-------------------------------|----------------------------------|
|                               | 25 mM Hepes, pH 7.5  
0.25 M NaCl  
1 mM MgCl₂  
1 mM EGTA  
20 mM p-glycerol phosphate  
1 mM sodium vanadate  
10 mM NaF  
5% glycerol  
0.5% Triton X-100  
5 mM DTT  
Protease inhibitor cocktail tablet (Roche)  
Phosphatase inhibitor cocktail tablet (Roche) |
| **Nitrocellulose transfer buffer, 10x** | 30.3 g Tris base  
144 g glycine  
1000 mL water |
| **Nitrocellulose transfer buffer, 1x** | 100 mL 10X nitrocellulose transfer buffer  
100 mL methanol  
800 mL MilliQ water |
| **Phosphate Buffered Saline (PBS), 1x** | 137 mM NaCl  
2.7 mM KCl  
10 mM Na₂HPO₄  
2 mM KH₂PO₄  
1000 mL MilliQ water |
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjusted to pH 7.4</td>
<td>5 mL 4X Tris-Cl/SDS pH 6.8&lt;br&gt;5 mL glycerol&lt;br&gt;1 g SDS&lt;br&gt;0.93 g dithiothreitol (DTT)&lt;br&gt;0.15 mg bromophenol blue</td>
</tr>
<tr>
<td>SDS sample buffer, 6x</td>
<td>30.2 g Tris base&lt;br&gt;144 g glycine&lt;br&gt;10 g SDS&lt;br&gt;1000 mL MilliQ water</td>
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<tr>
<td>SDS-PAGE electrophoresis buffer, 10x</td>
<td>100 mL 10X SDS-PAGE electrophoresis buffer&lt;br&gt;900 mL of MilliQ water</td>
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<tr>
<td>SDS-PAGE electrophoresis buffer, 1x</td>
<td>6.05 g Tris base (0.5 M Tris-Cl)&lt;br&gt;0.4 g SDS (0.4% SDS)&lt;br&gt;100 mL of MilliQ water&lt;br&gt;Adjusted to pH 6.8</td>
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<tr>
<td>Tris-Cl/SDS pH 6.8, 4x</td>
<td>91 g Tris base (1.5 M Tris-Cl)&lt;br&gt;0.4 g SDS (0.4% SDS)&lt;br&gt;100 mL of MilliQ water&lt;br&gt;Adjusted to pH 8.8</td>
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<tr>
<td>Trypsin-EDTA solution</td>
<td>0.25% (w/v) Trypsin&lt;br&gt;0.53 mM EDTA</td>
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2.1.8 Primers

<table>
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<th>Primer Name</th>
<th>Primer sequence* (5' to 3')</th>
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<tbody>
<tr>
<td>CEN2 sense (BamHI)</td>
<td>ATATAAGGATCCATGGCCTCAAATTTAACAGAG</td>
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<tr>
<td>CEN2 antisense (Xhol)</td>
<td>ATATACTCGAGTTAAATAGAGCGGTGGTCTTTTTT</td>
</tr>
<tr>
<td>CFL1 WT sense (HindIII)</td>
<td>ATATAAGCTTATGGGCTCAGGTGGTGGGTG</td>
</tr>
<tr>
<td>CFL1 S3A sense (HindIII)</td>
<td>ATATAAGCTTATGGGCCGCGGTGGGTGGTG</td>
</tr>
<tr>
<td>CFL1 S3E sense (HindIII)</td>
<td>ATATAAGCTTATGGGCCGAGGTGGGTGGTG</td>
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<td>CFL1 WT antisense (NotI)</td>
<td>ATATAGCGGGCGCTCAAAAGGCTTTG</td>
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<td>DYNClLI1 sense (BamHI)</td>
<td>ATATATAAGGATCCATGGGCGCGGTGGG</td>
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<tr>
<td>DYNClLI1 antisense (Xhol)</td>
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<td>LIMK1 WT sense (HindIII)</td>
<td>ATATAAGCTTATGGGAGGGCTGATCTTTGGT</td>
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<tr>
<td>LIMK1 WT antisense (KpnI)</td>
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<td>LIMK1 D460A sense</td>
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<tr>
<td>LIMK1 D460A antisense</td>
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<td>LIMK1 T508A antisense</td>
<td>GCAAGAGCAGCGGCGGTGCTTTCTGGG</td>
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<tr>
<td>LIMK1 T508E sense</td>
<td>GGGGTTGGGACCAGCGGCGGTGCTTTCTGGG</td>
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<tr>
<td>LIMK1 T508E antisense</td>
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<tr>
<td>LIMK1 T508EE sense</td>
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<td>RNA Sequence (5’ to 3’)</td>
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<tr>
<td>DYNC1LI1 siRNA sense</td>
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<tr>
<td>DYNC1LI1 siRNA antisense</td>
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<td>DYNC1LI2 siRNA sense</td>
<td>CACUUUCUAAACAGGGUGGAGCAAAUU</td>
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*Restriction endonuclease sites are underlined and mutation site are highlighted in red.

### 2.1.9 Stealth siRNA

<table>
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</tr>
<tr>
<td>DYNC1LI2 siRNA sense</td>
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<td>siRNA</td>
<td>Sequences</td>
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<tr>
<td>DYNC1L2</td>
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<td>LIMK1-1</td>
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<td>LIMK1-2</td>
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<tr>
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### 2.1.10 Plasmids and gene constructs

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<th>Plasmid</th>
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<th>Mutation</th>
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<td>S3A</td>
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<td>Cytoplasmic dynein 1 light intermediate chain 1 (NM_016141.3)</td>
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<tr>
<td>pXJ-FLAG-DYNC1LI2</td>
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<td></td>
<td>1 to 219</td>
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</table>

*The accession numbers (bolded) of the respective genes were obtained from NCBI Gene database*
2.2 Methods

2.2.1 Cell culture

HeLa cells were maintained in Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 2.2 g/L sodium bicarbonate and 2 mM L-glutamine. HEK 293 and U2-OS cells were maintained in Dulbecco's modified Eagle's medium high glucose (DMEM) (Sigma-Aldrich) supplemented with 10% FBS and 3.7 g/L sodium bicarbonate. All cells were cultured in humidified 95% air, 5% CO₂ incubator at 37°C.

For sub-culturing, cells were washed twice with 1X phosphate buffered saline (PBS) and incubated with 0.25% (w/v) Trypsin-0.53 mM EDTA solution until cells rounded up. Complete growth media was then added to stop the reaction and used to flush trypsinized cells off culture dish. Cells were pelleted by centrifugation and re-suspended in fresh complete growth media. Cells were then sub-cultured at a ratio between 1:5 and 1:7.

For cell preservation, cells were trypsinized and re-suspended in FBS supplement with 10% DMSO at 1 x 10⁶ cells/mL density. Cells were cooled at a rate of 1°C/min until -80°C and transferred into liquid nitrogen tank for long term storage. For thawing, frozen cells were brought to 37°C and washed once with pre-warmed complete growth media. Cells were cultured and maintained as described above.

2.2.2 Molecular cloning

Total RNA were isolated from cells using Trizol (Invitrogen) and used to synthesize cDNA using Superscript VILO cDNA Synthesis Kit (Invitrogen) according to manufacturer's protocol. Primers with appropriate restriction enzyme recognition site were designed to amplify desired DNA sequence from synthesized cDNA by
polymerase chain reaction (PCR) using TaKaRa Ex TaqTM polymerase (TaKaRa). Amplified products were subjected to agarose gel electrophoresis and purified with QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer’s protocol. Plasmid vectors and purified PCR products were incubated with the appropriate restriction enzymes (New England Biolabs) at 37°C for 2 hours. Digested plasmid and PCR products were subjected to agarose gel electrophoresis and purified with QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer’s protocol.

Gel purified plasmid vector and PCR product were mixed at a molar ratio of 1:3 for ligation reaction using T4 DNA ligase (New England Biolabs). Ligation mixtures were then incubated in 16°C for at least 8 hours. After incubation, the ligation reaction were transformed into chemical competent DH5α cells by heat-shock method, plated on Lysogeny Broth (LB) agar plates supplemented with either 100 µg/mL ampicillin or 50 µg/mL kanamycin. The agar plates were then incubated at 37°C for at least 12 hours before checking for colony growth. Colonies were picked, inoculated in LB broth supplemented with appropriate antibiotics selection and plasmids were purified using AxyPrep™ Plasmid Miniprep Kit (Axygen) according to manufacturer’s protocol. Purified plasmids were subjected to restriction digestion and agarose gel electrophoresis to screen for plasmid with the appropriate insert size ligated. The identities of the inserts were verified by sequencing and positive constructs 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

The respective phospho-dead or phospho-mimic mutants were generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutagenic oligonucleotide primers were designed using QuikChange Primer Design Program. Mutagenesis reactions were carried out using the thermal cycling parameters as stated in manufacturer’s protocol. Parental DNA templates were removed by incubating the mutagenesis reaction with DpnI at 37°C for 1 hour. 5 µL of DpnI-digested
mutagenesis reaction was used for transformation into DH5α competent cells using heat shock method and plated on suitable antibiotic selection agar plates. Agar plates were then incubated at 37°C for at least 12 hours before checking for colony growth. Colonies were picked, incubated in LB broth with suitable antibiotics, and plasmids were purified using AxyPrep™ Plasmid Miniprep Kit (Axygen) as described by manufacturer’s protocol. Purified plasmids were then sequenced 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 Real-time quantitative PCR

Total RNA were isolated from cells using Trizol (Invitrogen) and 2 μg of total RNA were used to synthesize cDNA using Superscript VILO cDNA Synthesis Kit (Invitrogen) according to manufacturer’s protocol. The newly synthesized cDNA samples were diluted ten-fold and used for quantitative PCR (qPCR) analysis. StepOne Plus real time PCR machine (Applied Biosystems) was set to Fast SYBR green quantification program to run the real time qPCR reactions. TATA binding protein (TBP) was included as endogenous loading control for gene expression calculation. Melting curve analysis was performed after qPCR reactions to determine primer specificity and check for contamination. No template controls and no reverse transcription controls were included to eliminate the possibility of DNA contamination. After qPCR reactions and melting curve analysis, ΔΔCT method was used to calculate gene expression level. The calculated ΔΔCT values were then normalized with TBP expression level and plotted. Three technical and biological qPCR replicates were carried out for all samples.

### 2.2.5 siRNA and plasmid transfections

For all transfections, cells were seeded onto 35-mm dishes 24 hours prior to transfection. For siRNA transfection, 150 pmol of Stealth siRNA (Invitrogen) were mixed with 3 μL LipofectAMINE-2000 (Invitrogen) and incubated at room
temperature for 20 minutes. The siRNA/LipofectAMINE mixture was then added to and incubated with the cells for 48 hours. For plasmid transfection, 1 µg of plasmid were mixed with 3 µL LipofectAMINE-2000 and incubated at room temperature for 20 minutes. If two or more plasmid constructs were transfected into cells, the amount of plasmids was adjusted such that the total amount of plasmids transfected would be 1 µg. The DNA/LipofectAMINE mixture was then added to and incubated with the cells for 24 or 48 hours as described. For rescue experiments, cells were first transfected with siRNA for 24 hours and subsequently transfected with respective plasmid constructs. 24 hours after plasmid transfection, cells were processed for subsequent experiments. For rescue experiments, GST-FLAG empty vector was used as control for comparison. We utilized FLAG-tagged and GST-tagged constructs for our rescue studies. By utilizing GST-FLAG empty vector as control, it gave us the flexibility to examine the expression of the various tagged constructs with either anti-FLAG or anti-GST antibodies during western blot analysis.

2.2.6 Cell synchronization and chemical treatment

Double Thymidine block were used to synchronize cells at G1/S-phase. Briefly, cells were incubated with 2 mM Thymidine (Sigma) for 18 hours to block the cells at G1/S-phase. These cells were then release from the first Thymidine block by washing the cells thrice with 1X PBS and incubated with fresh media for 10 hours. After 10 hours, cells were again incubated with 2 mM Thymidine for 18 hours to further enhance the G1/S-phase synchronization. Cells were then released from the second Thymidine block and harvested/utilized for subsequent experiments.

1 µM of Compound 22 and 25 µM of Ciliobrevin D were added to G1/S-phase synchronized cells 8 hours after the second Thymidine release to inhibit LIMK and cytoplasmic dynein activity, respectively. HeLa cells were treated with C3-Transferase, Y27623, Jasplakinolide and Cytochalasin B when necessary. Treatment with the above mention chemicals were limited to 2 hours to minimize defects been carried over from Interphase to the mitotic phase.
2.2.7 Indirect immuno-fluorescence microscopy

Cells were seeded onto acid-washed glass coverslips and treated with the respective chemicals, siRNA and/or plasmid constructs as stated. For centrosomal protein immuno-staining, cells were fixed with cold methanol for 5 minutes at -20°C and re-hydrated with 1X PBS for 30 minutes. To stain other cellular structures, cells were fixed with 3.6% paraformaldehyde/1X PBS for 15 minutes at room temperature. Fixed cells were washed with 1X PBS to remove the excess paraformaldehyde and permeabilized with 0.1% Triton-X/1X PBS for 10 minutes. Permeabilized cells were incubated with 4% bovine serum albumin (BSA) and incubated with the respective primary antibody at 4°C overnight in a humidified chamber. Cells were washed thrice with 0.1% Triton-X/1X PBS and incubated with secondary antibodies at room temperature for 1 hour. Cells were washed thrice with 0.1% Triton-X/1X PBS after secondary antibody incubation. Immuno-stained samples were then counterstained with DAPI contained in mounting medium (Vectashield). All primary and secondary antibodies were diluted in 0.5% Triton-X/1X PBS at a ratio of 1:500 and 1:1000, respectively. All immuno-stained samples were examined using Axio Observer.D1 microscope (Zeiss) equipped with either EC Plan-Neofluar 40x/1.30 or Plan-Apochromat 63x/1.40 oil immersion objective lens (Carl Zeiss). AxioVision software was used to capture images using a CoolSNAP HQ2 camera. Images were then analyzed and processed using Image-J software.

2.2.8 Cell lysis, Western blotting and PhosTag SDS-PAGE

Cells were washed once with ice-cold 1X PBS and lysed with mammalian cell lysis buffer supplemented with protease and phosphatase inhibitor (Roche). The cell lysates were cleared by centrifugation at 14,000 rpm at 4°C for 10 minutes and Bradford reagent (Bio-Rad) was used to determine the protein concentration. Between 35 µg and 60 µg of total protein were treated with 1X SDS sample buffer and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membranes and blocked in 5% skim milk/1X TBS for 1 hour. Membranes were then incubated with the appropriate
primary and secondary antibodies diluted in 2.5% skim milk/1X TBS. All primary were diluted at a ratio of 1:1000 and incubated with the membranes at 4°C overnight with constant rotation. All secondary antibodies were diluted at a ratio of 1:5000 and incubated with the membranes at room temperature for 1 hour with constant rotation. Membranes were washed thrice with 0.05% Tween 20 in 1X TBS (TBST) after each round of antibody incubations. Membranes were subjected to chemiluminescence detection using ECL Plus Detection Kit (Amersham) and exposed onto Super RX Fuji X-ray film (Fuji). The films were developed using Kodak X-OMAT ME-1 processor.

For PhosTag SDS-PAGE, HeLa cell were synchronized and treated with the appropriate siRNA as described. Cells were washed once with ice-cold 1X TBS and lysed with mammalian cell lysis buffer supplemented with protease and phosphatase inhibitor (Roche). The cell lysates were cleared by centrifugation at 14,000 rpm at 4°C for 10 minutes and Bradford reagent (Bio-Rad) was used to determine the protein concentration. 8% PhosTag SDS-PAGE resolving gel and stacking gel were prepared according to manufacturer’s protocol (Wako Pure Chemical, Japan). 20 µg of total protein were treated with 1X SDS sample buffer and subjected to PhosTag SDS-PAGE. Proteins were transferred onto activated PVDF membrane and blocked in 5% skim milk/1X TBS for 1 hour. Membranes were incubated with the appropriate antibodies and washed as described above. Membranes were then subjected to chemiluminescence detection using ECL Plus Detection Kit (Amersham) and exposed onto Super RX Fuji X-ray film (Fuji). The films were developed using Kodak X-OMAT ME-1 processor.

2.2.9 Centrosome duplication assay

U2-OS cells were grown on acid-washed glass coverslips and transfected with either control or LIMK1 siRNA as described above. 24 hours after siRNA transfection, U2-OS were incubated with 4 mM Hydroxyurea (HU) for 40 hours to synchronize the cells in S-phase. The synchronization and siRNA knock-down efficacy were
analyzed with Fluorescence-activated cell sorting (FACS) and western blotting, respectively. After synchronization, cells were immuno-stained with γ-Tubulin as described above. 100 randomly chosen fields were imaged and the number of γ-Tubulin foci in each cell was then calculated. Experimental data from 3 independent repeats were statistically analyzed and plotted.

2.2.10 Centrosome defocusing, centrosomal protein intensity and astral microtubule intensity quantification

To quantify centrosome defocusing, the centrosome spread length was measured using Image-J software as illustrated in (Figure 2-1A). Briefly, fixed cells were immuno-stained for Pericentrin to visualize the centrosome. A line, parallel to the metaphase plate, was drawn on the Pericentrin foci and the length of the line was determined as the centrosome spread length.

For centrosomal protein foci intensity quantifications, fixed cells were immuno-stained with the respective antibodies and imaged as describe above. The centrosomal protein foci was outlined and background corrected integrated fluorescence intensity was calculated for each individual centrosomal protein foci using Image-J software (Figure 2-1B). The calculated intensity was then normalized against the area of the centrosomal protein foci. 300 cells from three independent biological repeats were collected for analysis. All immuno-stained samples from 3 independent biological repeats were imaged on the same day.

For astral microtubule intensity quantifications, fixed cells were immuno-stained with α-Tubulin antibody to visualize the mitotic spindle and imaged as described above. The background corrected integrated fluorescence intensities of the kinetochore microtubules and total microtubule were then calculated using Image-J software. The astral microtubule was then calculated by subtracting the intensity of kinetochores microtubules from the total microtubule intensity (Figure 2-1C). 300 cells from three independent biological repeats were collected analyzed and plotted for all
quantification. All immuno-stained samples from 3 independent biological repeats were imaged on the same day.

Figure 2-1. Illustration on the method used to calculate centrosome spread length, centrosomal protein intensity and astral microtubule intensity. (A) To quantify centrosome defocusing, a line, parallel to the metaphase plate, was drawn on the Pericentrin foci and the length of the line was determined as the centrosome spread length. (B) The centrosomal protein foci was outlined and background corrected integrated fluorescence intensity was calculated for each

Corrected Fluorescence Intensity = \[ \frac{I_C - \text{(Average } I_{Bj} \times \text{Area of centrosome})}{\text{Area of centrosome}} \]

Astral microtubule Intensity = (Background corrected Total microtubule Intensity - Background corrected Kinetochoore microtubule Intensity)
individual centrosomal protein foci. The calculated intensity was then normalized against the area of
the centrosomal protein foci. (C) For astral microtubule intensity quantifications, the background
corrected integrated fluorescence intensities of the kinetochore microtubules and total microtubule
were calculated. The astral microtubule was then calculated by subtracting the intensity of
kinetochores microtubules from the total microtubule intensity.

2.2.11 Mitotic spindle orientation and positioning quantification

To quantify the mitotic spindle orientation and positioning, HeLa cells were first
seeded onto Fibronectin-coated glass coverslips and treated with the respective
chemicals, siRNA and/or plasmid constructs. For spindle orientation, treated cells
were immuno-stained with Pericentrin to visualize the mitotic spindle poles.
Metaphase cells (identified through chromosome arrangement) were imaged for z-
stack at 0.5 μm distance apart. The spindle orientation, relative to the substratum,
was then determined by calculating the spindle angle (θ) using inverse trigonometry
function, θ = tan⁻¹ (A / B), where θ represented the metaphase spindle angle, in
degrees, in relation to the substratum; A represented z axis distance between
spindle poles in μm; and B represented x-y axis distance between spindle poles in
μm (Figure 2-2A). 120 cells from three independent biological repeats were collected
analyzed and the spindle angles were plotted as box-and-whiskers diagram.

For spindle positioning, treated HeLa cells were immuno-stained with α-Tubulin to
visualize the mitotic spindle. In addition, these cells were stained with Phalloidin to
visualize the cell boundary. The centroid of the mitotic cells and spindle were then
determined using ImageJ software. Spindle positioning was then determined by
calculating how far the spindle centroid deviated from the metaphase cell centroid
(Figure 2-2B). 120 cells from three independent biological repeats were collected for
analysis.
Figure 2-2. Illustration on the method used to calculate spindle orientation and positioning. (A) Metaphase cells were imaged for z-stack at 0.5 μm distance apart. The spindle orientation, relative to the substratum, was determined by calculating the spindle angle (θ) using inverse trigonometry function, $\theta = \tan^{-1}\left(\frac{A}{B}\right)$, where θ represented the metaphase spindle angle, in degrees, in relation to the substratum; A represented z-axis distance between spindle poles in μm; and B represented x-y axis distance between spindle poles in μm. (B) The centroid of the mitotic cells and spindle were determined using ImageJ software. Spindle positioning was then determined by calculating how far the spindle centroid deviated from the metaphase cell centroid.

2.2.12 Fluorescence-activated cell sorting

Fluorescence activated cell sorting (FACS) were used to analyze the DNA content of cells. First, HeLa or U2-OS cells were first treated with the respective chemicals or siRNAs for the indicated amount of time. These cells were then trypsinized, washed twice with ice-cold 1X PBS and fixed with 75% ethanol on ice for at least 1 hour. Fixed cells were then washed twice with ice-cold 1X PBS and stained with Propidium Iodide at 37°C for at least 1 hour. Stained samples were then analyzed with X-20 Fortessa (BD Biosciences) using FACSDiva software. For each condition, 3
technical and biological repeats were performed, and FACS plots shown in Results sections were representative plots for each treatment.

2.2.13 Glutathione S-transferase pull-down assay and Immuno-precipitation

HEK 293 cells were transfected with the respective plasmid constructs for 24 hours as described. Cells were lysed in mammalian cell lysis buffer, and lysate were cleared and quantified as described above. 40 µg of total lysate were kept to determine the expression of the respective constructs (Input) by SDS-PAGE and western blot analysis. The remaining supernatant was incubated with 50 µL of Glutathione Sepharose® 4B slurry beads (Amersham Biosciences) for 3 hours at 4°C with constant rotation. Beads were washed thrice with 1 mL of mammalian cell lysis buffer. Bound proteins were recovered from beads by adding 50 µL of 2X SDS sample buffer and heated at 100°C for 10 minutes. Supernatants were analyzed by SDS-PAGE and western blot.

For immuno-precipitation assay, HeLa cells were first synchronized to M-phase using nocodazole. Cells were washed once with ice-cold 1X PBS and lysed with mammalian cell lysis buffer supplemented with protease and phosphatase inhibitor (Roche). The cell lysates were cleared by centrifugation at 14,000 rpm at 4°C for 10 minutes and Bradford reagent (Bio-Rad) was used to determine the protein concentration. 40 µg of total lysate were kept to determine the endogenous protein level (Input) by SDS-PAGE and western blot analysis. The remaining cell lysate were pre-cleared by incubation with irrelevant antibodies slurry beads for 5 hours at 4°C with constant rotation. DYNC1LI1, DYNC1LI2 or Control antibodies were immobilized by incubating the antibodies with Protein A slurry beads for 5 hours at 4°C with constant rotation. Control antibodies (irrelevant IgG antibody) were included in the immuno-precipitation to serve as negative control. The pre-cleared lysate were then incubated with the immobilized antibodies for immuno-precipitation assay at 4°C for 5 hours with constant rotation. Beads were washed four times with 1 mL of mammalian cell lysis buffer. Bound proteins were recovered from beads by adding
50 μL of 2X SDS sample buffer and heated at 100°C for 10 minutes. Supernatants were analyzed by SDS-PAGE and western blot.

2.2.14 Statistical Analysis

Unpaired student’s t-test was used for all statistical analysis. A p-value of less than 0.05 was considered to be statistically significant. All results were represented by mean ± standard deviation of at least three independent experiments.

2.2.15 SILAC based mass spectrometry (SILAC-MS)

SILAC labeling

For SILAC-MS, HeLa cells were first culture in MEM media containing “heavy” or “light” arginine and lysine (Figure 2-3). Briefly, MEM media lacking arginine and lysine (MEM-Arg,-Lys) were purchased from Biowest (Miami, FL, USA). The “heavy” [13C6- Arginine and 13C6- Lysine] amino acids were purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA, USA) while the “Light” [12C6- Arginine and 12C6- Lysine] amino acids were purchased from Sigma-Aldrich. Dialyzed fetal bovine serum was purchased from Invitrogen. To constitute the “heavy” media, MEM-Arg,-Lys were supplemented with 13C6- Arginine and 13C6- Lysine, and dialyzed fetal bovine serum. To constitute the “light” media, MEM-Arg,-Lys were supplemented with 12C6- Arginine and 12C6- Lysine, and dialyzed fetal bovine serum. HeLa cells were first cultured and maintained in “heavy” and “light” media for 7 passages, allowing the proteins to be “labeled” with light and heavy amino acids. Labeled cells were then synchronized to M-phase using 50 ng/mL of nocodazole for 16 hours. Synchronized cells were then treated with either Compound 22 or DMSO for 1 hour.
Protein extraction, digestion and fractionation

For protein extraction, cells were washed with ice cold 1X PBS five times and lysed in protein lysis buffer containing 8 M urea (Sigma-Aldrich), 20 mM HEPES (Merck), complete protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor cocktail (Roche). Protein concentration was determined by Bicinchoninic Acid (BCA) protein assay. Equal amounts of protein (10 mg each) from heavy and light samples were mixed at a 1:1 ratio. Proteins were reduced with 10 mM dithiothreitol at 33°C for 1 hour, followed by alkylation with 55 mM of iodoacetamide at room temperature for 45 minutes. The reduced and alkylated samples were then diluted 8 times with 20 mM HEPES (Merck) and digested in-solution by trypsin (Sigma-Aldrich) at 1:100 dilution. Peptide samples were adjusted to pH 2 using 10% formic acid and desalted using SEP-PAK C18 cartridges (Waters, Milford, MA), and vacuum dried.

The resulting peptides were fractionated using Electrostatic repulsion hydrophilic interaction chromatography (ERLIC) method (Alpert, 2008). Briefly, the peptides were dissolved in Buffer A (72% acetonitrile + 0.5% formic acid) and injected into a PolyLC PolyWAX LP column (4.6 x 200 mm, 5 µm particle size, 300 Å pore size) mounted on a Shimadzu Prominance UFLC unit (Shimadzu Corporation, Kyoto, Japan). The 40 minutes gradient was generated using a combination of Buffer A1 (72% acetonitrile + 0.5% formic acid) and Buffer B1 (10% acetonitrile + 1.0% formic acid). The gradient was composed of 5 minutes 100% Buffer A1, followed by 30 minutes gradient from 0% to 100% Buffer B1, and ended with 5 minutes 100% Buffer B1. The first five collections were combined into one tube. The subsequent collections, containing phospho-peptides, were combined into 15 fractions for phospho-peptide analysis. The combined first five fractions were further fractionated into 20 fractions for whole proteome analysis. For the proteome fractionation, a different 40 minutes gradient was generated using a combination of Buffer A2 (85% acetonitrile + 10 mM ammonium acetate + 1% formic acid) and Buffer B2 (30% acetonitrile + 0.1% formic acid). The gradient was composed of 5 min 100% Buffer A2, followed by 30 minutes gradient from 0% to 100% Buffer B2, and ended with 5 min 100% Buffer B2. All 35 fractions were desalted using SEP-PAK C18 cartridges.
(Waters, Milford, MA); vacuum dried and stored at -80 °C prior to LC-MS/MS analysis.

Figure 2-3. Illustration of SILAC mass spectrometry experimental workflow. HeLa cells were cultured in media containing either “heavy” or “light” for seven generation. These cells were then synchronized with nocodazole for 16 hours before treating the synchronized “light” cells with 1 µM of...
Compound 22 and “heavy” cells with DMSO. The cell lysates for each treatment were then collected for phospho-protein/peptide enrichment and analyzed using mass spectrometry.

**Phospho-Peptide enrichment using ERLIC and TiO2**

The phosphopeptide was firstly enriched using ERLIC, followed by TiO2. Briefly, the peptide was injected to a PolyLC PolyWAX LP column (4.6 × 200mm, 5 μm particle size, 300 Å pore size) mounted on a Shimadzu Prominence UFLC unit (Shimadzu Corporation). The 40 minutes gradient was the combination of a Buffer A (72% acetonitrile + 0.5% formic acid) and a Buffer B (10% acetonitrile + 1.0% formic acid). The first five fractions were combined and vacuum-dried for TiO2. The following collections were combined into 22 fractions and vacuum dried for LC-MS/MS analysis.

For TiO2 enrichment, firstly, TiO2 beads (GL Science) were dissolved in Buffer A (50% acetonitrile + 0.1% trifluoroacetic acid). Peptide sample was dissolved in TiO2 Loading Buffer (50% acetonitrile + 0.1% trifluoroacetic acid + 35 mg/ml 2, 5-dihydroxy- benzoic acid). Equal amount of beads were added into each sample, shaking at room temperature for 1 hour. After centrifuge, supernatant was collected and vacuum dried, store at -80°C. Phosphopeptides were washed with TiO2 Loading Buffer thrice and eluted with elusion buffer (Concentrated NH4OH) thrice. All the eluted samples were pH-adjusted to pH 2 using 10% formic acid. Phosphopeptide was desalted using SEP-PAK C18 cartridges (Waters) and vacuum dried for LC-MS/MS analysis.

**LC-MS/MS analysis and Proteomic analysis**

Digested peptides were analyzed in an LTQ-FT Ultra mass spectrometer (Thermo Fisher, Waltham, Massachusetts, USA) coupled to a Prominence™ HPLC unit.
(Shimadzu, Kyoto, Japan) (Guo et al., 2012) by Associate Professor Sze Siu Kwan, Newman's laboratory group. For proteomics analysis, the MS raw files were converted to mzXML format and mgf format using Trans-Proteome Pipeline. Protein database search was performed by uploading mgf files to an in-house Mascot cluster server (version 2.2.07) (Matrix Science, Boston, MA) against a concatenated target and decoy version of manually annotated non-redundant UniProt Knowledgebase database (40516 sequences, downloaded on 8 October 2010). The decoy sequences were generated by reversing the UniProt protein sequences. The search was limited to a maximum of 2 missed trypsin cleavages; mass tolerance of 20 ppm for peptide precursors; and 0.35 Da mass tolerance for fragment ions. Fixed modification was carboxymethyl at Cys residues, while variable modifications include oxidation at methionine residues, phosphorylation at serine, threonine and tyrosine, heavy lysine and arginine. False discovery rates of 1%. The ratio for SILAC pairs was calculated based on the area of extracted ion chromatograph (XIC) of each peptide. The calculated ratio was then expressed as $\log_2$ when applicable.
Chapter 3. Results

3.1 The role of LIMK1 at the centrosome.

Although LIMK1 is reported to localize at the mitotic centrosome, its role at the centrosome is still largely unknown (Chakrabarti et al., 2007; Sumi et al., 2006). In this project, we set out to investigate the function of LIMK1 at the centrosome during mitosis. We then proceeded further to examine the possible mechanism, involving LIMK1, responsible in regulating mitotic centrosome integrity.

3.1.1 LIMK1 associates with centrosome during mitosis

To investigate the role of LIMK1 during mitosis, we first examined the localization pattern of LIMK1 in M-phase cells by immuno-fluorescence staining (Figure 3-1). Different stages of mitosis were identified by the chromosome arrangements, which were visualized by 4', 6'-diamidino-2-phenylindole (DAPI) staining. In addition, α-Tubulin antibody was used to immuno-stain the mitotic spindle. At G2/M-phase, weak LIMK1 positive foci were observed near the nuclei of cells undergoing nuclear envelop breakdown (NEB) (Figure 3-1, top panel). In addition, microtubules were observed to be radiating out from the LIMK1 positive foci. These observations suggest that endogenous LIMK1 begins to localize at the centrosome at the onset of G2- to M-phase transition. LIMK1 localization pattern at the mitotic centrosome continued as the cell progressed from prophase to metaphase (Figure 3-1). When the cell entered into anaphase, LIMK1 positive immuno-fluorescence staining was now observed at both the mitotic centrosome and the site of cleavage furrow formation (Figure 3-1, indicated by the white arrow). At telophase, LIMK1 localization to the centrosome was lost and LIMK1 was observed to accumulate between the two newly formed nuclei (Figure 3-1, indicated by the white arrow). The localization pattern at the site of cleavage furrow formation is consistent with earlier studies on the role of LIMK1 in acto-myosin contractile ring formation during cytokinesis (Amano et al., 2002; Kaji et al., 2008; Yang et al., 2004b).
From our immuno-fluorescence staining, we speculated that LIMK1 localization at the mitotic centrosome is dynamic and LIMK1 level at the centrosome changes as the cell progresses through the M-phase. Therefore, we proceeded to measure the fluorescence intensities of the LIMK1 at the mitotic centrosome through M-phase to determine if the localization pattern supported our hypothesis (Figure 3-2). From our fluorescence intensity data, we observed that LIMK1 accumulation at the mitotic centrosome increased as the cells progressed from G2/M-phase to pro-metaphase. At pro-metaphase and metaphase, LIMK1 accumulation at the centrosome was at its maximum and reached a plateau phase. After metaphase, LIMK1 localization at the centrosome began to decrease rapidly and was almost lost during telophase.

In summary, our localization study is consistent with earlier reports which demonstrate that LIMK1 accumulates at the centrosome from prophase till metaphase (Chakrabarti et al., 2007; Sumi et al., 2006). LIMK1 then begins to dissociate from the centrosome and localizes at the site of cytokinesis, where it regulates the formation of acto-myosin contractile ring during cytokinesis.
Figure 3-1. Localization pattern of LIMK1 during M-phase. HeLa cells were seeded onto acid-washed glass coverslips and subjected to indirect immuno-fluorescence staining. The cells were immuno-stained with LIMK1 and α-Tubulin antibody to visualize endogenous LIMK1 and mitotic
spindle, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images for each M-phase were processed using ImageJ and presented. Scale Bar: 10 µm.

Figure 3-2. LIMK1 fluorescence intensity at the centrosome during various stages of M-phase. HeLa cells were treated as described in Figure 3-1. The fluorescence intensity of LIMK1 at centrosome was measured and calculated as described in Methods section. The calculated intensity was normalized against the area of the selected foci and the mean LIMK1 fluorescence intensities were plotted. For each stage of M-phase, 300 cells from 3 independent repeats were included for the analysis. The error bars represent standard deviation. a.u. represents arbitrary unit.

3.1.2 RNAi-mediated LIMK1 depletion leads to multi-polar spindle formation and centrosome defocusing

From our indirect immuno-fluorescence staining data, we observed that LIMK1 was localized to the centrosome in M-phase. This observation led us to hypothesize that LIMK1 is involved in regulating functions that are performed by the mitotic centrosome. Therefore, we set out to investigate the possible roles of LIMK1 at the centrosome during M-phase. We first designed two siRNAs targeting the 3’ untranslated region (3’-UTR) of LIMK1 and a siRNA targeting Luciferase as negative control. The specificity and efficacy of the siRNAs in targeting LIMK1 were then
examined by both real-time quantitative polymerase chain reactions (qPCR) and western blot analysis. HeLa cells were transfected with LIMK1 or control siRNA for 48 hours, and endogenous LIMK1 mRNA and protein levels were determined (Figure 3-3). Firstly, our qPCR data demonstrated that both siRNA could significantly reduce the endogenous LIMK1 mRNA level (Figure 3-3A), suggesting that our siRNAs is effective in targeting and reducing LIMK1 mRNA. As protein is the final functioning unit of most gene coding sequences in the mammalian cell, we proceeded to examine the endogenous LIMK1 protein level using western blot. Consistent with our qPCR data, both LIMK1 siRNAs significantly reduced the amount of endogenous LIMK1 protein compared to control (Figure 3-3B). Both LIMK1 siRNAs did not affect the endogenous protein levels of LIMK2, another member of the LIM-kinase family, suggesting that both siRNAs specifically target LIMK1. Although siRNA-mediated depletion is a useful method to understand gene function, siRNA treatment has been reported to result in off-target effects, leading to false positive phenotypes (Cullen, 2006; Jackson and Linsley, 2010). Pooling siRNAs that target different region of a target gene is shown to be an effective strategy to significantly reduce off-target effects (Kittler et al., 2007). Therefore, we pooled both LIMK1 siRNA together for transfection in our subsequent experiments unless otherwise stated.

Next, we examined the effect of LIMK1 depletion on the mitotic centrosome. We focused our effort on metaphase centrosome, as our fluorescence intensity data showed that LIMK1 centrosome localization peaked during pro-metaphase/metaphase (Figure 3-2). HeLa cells were transfected with LIMK1 or control siRNA, and immuno-stained with Pericentrin and α-Tubulin to visualize the mitotic centrosome and spindle, respectively. Metaphase cells were identified through chromosome arrangements which were stained by DAPI. Majority of control siRNA transfected (mean = 97% ± 5.1%) metaphase cells contained two centrosomes that were focused into two compact foci positioned at either side of the metaphase chromosomes (Figure 3-4A and B). In contrast, only about 28% (± 6%) of LIMK1 siRNA treated metaphase cells formed bipolar spindle. Most of the LIMK1-depleted metaphase cells (mean = 72% ± 6.0%), compared to an average of 3% (± 5.1%) of control metaphase cells, were observed to contain more than two spindle poles (multi-polar spindles) (Figure 3-4A and B). Astral and kinetochore microtubules
were observed to radiate out from each of the spindle pole in the mitotic cells forming multi-polar spindles, suggesting that they are functional centrosome.

Figure 3-3. LIMK1 siRNA specificity and efficacy. (A) HeLa cells were transfected with the respective siRNA for 48 hours and synchronized to M-phase using nocodazole. The total RNA was then harvested for qPCR analysis to determine LIMK1 mRNA levels. RQ represents relative quantification. (B) HeLa cells were transfected with the respective siRNA for 48 hours and synchronized to M-phase using nocodazole. The cell lysates were then harvested for western blot analysis. LIMK1, LIMK2 and various centrosome associated protein antibodies were used to determine their endogenous protein levels. GAPDH was probed to serve as loading control.

Although majority of LIMK1-depleted cells formed multi-polar spindles, around 28% of these metaphase cells were observed to form bipolar spindles. However, the centrosomal material of these mitotic cells appeared to be diffused around the spindle pole (centrosome defocusing) (Figure 3-4A). We proceeded to measure the Pericentrin spread length to quantify the centrosome defocusing defect (Figure 3-4C). In control siRNA transfected cells, the mean Pericentrin spread length was 2.14 µm (± 0.49 µm). In contrast, the mean Pericentrin spread length of LIMK1 siRNA treated
cells [4.07 µm (± 1.60 µm)] was significantly longer compared to that of control cells. This observation suggests that the mitotic centrosome is not able to form compact and focused spindle poles when LIMK1 is depleted.

Pericentriolar material (PCM) proteins, such as Aurora kinase A (AurkA), γ-Tubulin, Nuclear mitotic apparatus protein (NuMA), Pericentrin and Polo-like kinase 1 (PLK1), are reported to be essential for proper centrosome maturation and spindle apparatus formation (Bruinsma et al., 2014; Carmena and Earnshaw, 2003; Radulescu and Cleveland, 2010). In addition, inhibiting the functions of these proteins has been reported to negatively affect spindle apparatus formation (Lee and Rhee, 2011; Oshimori et al., 2006). To eliminate the possibility that the mitotic spindle defects observed in our study were due to non-specific targeting of other PCM proteins, we proceeded to examine the endogenous protein levels of AurkA, NuMA, γ-Tubulin, PLK1, and Pericentrin using western blot (Figure 3.3B). Our western blot data demonstrated that both LIMK1 siRNAs did not reduced the protein levels of the above mentioned centrosome associated proteins (Figure 3-3B). This observation suggests that the mitotic spindle defects observed in LIMK1-depleted cells are not due to non-specific targeting of PCM proteins.

Taken together, LIMK1 depletion leads to multi-polar spindle and centrosome defocusing. Our data also suggests that LIMK1 may play an important role in regulating mitotic centrosome integrity.
Figure 3-4. LIMK1 depletion leads to mitotic spindle defects. (A) HeLa cells were seeded onto acid-washed glass coverslips and transfected with the respective siRNAs for 48 hours. The cells were immuno-stained with Pericentrin and α-Tubulin antibodies to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images for each treatment and phenotype were processed by ImageJ and presented. Scale Bar: 10 µm. (B) Cells were treated as described in (A)
and the number of metaphase cells displaying bipolar and multi-polar spindles were counted. The mean proportion of metaphase cell displaying the respectively phenotypes were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. *** represents p \leq 0.001. (C) Cells were treated as described in (A) and the metaphase centrosome spread length was measured as described in Methods section. The mean centrosome spread length were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. **** represents p \leq 0.0001.

3.1.3 Multi-polar spindle phenotype observed in LIMK1-depleted cells is not caused by centrosome duplication dysfunction.

Centrosome over-duplication can lead to the formation of multi-polar spindle during mitosis (Maiato and Logarinho, 2014). In our earlier experiments, we observed multi-polar spindles formation in LIMK1-depleted cells. This led us to hypothesize that LIMK1 depletion could have a negative impact on centrosome duplication pathway. Therefore, we proceeded to investigate if LIMK1 was involved in regulating centrosome duplication by using centrosome duplication assay in U2-OS cells. U2-OS cells were chosen for this assay as this cell-line is able to continuously undergo centrosome duplication even after prolong S-phase arrest (Liu and Erikson, 2002). In addition, this method has been utilized successfully in previous studies to identify key proteins that are involved in regulating centrosome duplication (Lin et al., 2013). U2-OS cells were first transfected with either LIMK1 or control siRNAs, synchronized to S-phase with hydroxyurea, and immuno-stained with γ-Tubulin to visualize centrosomes (Figure 3-5C). The siRNA and synchronization efficacy was investigated using western blot and FACS analysis, respectively (Figure 3-5A and B). The number of γ-Tubulin foci per cell for LIMK1 and control siRNA treated cells were then compared and analyzed. Our centrosome duplication assay data indicated that there was no significant increase in the number of γ-Tubulin foci per cell in LIMK1 siRNA treated U2-OS cell, compared to control cells (Figure 3-6), suggesting that LIMK1 depletion does not affect the centrosome replication process.
Figure 3-5. LIMK1 is not involved in regulating centrosome duplication. (A and B) U2-OS cells were transfected with the respective siRNA and synchronized to S-phase with 4 mM Hydroxyurea. Cells were then harvested for western blot analysis (A) and probed with LIMK1 antibody to determine the endogenous LIMK1 protein level. Endogenous Actin protein level was probed to serve as loading control. In addition, synchronized cells were harvested for FACS analysis (B) to investigate the efficacy of synchronization. (C) U2-OS cells were seeded onto acid-washed glass coverslips and treated as described in (A). Transfected cells were then immuno-stained with γ-Tubulin antibody to visualize centrosome. DNA was stained with DAPI to visualize nucleus. Immuno-stained samples were then observed under 40X objective lens. Representative images for each treatment were processed using ImageJ and presented. Scale Bar: 10 µm.
Figure 3.6. Quantification of centrosome duplication assay. U2-OS cells were treated as described in Figure 3-5C and 100 random fields were observed for each triplicate. The proportions of cells with the respective number of γ-Tubulin foci per cell were calculated and the mean proportion of cells was plotted. The error bars represent standard deviation. n.s represents $p \geq 0.05$.

To confirm the result of the centrosome duplication assay, we proceeded further to investigate the number of centriole pair in HeLa cells at early prophase. The process of centrosome duplication is completed by early prophase (Bettencourt-Dias and Glover, 2007). Therefore, the presence of extra centrioles at early prophase would suggest to us that the centrosome was over-duplicated. HeLa cells were transfected with LIMK1 or control siRNA for 48 hours and immuno-stained with Centrin 3 to visualize centrioles in early prophase cells (Figure 3-7A). Early prophase cells were identified by chromosome morphology, which was visualized by DAPI staining. From our immuno-staining data, majority of the LIMK1 and control siRNAs transfected cell contained only 2 pairs of centrioles [95.7% (±1.9%) and 96.1% (±1.4%), respectively; $p \geq 0.05$] (Figure 3-7B). In addition, the proportion of cells with extra centriole pairs in LIMK1 knocked down cells was not significantly different from control cells [4.3% (±1.9%) vs 3.9% (± 1.4%); $p \geq 0.05$]. This observation is in agreement with our
earlier centrosome duplication assay data, which suggests that LIMK1 depletion do not cause abnormal centrosome duplication.

Taken together, our data demonstrates that LIMK1 depletion does not caused centrosome over-duplication, suggesting that LIMK1 is not involved in regulating centrosome replication. Therefore, the multi-polar spindles observed in LIMK1-depleted cells are not due to centrosome over-duplication.

3.1.4 Multi-polar spindle phenotype observed in LIMK1-depleted cell is not due to cytokinesis failure

Cytokinesis failure could potentially lead to abnormal number of centrosome during subsequent cell cycle, giving rise to the formation of multi-polar spindles (Maiato and Logarinho, 2014). Given the importance of LIMK1-Cofilin pathway in the formation of acto-myosin contractile structure during cytokinesis, we proceeded to investigate if depleting LIMK1 would lead to cytokinesis failure (Amano et al., 2002; Yang et al., 2004b). In addition to abnormal number of centrosome, cytokinesis failure leads to the formation of multi-nucleate cells (Amano et al., 2002). This would then result in the formation of cells with abnormal DNA content, which can be detected by observing for the appearance of 6N or 8N DNA profile peaks during fluorescence activated cell sorting (FACS) analysis. For FACS DNA profile analysis, HeLa cells were transfected with the respective siRNAs, fixed and stained with Propidium iodide at specified time-points. Cells treated with Cytochlasin B were used as positive control as previous studies has shown that Cytochlasin B treatment resulted in cytokinesis failure (Krishan, 1972). The siRNA knock-down efficacy was examined using western blot analysis (Figure 3-8A).
Figure 3-7. LIMK1 depletion does not affect centriole numbers in G₂/M-phase HeLa cells. (A) HeLa cells were seeded onto acid-washed glass coverslips and transfected with the respective siRNAs for 48 hours. Cells were then immuno-stained with Centrin 3 antibody to visualize centrioles. Nucleus and chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens and early prophase cells were identified by nucleus morphology. Representative images for each treatment were processed using ImageJ and presented. Scale Bar: 10 µm. (B) HeLa cells were treated as described in (A) and the number of cells with the respective number of centriole pairs was counted. The mean proportion of cells displaying the respective number of centriole pairs were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. n.s represents p ≥ 0.05.
From our FACS data, the DNA content profile of LIMK1 and control siRNA treated cells was not significantly different from each other at all indicated time-points (Figure 3-8B). In agreement with earlier reports, treatment with Cytochalasin B led to the formation of multi-nucleated cells (indicated by the presence of 6N and 8N peaks) in our study (Krishan, 1972). Unlike cells treated with Cytochalasin B, the DNA content profile of LIMK1 siRNA treated cells did not displayed the presence of 6N or 8N DNA content even at 72 or 96 hours post siRNA transfection (Figure 3-8B). These FACS DNA profile data suggests that LIMK1-depleted cells are able to complete cytokinesis and do not form multi-nucleated cells.

In addition to FACS DNA content analysis, we also examined the presence of multi-nucleated cells and abnormal number of centrosome in interphase cells using immuno-staining. γ-Tubulin antibody and DAPI stain were used to visualize centrosome and nucleus, respectively (Figure 3-9A). Microtubule staining was used to identify individual cell boundary. From our immuno-staining data, we observed that the number of LIMK1 siRNA treated interphase cells with multi-nuclei or abnormal number of centrosome did not deviated significantly from control interphase cells (Figure 3-9B and C).

Taken together, our FACS and immuno-staining data suggests LIMK1 depletion does not cause defects in cytokinesis. Therefore, the multi-polar spindle phenotypes that are observed in LIMK1-depleted mitotic cells are not due to cytokinesis failure.
Figure 3-8. LIMK1 depletion does not lead to abnormal DNA content. (A) HeLa cells were treated with the respective chemical or siRNAs. Treated cells were then harvested every 24 hours for western blot analysis to determine the endogenous LIMK1 protein level. Endogenous GAPDH level was probed to serve as loading control. (B) HeLa cells were treated as described in part (A) and harvested for FACS analysis every 24 hours. The above plots are representative DNA content plot for each treatment and time-point.
Figure 3-9. LIMK1 depletion did not result in the formation of multi-nucleated cells and abnormal centrosome number. (A) HeLa cells were seeded onto acid-washed glass coverslips and transfected with respective siRNAs for 48 hours. Cells were then immuno-stained with γ-Tubulin and α-Tubulin antibody to visualize centrosome and microtubules. Nucleus was stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images for each treatment were processed with ImageJ and presented. Scale Bar = 10 µm. (B and C) HeLa cells were treated as described in (A) and 100 random fields were selected for analysis. The number of cells displaying more than 1 γ-Tubulin foci (B) and multi-nuclei (C) phenotypes were counted. The mean number of cells were then calculated and plotted. Both graphs represent data from 3 independent replicates, n = 300. The error bars represent standard deviation. n.s represents p ≥ 0.05.
3.1.5 LIMK1 depletion leads to pericentriolar material (PCM) fragmentation

Abnormal pericentriolar material (PCM) or pre-mature centrioles fragmentation during M-phase can lead to the formation of multi-polar spindles during mitosis (Maiato and Logarinho, 2014; Wang et al., 2008). Therefore, we decided to investigate if LIMK1 depletion would result in PCM or centrioles fragmentation using immuno-staining. LIMK1 and control siRNA treated HeLa cells were immuno-stained with PCM protein (Pericentrin) and centriole (Centrin 3) markers. We had classified the mitotic spindle pole morphology into four phenotype categories to determine if LIMK1 depletion led to PCM fragmentation (Figure 3-10A). In Phenotype 1, cell formed two spindle poles and each pole contained a pair of centrioles (normal bipolar). In Phenotype 2, cell formed extra mitotic spindle poles but only two of the spindle poles contained a pair of centrioles. In Phenotype 3, cell formed extra mitotic spindles and all the spindle poles contained centrioles. Lastly, cell displaying Phenotype 4 contained extra mitotic spindle poles and centrioles. However, not all the spindle poles contained centrioles in cells displaying Phenotype 4. If majority of mitotic cells display Phenotype 2, it would suggest that the multi-polar spindle is due to PCM fragmentation. In contrast, if majority of mitotic cells display Phenotype 3 or 4, then it would suggest that multi-polar spindle formation is due to pre-mature centriole fragmentation, leading to pre-mature initiation of centriole duplication and formation of extra spindle poles.

In control siRNA treated cells, about 96.2% (± 0.9%) of metaphase cells formed bipolar spindle (Phenotype 1) and only small percentage of control metaphase cells displayed Phenotype 2, 3 or 4 (Figure 3-10B). In contrast, only about 27.9% (± 5.9%) of LIMK1-depleted mitotic cells displayed Phenotype 1 (Figure 3-10B). Consistent with earlier observations, LIMK1-depleted cells that formed bipolar spindle displayed centrosome defocusing defect. About 56.0% (±1.5%) of LIMK1-depleted mitotic cells contained extra PCM foci with only two of them containing a pair of centriole, suggesting that these cells displays Phenotype 2 (Figure 3-10B). In contrast, only a very small percentage of LIMK1 siRNA treated cells displayed Phenotype 3 and 4 [7.0% (±1.8%) and 9.0% (±3.1%), respectively] (Figure 3-10B). To further confirm our
hypothesis that LIMK1 depletion leads to PCM fragmentation, we repeated the same assay by staining with γ-Tubulin, another PCM marker (Figure 3-11A). In agreement with our Pericentrin immuno-staining data, we made the similar observations and obtained the similar results when we repeated the experiment with γ-Tubulin immuno-staining (Figure 3-11B).

Taken together, our data suggests that majority of the LIMK1-depleted cells forms multi-polar spindle due to PCM fragmentation during mitosis. Although pre-mature centriole fragmentation contributes to the formation of multi-polar spindle, it is not the major contributing factor in LIMK1-depleted mitotic cells.
Figure 3-10. LIMK1 depletion leads to PCM fragmentation (Pericentrin staining). (A) HeLa cells were seeded onto acid-washed glass coverslips and transfected with either control or LIMK1 siRNA for 48 hours. Treated cells were then harvested for immuno-fluorescence staining with Pericentrin and
Centrin 3 antibodies to visualize centosome and centrioles, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of the various mitotic spindle phenotypes were processed using ImageJ and presented. Scale Bar = 10 µm. (B) HeLa cells were treated as described in part (A). The number of metaphase cells displaying the respective phenotypes was counted. The mean proportion of cells displaying the respective mitotic spindle phenotypes were then calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation.

3.1.6 LIMK1 depletion results in reduced centrosomal protein localization at the mitotic spindle poles

During our initial phenotype characterization of LIMK1-depleted mitotic cells, we observed that the Pericentrin foci fluorescence intensity at the centrosome seems weaker compared to control cells (Figure 3-4C and 3-13B). We decided to measure and calculate the Pericentrin foci fluorescence intensity at each spindle pole in both control and LIMK1 siRNA treated metaphase cells. Our fluorescence intensity analysis revealed that the fluorescence intensity of Pericentrin at the mitotic centrosome decreased by about 70% in LIMK1-depleted cells as compared to control cells (Figure 3-13B and 3-15D). This data suggests that the Pericentrin accumulation at mitotic centrosome is negatively affected in LIMK1 siRNA treated cells.
Figure 3-11. LIMK1 depletion leads to PCM fragmentation (γ-Tubulin staining). (A) HeLa cells were seeded onto acid-washed glass coverslips and transfected with either control or LIMK1 siRNA for 48 hours. Treated cells were then harvested for immuno-fluorescence staining with γ-Tubulin and
Centrin 3 antibodies to visualize centrosome and centrioles, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of the various mitotic spindle phenotypes were processed using ImageJ and presented. Scale Bar = 10 µm. (B) HeLa cells were treated as described in part (A). The number of metaphase cells displaying the respective phenotypes was counted. The mean proportion of cells displaying the respective mitotic spindle phenotypes were then calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation.

We proceeded further to investigate if other centrosomal protein accumulation at metaphase centrosome was also affected after LIMK1 siRNA treatment. We decided to focus our efforts on Aurora kinase A (AurkA), γ-Tubulin, Nuclear mitotic apparatus protein (NuMA), Polo-like kinase 1 (PLK1), and Gamma-tubulin complex component 2 (TubGCP2), as these proteins are known to regulate mitotic spindle formation and/or centrosome integrity (Carmena and Earnshaw, 2003; Radulescu and Cleveland, 2010; Zimmerman et al., 2004; Zitouni et al., 2014). Similar to our Pericentrin fluorescence data, the fluorescence intensities of AurkA, γ-Tubulin, NuMA, and PLK1 at spindle poles also decreased in LIMK1-depleted cells, compared to control (Figure 3-15). This observation suggests that the accumulation of AurkA, γ-Tubulin, NuMA, and PLK1 at mitotic centrosome is also negatively affected in LIMK1-depleted cells. Interestingly, the fluorescence intensity of TubGCP2 at metaphase centrosome was not significantly reduced in LIMK1 siRNA treated cells, compared to control cells (Figure 3-14B and 3-15F). This observation suggests that TubGCP2 accumulation at spindle poles is not affected when LIMK1 is depleted.

Taken together, our fluorescence intensity measurement suggests that LIMK1 depletion negatively affects the accumulation of some centrosomal proteins that are important for regulating spindle pole formation and integrity, at the mitotic spindle poles.
Figure 3-12. LIMK1 depletion reduces AurkA and γ-Tubulin accumulation at the mitotic centrosome. HeLa cells were seeded onto acid-washed glass coverslips and transfected with respective siRNAs for 48 hours. Transfected cells were then immuno-stained with either AurkA (A) or γ-Tubulin (B) to visualize mitotic centrosome. Mitotic spindle were immuno-stained with α-Tubulin. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of each treatment and phenotype were processed using ImageJ and presented. Scale bar = 10 µm.
Figure 3-13. LIMK1 depletion reduces NuMA and Pericentrin accumulation at the mitotic centrosome. HeLa cells were seeded onto acid-washed glass coverslips and transfected with respective siRNAs for 48 hours. Transfected cells were then immuno-stained with either NuMA (A) or Pericentrin (B) to visualize mitotic centrosome. Mitotic spindle were immuno-stained with α-Tubulin. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of each treatment and phenotype were processed using ImageJ and presented. Scale bar = 10 µm.
Figure 3-14 LIMK1 depletion reduces PLK1, but not TubGCP2, accumulation at the mitotic centrosome. HeLa cells were seeded onto acid-washed glass coverslips and transfected with respective siRNAs for 48 hours. Transfected cells were then immuno-stained with either PLK1 (A) or TubGCP2 (B) to visualize mitotic centrosome. Mitotic spindle were immuno-stained with α-Tubulin. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of each treatment and phenotype were processed using ImageJ and presented. Scale bar = 10 μm.
Figure 3-15. Quantification of centrosomal proteins intensities at mitotic spindle poles after LIMK1 siRNA treatment. HeLa cells were treated as described in Figure 3-12 – 3-14. The fluorescence intensity of AurkA (A), γ-Tubulin (B), NuMA (C), Pericentrin (D), PLK1 (E), and TubGCP2 (F) were measured and calculated as described in Methods section. The calculated intensity was normalized against the area of the selected foci and plotted Experiment was performed in triplicates; n=300. The error bars represent standard deviation. a.u. represents arbitrary unit. **** represents p ≤ 0.0001. n.s represents p ≥ 0.05.

3.1.7 Kinase activity of LIMK1 is important for maintaining mitotic centrosome integrity

Earlier studies show that LIMK1 is hyper-activated during mitosis and its kinase activity is involved in regulating several processes during mitosis (Amano et al., 2002;
Sumi et al., 2002). These earlier findings led us to hypothesize that the kinase activity of LIMK1 could also be crucial for regulating centrosome integrity. To test this hypothesis, we generated and introduced a kinase-dead LIMK1 mutant (LIMK1-D460A) or an active LIMK1 mutant (LIMK1-T508EE) into LIMK1-depleted cells. For the active LIMK1 mutant, we created a point mutation at residue 508 [from threonine (T) to glutamate (E)] with an additional insertion of glutamate (E) residue immediately after Threonine 508. LIMK1-T508EE mutant has been demonstrated to have a higher kinase activity, compared to LIMK1-WT (Edwards and Gill, 1999). Both LIMK1 siRNAs target the 3’ un-translated region (UTR) of LIMK1 mRNA. Since our LIMK1 constructs contains only the open reading frame of LIMK1 mRNA, the 3’ UTR is not present in our constructs. This eliminates the need to create siRNA-resistant LIMK1 constructs in our rescue studies. We then examined if these mutant could rescue the defects observed in LIMK1-depleted cells. Cells co-transfected with control siRNA and GST-FLAG construct served as negative control for comparison. The siRNA knock-down efficiency and expression of wild-type (LIMK1-WT), kinase dead (LIMK1-D460A) and constitutively active (LIMK1-T508EE) LIMK1 mutants constructs were examined using western blot analysis (Figure 3-16A). To verify earlier report on the activity of LIMK1-T508EE, we examined the endogenous phospho-Cofilin level in cells transfected with various LIMK1 constructs (Figure 3-16B) (Edwards and Gill, 1999). Cofilin was selected for analysis as it is a downstream substrate of LIMK1 (Arber et al., 1998). From our data, we observed a significant decrease in phospho-Cofilin level in LIMK1 siRNA and GST-FLAG co-transfected cells (Figure 3-16B). The level of phosphorylated Cofilin was restored close to control level when LIMK1-WT was introduced into LIMK1-depleted cells (Figure 3-16B). Our data confirm earlier reports on the role of LIMK1 in regulating Cofilin activity through phosphorylation (Agnew et al., 1995; Arber et al., 1998). The level of phospho-Cofilin level was significantly higher in LIMK1-T508EE transfected cells when compared to Control siRNA and GST-FLAG co-transfected cells (Figure 3-16B). This observation suggests that LIMK1-T508EE is able to induce Cofilin phosphorylation and the mutant is a constitutively active mutant. Although LIMK1 is an up-stream kinase of Cofilin, we did not observe a complete lost of phospho-Cofilin level in cells co-transfected with LIMK1 siRNA and GST-FLAG (Arber et al., 1998). LIMK2 is reported to phosphorylate Cofilin and regulates actin dynamics (Sumi et al., 1999). Therefore,
LIMK2 might substitute the function of LIMK1 in regulating Cofilin activity through phosphorylation.

Figure 3-16. Western blot analysis to determine the expression of LIMK1 constructs and efficacy of LIMK1 siRNA. (A) HeLa cells were co-transfected with their respective combination of siRNAs and LIMK1 constructs for 48 hours. Transfected cells were then harvested for western blot analysis. Cell lysate were probed with LIMK1 and GST antibodies to detect the endogenous LIMK1 protein level and expression of LIMK1 constructs, respectively. GAPDH were probed to serve as loading control. (B) HeLa cells were treated as described in (A) and harvested for western blot analysis. Endogenous phospho-Cofilin and Cofilin were probed. The intensities of phospho-Cofilin and Cofilin were measured using ImageJ and normalized against loading control (GAPDH). The relative phospho-Cofilin ratio was calculated by dividing normalized phospho-Cofilin intensity with normalized Cofilin intensity. Experiment was performed in triplicates. The error bars represent standard deviation. a.u. represents arbitrary unit.* represents p ≤ 0.05. ** represent s p ≤ 0.01. n.s represents p ≥ 0.05.

The transfected cells were then immuno-stained with Pericentrin and α-Tubulin to visualize the mitotic centrosome and spindle apparatus, respectively. Metaphase cells were identified through chromosome arrangement, which were stained with DAPI. From our immuno-staining data, we observed that control cells formed two
focused centrosome at either side of the chromosome during metaphase (Figure 3-17). Only about 3.7% (± 1.7%) of control cells displayed multi-polar spindle phenotype (Figure 3-18A). In contrast, about 57.9% (± 5.2%) of cells co-transfected with LIMK1 siRNA and GST-FLAG displayed multi-polar spindle phenotype (Figure 3.18A). This number was significantly higher compared to control cells [57.9% (± 5.2%) vs 3.7% (± 1.7%); p ≤ 0.0001]. This observation was in agreement with our earlier data, which showed that LIMK1 depletion resulted in the formation of multi-polar spindle. When LIMK1-WT and LIMK1-T508EE were introduced into LIMK1-depleted cells, the proportion of metaphase cells displaying multi-polar spindle defects was significantly reduced [9.2% (± 3.8) vs 57.9% (± 5.2%); p ≤ 0.001 and 12.1% (± 2.8%) vs 57.9% (± 5.2%); p ≤ 0.001, respectively], compared to cells co-transfected with LIMK1 siRNA and GST-FLAG. In contrast, when cells were co-transfected with LIMK1 siRNA and LIMK1-D460A mutant, the proportion of metaphase cells displaying multi-polar spindle defects were not significantly reduced when compared to cells co-transfected with LIMK1 siRNA and GST-FLAG.

We then measured the centrosome spread length to determine if LIMK1 kinase activity is crucial for regulating mitotic centrosome integrity. From the measurements of centrosome spread length, we observed that the mean centrosome spread length of control cells was 2.25 μm (± 0.45 μm), suggesting that control cells are able to form two focused centrosome at either side of the chromosome during metaphase (Figure 3-17 and 3-18B). In contrast, the mean centrosome spread length of cells co-transfected with LIMK1 siRNA and GST-FLAG was significantly higher than control cells [4.75 μm (± 1.04 μm) vs 2.25 μm (± 0.45 μm); p ≤ 0.0001] (Figure 3.18B). When LIMK1-WT and LIMK1-T508EE were introduced into LIMK1 depleted cells, the mean centrosome spread length was significantly reduced [2.16 μm (± 0.31 μm) vs 4.75 μm (± 1.04 μm); p ≤ 0.0001 and 3.11 μm (± 0.88 μm) vs 4.75 μm (± 1.04 μm); p ≤ 0.0001, respectively], compared to cells co-transfected with LIMK1 siRNA and GST-FLAG. In agreement with the multi-polar spindle phenotype, the mean centrosome spread length of cells co-transfected with LIMK1 siRNA and LIMK1-D460A mutant were not significantly reduced when compared to cells co-transfected with LIMK1 siRNA and GST-FLAG [4.79 μm (± 1.22 μm) vs 4.75 μm (± 1.04 μm); p ≥ 0.05].
Taken together, the results from the rescue experiment are in agreement with our hypothesis, which suggests that LIMK1 kinase activity is important for regulating and maintaining mitotic centrosome integrity. Our observations also suggest a possible substrate of LIMK1 functioning downstream of the kinase in regulating mitotic centrosome integrity since active kinase can rescue the LIMK1-knockdown phenotype.

3.1.8 Inhibiting LIMK activity with Compound 22 leads to centrosome defocusing, but not multi-polar spindle.

To further demonstrate the importance of LIMK1 kinase activity in regulating centrosome integrity, we decided to utilize a chemical (Compound 22) which has the ability to inhibit LIMK kinase activity (Harrison et al., 2009). We first determined the effectiveness of Compound 22 in inhibiting LIMK activity by determining the levels of phosphorylated Cofilin, as it is a known substrate of LIMK. HeLa cells were treated with 1 μM of Compound 22 and cell lysates were harvested at specified time-point. The specified concentration was selected as previous report indicates that 1 μM is sufficient to inhibit LIMK kinase activity (Harrison et al., 2009). Cells treated with DMSO for 12 hours served as negative control. Western blot analysis showed that the levels of phosphorylated Cofilin were almost undetected when cells were treated with Compound 22 for 1 hour and remained undetected for up to 9 hours post drug treatment (Figure 3-19B). The failure to detect phosphorylated Cofilin was not due to changes in total Cofilin protein level, as western blot analysis did not revealed any changes to total Cofilin protein levels. 10 hours after Compound 22 treatment, the levels of phosphorylated Cofilin recovered back to those of control, suggesting that the chemical could remain active for up to 9 hours. We did not observed any changes to the phospho-Cofilin levels in DMSO treated cells, suggesting that the decrease in phospho-Cofilin levels was specific to Compound 22 treatment.
Figure 3-17. LIMK1 kinase activity is important for maintaining centrosome integrity. HeLa cells were seeded onto acid-washed glass coverslips and transfected with their respective combination of
siRNA and LIMK1 constructs for 48 hours. Transfected cells were then harvested for immuno-fluorescence staining with Pericentrin and α-Tubulin antibodies to visualize mitotic centrosome and spindle apparatus, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of the respective treatment and phenotype were processed with ImageJ and presented. Scale bar = 10 µm.

Figure 3-18. Quantification of mitotic cells displaying multi-polar spindle and centrosome defocusing. (A) Cells were treated as described in Figure 3-17 and the number of mitotic cells displaying multi-polar spindles was counted. The mean proportion of mitotic cell displaying multi-polar spindle phenotype was calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. (B) Cells were treated as described in Figure 3-17 and the mitotic centrosome spread length was measured as described in Methods section. The mean metaphase centrosome spread length was calculated and plotted. Experiment was performed in triplicates; n=300. For both plots, the error bars represent standard deviation. *** represents p ≤ 0.001; **** represent p ≤ 0.0001; n.s p ≥ 0.05.

Next, we investigated centrosome defocusing and multi-polar spindle formation in cells treated with LIMK inhibitor using indirect immuno-fluorescence microscopy. As the efficacy of Compound 22 lasted only 9 hours, we decided to synchronize HeLa cells to G1/S-phase using double Thymidine block method before treating them with
Compound 22. Western blot analysis showed that HeLa cells began to enter mitosis (indicated by the presence of Cyclin-B and phospho-Histone 3) 9 hours after released from the second Thymidine block (Figure 3-20A). As inhibiting LIMK1 during interphase has been shown to negatively affect cell cycle progression (Davila et al., 2003), we decided to treat the cells just before they entered into M-phase and limited our treatment with Compound 22 to 1 hour. Therefore, cells were first synchronized with double Thymidine block and treated with Compound 22 9 hours after the released from second Thymidine block. Cells were harvested for immuno-fluorescence staining after one hour of Compound 22 treatment. Cells treated with DMSO served as negative control for comparison. In agreement with LIMK1 siRNA treatment, the mean centrosome spread length was significantly higher compared to DMSO treated cells [5.73 µm (± 1.33 µm) vs 2.02 µm (± 0.22 µm); p ≤ 0.0001] (Figure 3-20A and 3-20C), suggesting that inhibiting LIMK kinase activity does indeed lead to centrosome defocusing. Surprisingly, we did not observed any significant difference in the number of cells forming multi-polar spindle after Compound 22 treatment, compared to control cells [8.9% (± 1.1%) vs 7.0% (± 2.3%); p ≥ 0.05). The discrepancy in phenotypes between Compound 22 and LIMK1 siRNAs treatment was not immediately clear. However, the difference in the duration of treatment/experimental times between Compound 22 treatment and siRNA transfection might contribute to the discrepancies. Besides the differences in the duration of treatment, the activity of Compound 22 is marginally more active towards LIMK2 when compared to LIMK1 (Harrison et al., 2009). This might also contribute to the discrepancy in the phenotypes between Compound 22 and LIMK1 siRNAs treatment.

Taken together, our data supports our hypothesis that inhibiting LIMK activity exerts a negative effect on mitotic centrosome integrity. Although we do not observe any multi-polar spindle defects, we can still detect centrosome defocusing when the cells are treated with Compound 22.
Figure 3-19. Western blot analysis of Thymidine synchronization and Compound 22 efficacy. (A) HeLa cells were synchronized to G₁/S-phase using double Thymidine block. Cell lysates were collected hourly after second Thymidine release and subjected to western blot analysis. Endogenous Cyclin-A (S- and G₂-phase marker), Cyclin-B (M-phase marker), Cyclin-D (G₁-phase marker), Cyclin-E (G₁/S-phase marker) and phospho-Ser10 Histone 3 (M-phase marker) levels were probed to track the cell cycle progression after second Thymidine release. (B) Asynchronized HeLa cells were treated with Compound 22 and harvested for western blot analysis. Cells treated with DMSO for 12 hours served as negative control. The endogenous level of phospho-Ser3 Cofilin was probed to determine the efficacy of Compound 22 activity on LIMKs. After probing for phospho-Cofilin level, the antibodies were stripped off from the membrane and total Cofilin levels were probed to serve as loading control.
Figure 3-20. Inhibiting LIMK with Compound 22 results in centrosome defocusing but not multi-polar spindle. (A) HeLa cells were seeded on acid-washed glass coverslips and treated with either DMSO or Compound 22 as described. Treated cells were immuno-stained with Pericentrin and α-Tubulin to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained cells were observed with 63x objective lens. Representative images for each treatment and phenotype were processed using ImageJ and presented. Scale bar = 10 µm. (B) Cells were treated as described in (A) and the number of mitotic cells displaying bipolar and multi-polar spindles were counted. The mean proportion of mitotic cell displaying the respective phenotypes were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. (C) Cells were treated as described in (A) and the metaphase centrosome spread length was measured as described in Methods section. The mean metaphase centrosome spread length were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. **** represents p ≤ 0.0001; n.s represents p ≥ 0.05.
3.1.9 LIMK1 kinase activity is important for centrosomal protein accumulation at the mitotic spindle poles.

Earlier, we showed that LIMK1 depletion reduced protein accumulation at the mitotic centrosome. We also demonstrated that only wild-type and constitutive active LIMK1 mutant rescued the mitotic centrosome integrity defects in LIMK1 siRNA treated cells. These observations led us to hypothesize that the kinase activity of LIMK1 could be crucial for centrosomal proteins accumulation at mitotic spindle poles. Again, we focused our effort on the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, PLK1, and TubGCP2 at mitotic spindle poles [Figure 3-21 (only γ-Tubulin staining were shown) and 3-22]. Cells co-transfected with control siRNA and GST-FLAG construct serve as negative control for comparison. From the fluorescence intensity analysis, we observed that the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 decreased significantly when cells were co-transfected with LIMK1 siRNA and GST-FLAG, compared to control cells (Figure 3-22A – E). When wild-type (LIMK1-WT) and constitutively active (LIMK1-T508EE) LIMK1 constructs were introduced into LIMK1-depleted cells, the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 at mitotic spindle poles were partially restored back to control level. In addition, the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 were significantly higher in LIMK1-WT and LIMK1-T508EE transfected cells compared to cells co-transfected with LIMK1 siRNA and GST-FLAG constructs (Figure 3-22A – E). This observation suggests that wild-type and constitutive active LIMK1 are able to restore the centrosomal protein levels at the mitotic spindle when endogenous LIMK1 are depleted. In contrast, kinase-dead LIMK1 mutant (LIMK1-D460A) did not restored the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 at mitotic spindle poles back to control level (Figure 3-22A – E). In addition, the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 were not significantly different from cells co-transfected with LIMK1 siRNA and GST-FLAG constructs. These observations suggest that the kinase activity of LIMK1 is important for regulating protein accumulation at mitotic centrosome. Consistent with earlier results, TubGCP2 fluorescence intensity was not affected when cells were transfected with LIMK1 siRNA or when various LIMK1 mutant constructs were
introduced into LIMK1-depleted cells, suggesting that LIMK1 does not regulate TubGCP2 accumulation during mitosis.

We proceeded further to confirm our findings by treating cells with Compound 22 and examined the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, PLK1, and TubGCP2 at the mitotic centrosome (Figure 3-23 and 3-24). Cells were treated with Compound 22 as described earlier and cells treated with DMSO served as negative control. In agreement with our earlier findings, we observed a slight decrease in the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 at metaphase spindle poles (Figure 3-25). However, the decrease in fluorescence intensities was not significantly lower when compared to those of control cells (Figure 3-25). Although the decrease was not significant, we could still observe a decreasing trend in fluorescence intensities of these proteins at the mitotic centrosome. Again, we could not detect any significant changes to the TubGCP2 fluorescence intensity at metaphase spindle poles, compared to that at the metaphase spindle poles of control cells. Our findings from cells treated with Compound 22 support the hypothesis that LIMK1 kinase activity is important for accumulation of some of the centrosomal protein at the mitotic spindle poles.
Figure 3-21 LIMK1 kinase activity is important for protein accumulation at the mitotic centrosome. HeLa cells were seeded onto acid-washed glass coverslips and co-transfected with
their respective combinations of siRNA and LIMK1 constructs for 48 hours. Transfected cells were then harvested for immuno-staining with γ-Tubulin and α-Tubulin antibodies to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images for the respective treatment and phenotype were processed with ImageJ and presented. Scale Bar: 10 µm.

**Figure 3-22. Quantification of centrosomal proteins intensities at the mitotic spindle poles.**

HeLa cells were treated as described in Figure 3-21. The fluorescence intensity of AurkA (A), γ-Tubulin (B), NuMA (C), Pericentrin (D), PLK1 (E), and TubGCP2 (F) at the mitotic centrosome were measured and calculated as described in Methods section. The calculated intensity was normalized against the area of the selected foci and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. a.u. represents arbitrary unit. **** represents p ≤ 0.0001; n.s represents p ≥ 0.05.
Figure 3-23. Treatment with Compound 22 does not significantly affect AurkA, γ-Tubulin and NuMA accumulation at the mitotic spindle poles. HeLa cells were seeded onto acid washed coverslips and synchronized to G1/S-phase using double Thymidine block. Synchronized cells were treated with either DMSO or Compound 22 9 hours after second Thymidine release for 1 hour. Treated cells were then harvested for immuno-fluorescence staining with AurkA (A), γ-Tubulin (B) and NuMA (C) to visualize the mitotic centrosome. Mitotic spindle were visualized by immuno-staining with α-Tubulin. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then
observed under 63X objective lens. Representative images for each treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm.
Figure 3-24. Treatment with Compound 22 does not significantly affect Pericentrin, PLK1 and TubGCP2 accumulation at mitotic spindle poles. HeLa cells were seeded onto acid washed coverslips and synchronized to G₁/S-phase using double Thymidine block. Synchronized cells were treated with either DMSO or Compound 22 8 hours after second Thymidine release. Treated cells were then harvested for immuno-fluorescence staining with Pericentrin (A), PLK1 (B) and TubGCP2 (C) to visualize mitotic centrosome. Mitotic spindle were visualized by immuno-staining with α-Tubulin. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images for each treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm.

Figure 3-25. Quantification of centrosomal proteins intensities at mitotic spindle poles. HeLa cells were treated as described in Figure 3-23 – 3-24. The fluorescence intensity of AurkA (A), γ-Tubulin (B), NuMA (C), Pericentrin (D), PLK1 (E) and TubGCP2 (F) at the mitotic centrosome were measured and calculated as described in Methods section. The calculated intensity was normalized against the area of the selected foci and plotted Experiment was performed in triplicates; n=300. The error bars represent standard deviation. The p-values were stated above the bar chart.
3.1.10 LIMK1-Cofilin and LIMK1-TPPP pathways are not involved in regulating mitotic centrosome integrity

Earlier we observed that the kinase-dead mutant of LIMK1 did not rescue the mitotic spindle defects observed in LIMK1 depleted cells, suggesting that the kinase activity of LIMK1 is important for maintaining mitotic centrosome integrity. In addition, it suggest that a substrate could potentially function downstream of LIMK1 in regulating mitotic centrosome integrity. Cofilin and Tubulin Polymerization Promoting Protein (TPPP/p25) are two known substrates of LIMK1 (Acevedo et al., 2007; Arber et al., 1998). In addition, both proteins are reported to function downstream of LIMKs to regulate certain mitotic processes (Heng et al., 2012; Kaji et al., 2008). Therefore, we decided to investigate if Cofilin and TPPP/p25 were involved in regulating mitotic centrosome integrity.

To investigate if Cofilin functions downstream of LIMK1 in regulating mitotic centrosome integrity, we generated FLAG tagged wild-type (Cofilin-WT), phospho-mimic (Cofilin-S3E), or phospho-dead (Cofilin-S3A) Cofilin constructs (Figure 3-26A). These constructs were then transfected into LIMK1 siRNA treated cells. Mitotic centrosome integrity of the transfected cells were then examined using immuno-fluorescence staining (Figure 3-27). Cell co-transfected with control siRNA and GST-FLAG construct serve as control for comparison. From indirect immuno-fluorescence microscopy, we did not observe a significant decrease in the proportion of cells displaying multi-polar spindle defects when the three Cofilin constructs were introduced into LIMK1-depleted cells, compared to cells co-transfected with LIMK1 siRNA and GST-FLAG (Figure 3-27 and 3-28A). In addition, we did not observe any significant decrease in the mean centrosome spread length when the three Cofilin constructs were introduced into LIMK1-depleted cells, compared to cells co-transfected with LIMK1 siRNA and GST-FLAG (Figure 3-27 and 3-28B). The observations suggest that the various Cofilin constructs are not able to rescue the centrosome defocusing defects in LIMK1 siRNA treated cells. Thus, we conclude that LIMK1-Cofilin signaling pathway is not involved in regulating mitotic centrosome integrity.
Figure 3.26. Western blot analysis to determine the siRNA efficacy and expression of various FLAG tagged constructs. (A) HeLa cells were co-transfected with their respective combination of siRNA and Cofilin constructs. Transfected cells were then harvested for western blot analysis. Cell lysate were probed with LIMK1 and FLAG antibodies to detect the endogenous LIMK1 protein level and expression of Cofilin constructs, respectively. GAPDH protein level was probed to serve as loading control. (B) HeLa cells were co-transfected with the respective combinations of siRNAs. Transfected cells were then harvested for western blot analysis to determine the endogenous level of LIMK1 and TPPP/p25. GAPDH protein level was probed to serve as loading control. (C) HeLa cells were co-transfected with their respective combination of siRNA and TPPP/p25 constructs. Transfected cells were then harvested for western blot analysis. Cell lysate were probed with LIMK1 and FLAG antibodies to detect the endogenous LIMK1 protein level and expression of TPPP/p25 constructs, respectively. GAPDH protein level was probed to serve as loading control.
Figure 3.27 Cofilin does not function downstream of LIMK1 in regulating centrosome integrity. HeLa cells were seeded onto acid-washed glass coverslips and transfected with the respective combinations of siRNA and Cofilin constructs. Transfected cells were then immuno-stained with Pericentrin and α-Tubulin to visualize centrosome and mitotic spindle, respectively. Mitotic
chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of the respective treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm.

**Figure 3-28. Quantification of cells displaying multi-polar spindle and centrosome defocusing.**

**(A)** Cells were treated as described in Figure 3-27 and the number of mitotic cells displaying multi-polar spindles was counted. The mean proportion of mitotic cell displaying the multi-polar spindle phenotype were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. **(B)** Cells were treated as described in Figure 3-27 and the metaphase centrosome spread length was measured as described in Methods section. The mean metaphase centrosome spread length were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. **** represents p ≤ 0.0001; n.s represent p ≥ 0.05.

TPPP/p25, another known substrate of LIMK1, is found to be involved in regulating astral microtubule dynamics during mitosis (Acevedo et al., 2007; Heng et al., 2012). Given the importance of astral microtubule in proper bipolar spindle formation, we decided to investigate if TPPP/p25 functions downstream of LIMK1 in regulating mitotic centrosome integrity (Carreno et al., 2008). It has been reported that LIMK1-
mediated phosphorylation of TPPP/p25 inactivates its tubulin polymerization activity (Acevedo et al., 2007). Previous work from our laboratory shows that silencing TPPP/p25 rescues the mis-orientation of the mitotic spindle caused by inhibition of Rho-ROCK-LIMK2 pathway (Heng et al., 2012). Since we do not have information on the specific residues on TPPP/p25 which are phosphorylated by LIMK1, we designed siRNA against TPPP/p25 to replicate the effect of loss of TPPP/p25 function. The efficacy of TPPP/p25 siRNA was determined by western blot analysis (Figure 3-26B). HeLa cells were co-transfected with the various combinations of LIMK1 and TPPP/p25 siRNAs, and mitotic centrosome integrity was examined using indirect immuno-fluorescence microscopy (Figure 3-29). Cell transfected twice with control siRNA serve as control for comparison. We did not observe a significant decrease in the proportion of cells displaying multi-polar spindle defects when LIMK1 and TPPP/p25 were co-depleted, compared to when LIMK1 was depleted alone [64.0% (± 7.8%)% vs 7.0% (± 2.0%); p ≥ 0.05] (Figure 3-29 and 3-30A). TPPP/p25 siRNA treatment alone did not lead to significant increase in the number of cells forming multi-polar spindle, compared to control cells [7.0% (± 2.0%) vs 7.0% (± 2.0%); p ≥ 0.05], suggesting that TPPP/p25 is not involved in regulating centrosome integrity. In addition, we did not observe any significant decrease in the mean centrosome spread length when LIMK1 and TPPP/p25 were co-depleted, compared to when LIMK1 was depleted alone [7.95 µm (± 3.01 µm) vs 8.01 µm (± 3.11 µm); p ≥ 0.05] (Figure 3-30B). TPPP/p25 siRNA treatment alone did not lead to any significant increase to the mean metaphase centrosome spread length, compared to control cells [1.89 µm (± 0.34 µm) vs 1.83 µm (± 0.26 µm); p ≥ 0.05].

Phosphorylation is an important post-translational modification which regulates the activity of TPPP/p25 (Acevedo et al., 2007; Hlavanda et al., 2007). Although the exact residues on TPPP/p25 phosphorylated by LIMK1 are not yet identified, Threonine 14 (Thr14) and Serine 18 (Ser18) are identified as the major phosphorylation site that regulates TPPP/p25 activity (Hlavanda et al., 2007). Therefore, we decided to generate FLAG tagged wild-type (TPPP/p25-WT), phospho-dead (TPPP/p25-T14A/S18A), and phospho-mimic (TPPP/p25-T14E/S18E) TPPP/p25 mutant constructs. These constructs were then transfected into LIMK1-depleted cells and mitotic centrosome integrity was examined using indirect immuno-
fluorescence microscopy (Figure 3-31). Cells co-transfected with control siRNA and GST-FLAG construct served as control for comparison. We did not observe any significant decrease in the proportion of cells displaying multi-polar spindle defects when the three TPPP/p25 constructs were introduced into LIMK1 depleted cells, compared to cells co-transfected with LIMK1 siRNA and GST-FLAG (Figure 3-31 and 3-32A). In addition, we did not observe any significant decrease in the mean centrosome spread length when the three TPPP/p25 constructs were introduced into LIMK1-depleted cells, compared to cells co-transfected with LIMK1 siRNA and GST-FLAG (Figure 3-31 and 3-32B). These observations suggest that the various TPPP/p25 constructs are not able to rescue the centrosome defocusing defects observed in LIMK1 siRNA treated cells. Thus, we conclude that LIMK1-TPPP/p25 signaling pathway is not involved in regulating mitotic centrosome integrity.

In summary, our findings from Cofilin and TPPP/p25 rescue experiments suggest that both Cofilin and TPPP/p25 do not function downstream of LIMK1 in regulating mitotic centrosome integrity. It is possible that a novel substrate might be functioning downstream of LIMK1 to regulate mitotic centrosome integrity.
**Figure 3-29. Co-depletion of LIMK1 and TPPP/p25 does not rescue the mitotic defects.** HeLa cells were seeded onto acid-washed glass coverslips and transfected with the respective combinations of siRNA. Transfected cells were then immuno-stained with Pericentrin and α-Tubulin to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images from respective treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm.

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Figure 3-30. Quantification of cells displaying multi-polar spindle and centrosome defocusing.

(A) Cells were treated as described in Figure 3-29 and the number of mitotic cells displaying multi-polar spindles was counted. The mean proportion of mitotic cell displaying the multi-polar spindle phenotype were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. 

(B) Cells were treated as described in Figure 3-29 and the metaphase centrosome spread length was measured as described in Methods section. The mean metaphase centrosome spread length were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. *** represents p ≤ 0.001; **** represents p ≤ 0.0001; n.s represents p ≥ 0.05.
Figure 3-31. Introducing TPPP/p25 into LIMK1-depleted cells does not rescue the mitotic defects. HeLa cells were seeded onto acid-washed glass coverslips and transfected with the
respective combinations of siRNA and TPPP/p25 constructs. Transfected cells were then immuno-stained with Pericentrin and α-Tubulin to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images from respective treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm.

Figure 3-32. Quantification of cells displaying multi-polar spindle and centrosome defocusing. (A) Cells were treated as described in Figure 3-31 and the number of mitotic cells displaying multi-polar spindles was counted. The mean proportion of mitotic cells displaying the multi-polar spindle phenotype were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. (B) Cells were treated as described in Figure 3-31 and the metaphase centrosome spread length was measured as described in Methods section. The mean metaphase centrosome spread length were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. *** represents $p \leq 0.001$; **** represents $p \leq 0.0001$; n.s represent $p \geq 0.05$. 
3.1.11 Rho-ROCK signaling does not function upstream of LIMK1 in regulating mitotic centrosome integrity

Rho-ROCK signaling is a well-studied pathway leading to the activation of LIMK1 (Ohashi et al., 2000). In addition, Rho-ROCK signaling is involved in several mitotic processes, such as cytokinesis, spindle orientation and maintaining mitotic cortical rigidity (Chircop, 2014; Heng and Koh, 2010; Narumiya and Yasuda, 2006). Therefore, we decided to examine if Rho-ROCK signaling pathway functioned upstream of LIMK1 in regulating mitotic centrosome integrity. We utilized C3-Transferase and Y27632 compound which are known to inhibit Rho and ROCK, respectively (Uehata et al., 1997; Wilde and Aktories, 2001). Prolong disruption of Rho-ROCK signaling during interphase has been shown to disrupt cell cycle progression and cause mitotic defects in subsequent M-phase (Chircop, 2014; Heng and Koh, 2010). Therefore, we limited our chemical treatments to 2 hours to minimize the possibility that defects from previous stages of cell cycle were being brought over to the M-phase. Cells treated with DMSO were used as control for comparison. Cells treated with the various chemicals were then harvested for immuno-fluorescence microscopy examination to observe any mitotic centrosome defects.

From the immuno-fluorescence examination, we observed centrosome defocusing when Rho-ROCK signaling was inhibited by C3-Transferase or Y27632 (Figure 3-33A). The mean metaphase centrosome spread length of Rho or ROCK inhibited cells was significantly higher than cells treated with DMSO (Figure 3-33C). However, we did not observe a significant increase in the proportion of metaphase cells displaying multi-polar spindle defects in C3 Transferase or Y27632, compared to control cells (Figure 3-33B). These findings suggest that inhibiting Rho-ROCK signaling results in centrosome defocusing but not multi-polar spindle formation.

We then proceeded to investigate if Rho or ROCK functions upstream of LIMK1 in regulating centrosomal protein accumulation on metaphase spindle poles. To achieve this objective, we examined the fluorescence intensity of centrosomal
protein at the spindle poles after Rho or ROCK inhibitor treatment. We focused our efforts on AurkB, γ-Tubulin, NuMA, Pericentrin, PLK1, and TubGCP2 fluorescence intensities as these proteins were examined in our earlier characterization of mitotic spindle apparatus defects in LIMK1-depleted cells (Figure 3-34 – Figure 3-36). Cells treated with DMSO were used as control for comparison. From the fluorescence intensities measurement, we observed that neither C3-Transferase nor Y27632 treatment significantly affect the fluorescence intensities of the above mentioned proteins at the metaphase centrosome, compared to control cells (Figure 3-37). This finding suggests that Rho-ROCK signaling is not involved in regulating centrosomal protein accumulation at the metaphase spindle poles.

Taken together, our data suggest that inhibiting Rho-ROCK signaling could potentially affect some aspects of centrosome integrity. However, inhibiting Rho-ROCK signaling does not fully replicates the mitotic spindle apparatus defects observed in LIMK1-depleted cells. Therefore, Rho-ROCK signaling might not function upstream of LIMK1 in regulating centrosome integrity. Our findings also suggest that other signaling pathways might be regulating the function of LIMK1 during mitosis.
Figure 3-33. Inhibiting Rho-ROCK signaling causes centrosome defocusing. (A) HeLa cells were seeded onto acid washed glass coverslips and treated with the respective chemicals for 2 hours. Treated cells were then harvested for immuno-fluorescence staining with Pericentrin and α-Tubulin to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images from the respective treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm. (B) Cells were treated as described in (A) and the number of mitotic cells displaying multi-polar spindles was counted. The mean proportion of mitotic cell displaying multi-polar
spindle phenotypes were calculated and plotted. Experiment was performed in triplicates; n=300. (C) Cells were treated as described in (A) and the metaphase centrosome spread length was measured as described in Methods section. The mean metaphase centrosome length were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. **** represents p ≤ 0.0001. n.s represents p ≥ 0.05.

3.1.12 DYNC1LI1 and 2 are able to rescue the centrosome integrity defects observed in LIMK1-depleted cells

Our earlier data suggest that LIMK1-depletion negatively affect AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 at mitotic centrosome (Figure 3-15A – E). However, centrosomal accumulation of TubGCP2 at the centrosome was not affected in LIMK1 siRNA treated cells (Figure 3-15F). Interestingly, all the centrosomal proteins, except for TubGCP2, we selected for our analysis were reported cargoes of cytoplasmic dynein 1 (Ma et al., 2010; Merdes et al., 2000; Merdes et al., 1996; Yeh et al., 2013; Young et al., 2000). Cytoplasmic dynein 1 light intermediate chain 1 and 2 (DYNC1LI1 and DYNC1LI2) are two components of the multiple subunits that make up cytoplasmic dynein 1 motor complex. DYNC1LI1 and DYNC1LI2 are reported to define cargo specificity of cytoplasmic dynein 1 (Palmer et al., 2009; Purohit et al., 1999; Schmoranzer et al., 2009; Tynan et al., 2000). In addition, both cytoplasmic dynein 1 light intermediate chains are reported to localize onto the mitotic spindle apparatus and regulate key M-phase processes (Horgan et al., 2011; Palmer et al., 2009; Sivaram et al., 2009). Therefore, we hypothesized that DYNC1LI1 and DYNC1LI2 might function downstream of LIMK1 to regulate the transportation of centrosomal proteins during mitosis. This event would in turn help to maintain the integrity of mitotic centrosome.
Figure 3-34. Rho-ROCK signaling does not affect AurkA and γ-Tubulin accumulation at the mitotic spindle poles. HeLa cells were seeded onto acid washed coverslips and treated with the stated chemical for 2 hours. Treated cells were then harvested for immuno-fluorescence staining with AurkA (A) and γ-Tubulin (B) to visualize mitotic centrosome. Mitotic spindle were visualized by immuno-staining with α-Tubulin. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images from respective treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm.
Figure 3-35. Rho-ROCK signaling does not affect NuMA and Pericentrin accumulation at the mitotic spindle poles. HeLa cells were seeded onto acid washed coverslips and treated with the stated chemical for 2 hours. Treated cells were then harvested for immuno-fluorescence staining with NuMA (A) and Pericentrin (B) to visualize mitotic centrosome. Mitotic spindle were visualized by immuno-staining with α-Tubulin. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images from respective treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 μm.
Figure 3-36. Rho-ROCK signaling does not affect PLK1 and TubGCP2 accumulation at the mitotic spindle poles. HeLa cells were seeded onto acid washed coverslips and treated with the stated chemical for 2 hours. Treated cells were then harvested for immuno-fluorescence staining with PLK1 (A) and TubGCP2 (B) to visualize mitotic centrosome. Mitotic spindle were visualized by immuno-staining with α-Tubulin. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images from respective treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 μm
Figure 3-37. Quantification of various centrosomal proteins intensities at the mitotic spindle poles. HeLa cells were treated as described in Figure 3-33 – 3-35. The fluorescence intensity of AurkA (A), γ-Tubulin (B), NuMA (C), Pericentrin (D), PLK1 (E), and TubGCP2 (F) were measured and calculated as described in Methods section. The calculated intensity was normalized against the area of the selected foci and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. n.s represents p ≥ 0.05.

To investigate if DYN1L1 and DYN1L2 functions downstream of LIMK1 in regulating mitotic centrosome integrity, we introduced FLAG tagged DYN1L1 and DYN1L2 constructs into LIMK1 siRNA treated cells. We then proceeded to observe for any mitotic centrosome defects using indirect immuno-fluorescence microscopy. Cell co-transfected with control siRNA and GST-FLAG construct served as control for comparison. The knock-down efficacy and expression of the various FLAG tagged constructs were examined with western blot analysis (Figure 3-38). From the microscopy data, we observed that the proportion of cells displaying multi-
polar spindle decreased when DYNC1LI1 and DYNC1LI2 were introduced into LIMK1-depleted cells [11.7% (± 3.4%) and 14.8% (± 6.5%), respectively] (Figure 3-39 and 3-40A). This decrease was significant when compared to cells co-transfected with LIMK1 siRNA and GST-FLAG [11.7% (± 3.4%) vs 72.0 (± 6.0%); p ≤ 0.001 and 14.8% (± 6.5%) vs 72.0 (± 6.0%); p ≤ 0.001] (Figure 3-40A). Besides reducing the number of cells displaying multi-polar spindle, introducing either DYNC1LI1 or DYNC1LI2 were able to reduce the mean metaphase centrosome spread length (Figure 3-40B). The reduction in mean metaphase centrosome spread length was significant when compared to cells co-transfected with LIMK1 siRNA and GST-FLAG (3.01 µm (± 0.32 µm) vs 5.34 µm (± 1.50 µm); p ≤ 0.0001 and 3.26 µm (± 0.41 µm) vs 5.34 µm (± 1.50 µm); p ≤ 0.0001). These observations suggest that DYNC1LI1 and 2 could rescue the defects in LIMK1-depleted cells. In addition, DYNC1LI1 and 2 could potentially be novel substrates functioning downstream of LIMK1 in regulating mitotic centrosome integrity.

Figure 3-38. LIMK1 siRNA efficacy and expression level of various FLAG-tagged constructs. HeLa cells were co-transfected with the respective combination of siRNA and DYNC1LIs constructs for 48 hours. Transfected cells were then harvested for western blot analysis. Cell lysates were probed with LIMK1 and FLAG antibodies to detect the endogenous LIMK1 protein level and expression of DYNC1LIs constructs, respectively. GAPDH were probed to serve as loading control.
Figure 3-39. Introducing DYNC1LI1 and 2 into LIMK1-depleted cells rescued the mitotic defects. HeLa cells were seeded onto acid-washed glass coverslips and transfected with their respective combination of siRNA and DYNC1LIs constructs for 48 hours. Treated cells were then harvested for immuno-fluorescence staining with Pericentrin and α-Tubulin antibodies to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images for the respective treatment and phenotypes were processed with ImageJ and presented. Scale Bar = 10 µm.
Figure 3-40. Quantification of cells displaying multi-polar spindle and centrosome defocusing. (A) Cells were treated as described in Figure 3-39 and the number of mitotic cells displaying multi-polar spindles was counted. The mean proportion of mitotic cell displaying multi-polar spindle phenotypes were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. (B) Cells were treated as described in Figure 3-39 and the metaphase centrosome spread length was measured as described in Methods section. The mean metaphase centrosome spread length were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. *** represent $p \leq 0.001$; **** represents $p \leq 0.0001$.

3.1.13 DYNC1LI1 and 2 restore the levels of centrosomal proteins at the spindle poles in LIMK1-depleted cells

Next, we investigated if DYNC1LI1 and 2 could restore the centrosomal protein levels at the spindle poles in LIMK1-depleted cells. To achieve that objective, we introduced either FLAG-tagged DYNC1LI1 or DYNC1LI2 into LIMK1 siRNA treated cells. The transfected cells were then used for indirect immuno-fluorescence microscopy and the fluorescence intensities of the centrosomal proteins were calculated as described in previous section [Figure 3-41 (only γ-Tubulin staining were shown)]. Cells co-transfected with control siRNA and GST-FLAG construct served as control for comparison. Similar to earlier experiments, we focused our
effort on the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, PLK1, and TubGCP2 at the mitotic spindle poles. From our fluorescence intensity measurement, we observed that the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin and PLK1 at spindle poles were partially restored to control level when DYNC1LI1 or DYNC1LI2 were introduced into LIMK1-depleted cells (Figure 3-42A – E). The restoration in fluorescence intensity levels was significant when compare to the fluorescence intensity of cells co-transfected with LIMK1 siRNA and GST-FLAG. Our observations suggest that introducing DYNC1LI1 and DYNC1LI2 restore centrosomal protein accumulation at the mitotic spindle poles in LIMK1 depleted cells. In addition, both DYNC1LIs could potentially function downstream of LIMK1 in regulating the transportation of centrosomal protein to spindle poles.

3.1.14 RNAi-mediated DYNC1LI1 and 2 depletion results in mitotic spindle apparatus defects similar to LIMK1 siRNA treatment

Earlier we showed that introducing DYNC1LI1 and 2 into LIMK1-depleted cells reduced metaphase centrosome defocusing and the proportion of metaphase cells forming multi-polar spindles. In addition, we demonstrated that both dynein light intermediate chains restored the centrosomal protein levels at mitotic spindle poles in LIMK1 siRNA treated cells. These data suggest that DYNC1LI1 and 2 could function downstream of LIMK1 in regulating centrosome integrity. Therefore, we decided to determine if knocking down endogenous DYNC1LI1 and 2 would lead to similar defective phenotype(s) as LIMK1 depletion.
Figure 3-41. Introducing DYNC1LI1 and 2 into LIMK1-depleted cells could restore PCM protein accumulation at the mitotic centrosome. HeLa cells were seeded onto acid-washed glass coverslips and transfected with their respective combination of siRNA and DYNC1LIs constructs for 48 hours. Treated cells were then harvested for immuno-fluorescence staining with γ-Tubulin and α-Tubulin antibodies to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of the respective treatment and phenotypes were processed with ImageJ and presented. Scale Bar = 10 µm.
Figure 3-42. Quantification of various centrosomal protein intensities at the mitotic spindle poles. HeLa cells were treated as described in Figure 3-41. The fluorescence intensities of AurK\(A\) (A), γ-Tubulin (B), NuMA (C), Pericentrin (D), PLK1 (E), and TubGCP2 (F) at mitotic centrosome were measured and calculated as described in Methods section. The calculated intensity was normalized against the area of the selected foci and plotted Experiment was performed in triplicates; \(n=300\). The error bars represent standard deviation. a.u. represents arbitrary unit. **** represents \(p \leq 0.0001\); n.s represents \(p \geq 0.05\).

Firstly, we designed siRNA specifically against either DYNC1LI1 or DYNC1LI2, and tested their specificity and efficiency using western blotting. Our western blot analysis revealed that both DYNC1LI1 and DYNC1LI2 siRNA were able to specifically reduce the endogenous protein level of their intended targets (Figure 3-43A). Next, we proceeded to knock down DYNC1LI1 and DYNC1LI2 in HeLa cells, and observed for centrosome defects using indirect immuno-fluorescence staining. Cells transfected with siRNA targeting Luciferase serve as negative control for
comparison. In control cells, we observed that about 6.0% (± 2.0%) of the control cells forms multi-polar spindles. In contrast, about 70.0% (± 3.0%) of DYNC1LI1 siRNA treated cells and 56.0% (± 3.0%) of DYNC1LI2 siRNA treated cells were observed to form multi-polar spindles. This increase was significantly higher when compared to Luciferase siRNA treated cells (70.0% (± 3.0%) vs 6.0% (± 2.0%), p ≤ 0.0001; 56.0% (± 3.0%) vs 6.0% (± 2.0%), p ≤ 0.001).

We next examined the mean metaphase centrosome spread length. Similar to our earlier findings, the mean metaphase centrosome spread length was about 1.9 μm (± 0.6 μm). When cells were treated with either DYNC1LI1 (5 μm ± 0.5 μm vs 1.9 μm ± 0.6 μm; p ≤ 0.0001) or DYNC1LI2 (5.8 μm ± 0.7 μm vs 1.9 μm ± 0.6 μm; p ≤ 0.0001) the mean metaphase centrosome spread length was significantly increased when compared to control cells. These observations suggest that depleting either one of the dynein light intermediate chains lead to centrosome defocusing.

Lastly, we proceeded to investigate if depleting DYNC1LI1 and DYNC1LI2 would result in lower centrosomal protein accumulation at the mitotic spindle poles. HeLa cells transfected with control, DYNC1LI1 or DYNC1LI2 siRNA, and collected to perform immuno-fluorescence staining using antibodies to the various centrosomal proteins [Figure 3-45 (only γ-Tubulin staining was shown)]. Similar to earlier experiments, we focused our effort on the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1. The fluorescence intensity of TubGCP2 would serve as a control because it was not reported to be a cargo of cytoplasmic dynein 1 during mitosis. From our fluorescence intensities measurement, we observed that the fluorescence intensities of the above mention proteins at the mitotic spindle poles were reduced in either DYNC1LI1 or DYNC1LI2 siRNA treated cells. This reduction in fluorescence intensities was significant compared to intensities of control cells. In agreement with our hypothesis, we did not observed any significantly changes to the fluorescence intensity of TubGCP2 at the mitotic centrosome when cells were treated with either DYNC1LI1 or DYNC1LI2 siRNA.
Thus, suggesting that cytoplasmic dynein light intermediate chain depletion has a negative effect on centrosomal protein accumulation at the mitotic spindle poles.

Taken together, our data suggest that either DYNC1LI1 or DYNC1LI2 depletion results in centrosome defocusing and multi-polar spindle formation. Similar to when LIMK1 is depletion; treating cells with either DYNC1LI1 or DYNC1LI2 siRNA also reduce the accumulation of proteins at the mitotic centrosome. These findings further supports our hypothesis that DYNC1LI1 and DYNC1LI2 function downstream of LIMK1 to regulate mitotic centrosome integrity.
Figure 3-43. DYNC1LI1 or DYNC1LI2 depletion leads to centrosome defocusing and multi-polar spindle formation. (A) HeLa cells were transfected with the respective siRNAs and harvested for
western blot analysis to determine endogenous DYNC1LI1 and DYNC1LI2 protein level. Endogenous GAPDH protein was probed to serve as loading control. (B) HeLa cells were seeded onto acid-washed glass coverslips and transfected with the respective siRNA for 48 hours. Treated cells were then harvested for immuno-fluorescence staining with Pericentrin and α-Tubulin antibodies to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images for each treatment and phenotype were processed with ImageJ and presented. Scale Bar = 10 µm.

Figure 3-44. Quantification of mitotic cells displaying multi-polar spindle and centrosome defocusing. (A) Cells were treated as described in Figure 3-43 and the number of mitotic cells displaying multi-polar spindles was counted. The mean proportion of mitotic cell displaying multi-polar spindle phenotype was calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. (B) Cells were treated as described in Figure 3-43 and the metaphase centrosome spread length was measured as described in Methods section. The mean metaphase centrosome spread length was calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. *** represent p ≤ 0.001; **** represents p ≤ 0.0001.
Figure 3-45. DYNC1LI1 and DYNC1LI2 depletion negatively affects PCM protein accumulation at mitotic centrosome. HeLa cells were seeded onto acid-washed glass coverslips and transfected with the respective siRNAs for 48 hours. Treated cells were then harvested for immuno-fluorescence staining with γ-Tubulin and α-Tubulin antibodies to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images for each treatment and phenotype were processed using ImageJ and presented. Scale Bar = 10 µm.
3.1.15 Inhibiting the motor activity of cytoplasmic dynein with Ciliobrevin D leads to multi-polar spindle formation and centrosome defocusing

Our findings in earlier section demonstrated that depleting DYNC1LI1 and DYNC1LI2, which are subunits of cytoplasmic dynein 1, resulted in centrosome defocusing and multi-polar spindle formation. In addition, we observed lower centrosomal protein accumulation at the centrosomes when DYNC1LI1 and DYNC1LI2 were depleted. This led us to hypothesize that cytoplasmic dynein 1...
could also be involved in regulating mitotic centrosome integrity via its ability to regulate the transportation of centrosomal proteins to the mitotic spindle poles. To test this hypothesis, we treated HeLa cells with Ciliobrevin D, a chemical reported to inhibit cytoplasmic dynein 1 motor activity (Firestone et al., 2012). HeLa cells were treated with 25 μM of Ciliobrevin D for 2 hours for our experiment as earlier study has shown that this treatment condition is able to inhibit cytoplasmic dynein 1 motor activity (Firestone et al., 2012). DMSO, the solvent used to solubilize the inhibitor, served as negative control. Ciliobrevin D and DMSO treated cells were then observed for any mitotic centrosome defects using indirect immuno-fluorescence microscopy (Figure 3-47A). Our microscopy data revealed that about 63% (± 14%) of cells treated with Ciliobrevin D forms multi-polar spindles, compared to about 7% (± 3%) of cells treated with DMSO (p ≤ 0.01) (Figure 3-47B). The mean metaphase centrosome spread length of Ciliobrevin D treated cells was also significantly longer than that of the control cells (3.6 μm ± 0.9 μm vs 2.2 μm ± 0.6 μm, p ≤ 0.0001) (Figure 3-47B). These findings suggest that inhibiting cytoplasmic dynein motor activity results in centrosome defocusing and multi-polar spindle formation.

Next, we measured the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 at the mitotic spindle poles to determine if Ciliobrevin D treatment affects protein accumulation at the mitotic centrosome (Figure 3-48 – 3-50). As TubGCP2 was not reported to be an M-phase cargo of cytoplasmic dynein 1, the fluorescence intensity of TubGCP2 at the mitotic spindle poles served as a control. Consistent with our hypothesis, the fluorescence intensities of AurkA, γ-Tubulin, NuMA, Pericentrin, and PLK1 at the mitotic spindle poles were significantly reduced when cells were treated with Ciliobrevin D compared to cells treated with DMSO (Figure 3-51A – E). The observations suggest that cytoplasmic dynein motor activity is important for accumulation of certain centrosomal proteins at the mitotic centrosomes. The fluorescence intensity of TubGCP2 at the mitotic spindle poles was not significantly affected by Ciliobrevin D treatment, confirming that TubGCP2 accumulation is not regulated by cytoplasmic dynein 1 (Figure 3-51F).
Taken together, these data suggest that cytoplasmic dynein motor activity is involved in regulating centrosome integrity and proper bipolar formation. In addition, cytoplasmic dynein is important for regulating the accumulation of centrosomal proteins at the mitotic spindle poles.

Figure 3-47. Inhibition of the dynein motor leads to mitotic spindle defects. (A) HeLa cells were seeded onto acid-washed glass coverslips and treated with Ciliobrevin D or DMSO for 2 hours. Treated cells were then immuno-stained with Pericentrin and α-Tubulin antibodies to visualize centrosome and mitotic spindle, respectively. Mitotic chromosomes were stained with DAPI. Immuno-
stained samples were then observed under 63X objective lens. Representative images for each treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 μm. (B) Cells were treated as described in (A) and the number of mitotic cells displaying multi-polar spindles was counted. The mean proportion of mitotic cell displaying multi-polar spindle phenotype was calculated and plotted. Experiment was performed in triplicates; n=300. (C) Cells were treated as described in (A) and the metaphase centrosome spread length was measured as described in Methods section. The mean metaphase centrosome spread length were calculated and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. ** represents p ≤ 0.01; **** represents p ≤ 0.0001.

3.1.16 DYNC1LI1 and DYNC1LI2 interact with the kinase domain of LIMK1.

As DYNC1LI1 and DYNC1LI2 were able to rescue the defects observed in LIMK1 knocked down cells, we hypothesized that both DYNC1LI1 and DYNC1LI2 could potentially function downstream of LIMK1. To test this hypothesis, we proceeded to examine if DYNC1LI1 and DYNC1LI2 were interacting partners of LIMK1. FLAG-tagged DYNC1LI1 and DYNC1LI2 were co-transfected with either GST or GST-tagged LIMK1 into HEK 293 cells. The cell lysate were then subjected to GST pull-down assay. HEK 293 cells co-transfected with GST-LIMK1 and FLAG-tagged Centrin 2 served as negative control. From the GST pull-down analysis, we observed that both FLAG-tagged DYNC1LI1 and DYNC1LI2 co-precipitated with LIMK1 (Figure 3-52 and 3-53), suggesting that DYNC1LI1 and DYNC1LI2 were found in the same complex as LIMK1 and could be potential interacting partners of LIMK1.
Figure 3-48. Inhibiting cytoplasmic dynein 1 motor activity negatively affects AurkA and γ-Tubulin accumulation at mitotic centrosome. HeLa cells were seeded onto acid washed coverslips and treated with either DMSO or Ciliobrevin D for 2 hours. Treated cells were then harvested for immuno-fluorescence staining with AurkA (A) and γ-Tubulin (B) to visualize mitotic centrosome. Mitotic spindle were visualized by immuno-staining with α-Tubulin. Mitotic chromosomes were visualized with DAPI stain. Representative images for each treatment and phenotype were processed using ImageJ and presented. Immuno-stained samples were then observed under 63X objective lens. Scale Bar: 10 µm.
Figure 3-49. Inhibiting cytoplasmic dynein 1 motor activity negatively affects NuMA and Pericentrin accumulation at mitotic centrosome. HeLa cells were seeded onto acid washed coverslips and treated with either DMSO or Ciliobrevin D for 2 hours. Treated cells were then harvested for immuno-fluorescence staining with NuMA (A) and Pericentrin (B) to visualize mitotic centrosome. Mitotic spindle were visualized by immuno-staining with α-Tubulin. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images for each treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm.
Figure 3-50. Inhibiting cytoplasmic dynein 1 motor activity negatively affects PLK1 accumulation at mitotic centrosome. HeLa cells were seeded onto acid washed coverslips and treated with either DMSO or Ciliobrevin D for 2 hours. Treated cells were then harvested for immuno-fluorescence staining with PLK1 (A) and TubGCP2 (B) to visualize mitotic centrosome. Mitotic spindle were visualized by immuno-staining with α-Tubulin. Mitotic chromosomes were visualized with DAPI stain. Immuno-stained samples were then observed under 63X objective lens. Representative images for each treatment and phenotype were processed using ImageJ and presented. Scale Bar: 10 µm.
Figure 3-51. Quantification of PCM proteins intensities at the mitotic centrosome. HeLa cells were treated as described in Figure 3-47 – 3-49. The fluorescence intensities of AurkA (A), γ-Tubulin (B), NuMA (C), Pericentrin (D), PLK1 (E), and TubGCP2 (F) at mitotic centrosome were measured and calculated as described in Methods section. The calculated intensity was normalized against the area of the selected foci and plotted. Experiment was performed in triplicates; n=300. The error bars represent standard deviation. **** represents $p \leq 0.0001$. n.s represents $p \geq 0.05$.

To narrow down the domain on LIMK1 which could potentially interact with DYNC1LI1 and DYNC1LI2, we generated two GST tagged LIMK1 constructs containing either the PDZ-LIM domains or the kinase domain (Figure 3-54). We then performed the same GST pull-down analysis to determine which domain of LIMK1 was responsible for the interaction with DYNC1LI1 and DYNC1LI2. From the GST-pull down analysis, we observed that FLAG-tagged DYNC1LI1 and DYNC1LI2 co-precipitated with LIMK1 kinase domain, but not with the PDZ-LIM domains (Figure 3-
This observation suggests that LIMK1 interacts with DYNC1LI1 and DYNC1LI2 through its kinase domain.

In our GST pull-down analysis, we observed that DYNC1LI1 co-precipitated with full length LIMK1 construct more efficiently when compared with kinase domain alone (Figure 3-52). Interestingly, the reverse was observed when the GST pull-down assay was performed with DYNC1LI2 (Figure 3-53). The LIM-PDZ domains of LIMKs are involved in regulating the interaction between LIMKs and its binding partners (Gorovoy et al., 2005; Yang et al., 2004b; Yokoo et al., 2003). Therefore, it is likely that the LIM-PDZ domains of LIMK1 play a similar role in our study. Part of the LIM-PDZ domain might be needed to mediate the interaction between LIMK1 and DYNC1LI1. Therefore, the kinase domain alone could not co-precipitate as efficiently as the full-length. However, the reverse is true for the interaction between LIMK1 and DYNC1LI2. The LIM-PDZ could have an inhibitory effect on the interaction between LIMK1 and DYNC1LI2. Therefore, the kinase domain alone is sufficient to co-precipitate DYNC1LI2 efficiently compared to full-length LIMK1.

Over-expression of exogenous fusion proteins has been reported to result in false positive interactions (Mellacheruvu et al., 2013). To verify the interaction between LIMK1 and DYNC1LIs, we proceeded to perform endogenous protein immunoprecipitation assay. As we were interested in the interaction between LIMK1 and DYNC1LIs during M-phase, HeLa cells were first synchronized to M-phase with nocodazole. Synchronized HeLa cell lysates were collected and incubated with either DYNC1LI1 or DYNC1LI2 IgG antibodies for immunoprecipitation assay (Figure 3-55). From the immunoprecipitation analysis, we observed that LIMK1 was co-immunoprecipitated with both DYNC1LI1 and DYNC1LI2 antibodies (Figure 3-55). LIMK2, a closely related protein kinase of LIMK1, was not co-immunoprecipitated. These data suggest that LIMK1, but not LIMK2, interacts with both DYNC1LI1 and DYNC1LI2 during M-phase. In addition, we observed a similar interaction affinity difference between LIMK1-DYNC1LI1 and LIMK1-DYNC1LI2 in our immunoprecipitation assay (Figure 3-52 – 3-53 and 3-54). This observation further supports our hypothesis that LIM-PDZ domains of LIMK1 play a role in
mediating the interaction between LIMK1 and the cytoplasmic dynein light intermediate chains.

**Figure 3-52. DYNC1LI1 interacts with the kinase domain of LIMK1.** HEK 293 cells were transfected with the respective combinations of LIMK1 and DYNC1LI1 constructs. 24 hours after transfection, cell lysates were harvested and subjected to GST-pull-down. Total lysates and GST-pull-down fractions were subjected to western blot analysis. Lysate and GST-pull-down fractions were probed with FLAG and GST antibodies to detect DYNC1LI1 and LIMK1 constructs, respectively.
Figure 3-53. DYNC1LI2 interacts with the kinase domain of LIMK1. HEK 293 cells were transfected with the respective combinations of LIMK1 and DYNC1LI2 constructs. 24 hours after transfection, cell lysates were harvested and subjected to GST-pull-down. Total lysates and GST-pull-down fractions were subjected to western blot analysis. Lysate and GST-pull-down fractions were probed with FLAG and GST antibodies to detect DYNC1LI2 and LIMK1 constructs, respectively.
Figure 3.54. Summary of interaction between DYNC1LI1 and DYNC1LI2, and various LIMK1 constructs. N and C represent N-terminus and C-terminus, respectively. The number above the constructs represents the amino acid residue number.

Figure 3.55. LIMK1 interacts with DYNC1LI1 and DYNC1LI2 during M-phase. HeLa cells were first synchronized to M-phase by treating the cells with nocodazole for 16 hours. After synchronization, cell lysates were collected and incubated with either DYNC1LI1 (left panel) or DYNC1LI2 (right panel) antibodies for endogenous immuno-precipitation assay. The immuno-precipitation assay were then subjected to western blot analysis and probed for endogenous LIMK1, LIMK2, and the respective DYNC1LIs. Non-immunized rabbit IgG antibodies were included as negative control for the immuno-precipitation assay.

3.1.17 LIMK1 depletion results in changes to DYNC1LI1 and DYNC1LI2 mitotic phosphorylation profile

Our results reported in the earlier sections showed that DYNC1LI1 and DYNC1LI2 could potentially function downstream of LIMK1 in regulating centrosome integrity. We had also shown that LIMK1 kinase activity was important for regulating centrosome integrity. Interestingly, both dynein light intermediate chains were also observed to interact with the kinase domain of LIMK1. Therefore, we hypothesized that DYNC1LI1 and DYNC1LI2 could be potential downstream substrates of LIMK1 during M-phase. To test this hypothesis, we decided to examine the phosphorylation profiles of DYNC1LI1 and DYNC1LI2 in LIMK1 siRNA treated cells. PhosTag poly-acrylamide gel electrophoresis (PhosTag PAGE) is a useful method to study the
phosphorylation status and profile of endogenous proteins (Messer et al., 2009). Metal ions present in PhosTag PAGE interact with the phosphate groups added onto phosphorylated proteins. This slows the migration rate of phosphorylated proteins through the PhosTag PAGE gel compared to un-phosphorylated proteins. In addition, the migration rate of phosphorylated proteins also depends on the number of phosphorylated residues present on the proteins. A protein with two or more phosphorylated residues migrates slower through the PhosTag PAGE gel compared to a protein with only one phosphorylated residue. The changes in migration through the PhosTag PAGE gel would provide us a good estimate on the phosphorylation profile of the protein of interest.

To view the phosphorylation profiles of DYNC1LI1 and DYNC1LI2, HeLa cells were first treated with either LIMK1 or control siRNA. Transfected cells were then synchronized to M-phase with nocodazole. Synchronized cell lysates were then harvested and subjected to PhosTag PAGE and western blot analysis. LIMK2 and AurkA siRNAs were used as additional negative controls to determine if any changes to DYNC1LI1 and DYNC1LI2 phosphorylation profiles were specific to LIMK1 depletion. The specificity and efficacy of the various siRNAs were examined using western blot analysis (Figure 3-56A). From the PhosTag PAGE analysis, we observed several slower migrating DYNC1LI1 and DYNC1LI2 bands that were present in M-phase, but not in interphase cell lysates (Figure 3-56B). This observation suggests that some residues or combination of residues on DYNC1LI1 and DYNC1LI2 are specifically phosphorylated during mitosis. When cells were transfected with either AurkA or LIMK2 siRNAs, the mitotic phosphorylation profiles of DYNC1LI1 and DYNC1LI2 were similar to cells treated with control siRNA (Figure 3-56B). This observation suggests that both AurkA and LIMK2 might not be an upstream kinase of DYNC1LIs. In contrast, the mitotic phosphorylation profiles of both dynein light intermediate chains were altered when cells were transfected with LIMK1 siRNA. This observation suggests that LIMK1 depletion affects the phosphorylation of DYNC1LI1 and DYNC1LI2. Together with the results obtained from GST pull-down assay, it is reasonable to speculate that dynein light intermediate chains are potential substrates of LIMK1.
Figure 3-56. LIMK1 depletion affects dynein light intermediate chains phosphorylation. HeLa cells were treated with the respective siRNAs and synchronized at M-phase with nocodazole. Synchronized cell lysate were then collected and the efficacies of the respective siRNAs were investigated using normal SDS-PAGE (A). The phosphorylation profiles of DYNC1L1 and 2 were investigated using PhosTag PAGE (B). Both acrylamide gels were then subjected to western blot analysis. Cyclin-B1 and phospho-Histone 3 levels serve as M-phase marker. Endogenous GAPDH levels served as loading control.
3.2 The role of LIMK1 in regulating spindle orientation and positioning

Our findings in previous section showed that LIMK1 is involved in regulating mitotic centrosome integrity. LIMK1 could potentially regulate centrosome integrity through its action on cytoplasmic dynein light intermediate chains. These subunits of cytoplasmic dynein would then regulate the accumulation of centrosomal proteins, which are important for regulating centrosome integrity, at the mitotic spindle pole.

In this part of the project, we went further to investigate if LIMK1 performed additional role during mitosis. Proper cortical actin cytoskeleton organization had been shown to be important for proper mitotic spindle orientation and positioning (Carreno et al., 2008). Given the importance of LIMK1 in regulating actin cytoskeleton dynamics (Manetti, 2012; Scott and Olson, 2007), we decided to investigate the role of LIMK1 in regulating mitotic spindle orientation and positioning.

3.2.1 Perturbing actin cytoskeleton dynamics results in mitotic spindle mis-positioning and mis-orientation

To investigate the role of actin cytoskeleton dynamics on mitotic spindle orientation, HeLa cells were treated with F-actin stabilizing drug (Jasplakinolide) or F-actin depolymerization drugs (C3-Transferase, Y27623, and Cytochalasin B). These treated cells were then subjected to indirect immuno-fluorescence staining with Pericentrin and subjected to z-stack microscopy analysis. Perturbing actin cytoskeleton dynamics of Interphase cells results in mitotic spindle formation defects (Huang and Ingber, 2002; Lee and Song, 2007; Reshetnikova et al., 2000). To minimize the possibility of these defects being carried over from Interphase to M-phase, we limited our drug treatment to two hours before immuno-staining. Metaphase cells treated with DMSO served as negative control. Fluorescent microscopy images of the z-stacks were taken and spindle orientations were then calculated as illustrated (Figure 3-57A).
Figure 3-57. Schematic explaining the evaluation of spindle orientation and positioning. (A) Immuno-stained samples were imaged for z-stack at 0.5 μm distance apart. The spindle orientation, relative to the substratum, was then determined by calculating the spindle angle (θ) using inverse trigonometry function, $\theta = \tan^{-1} (A / B)$, where θ 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. (B) To examine if the mitotic spindle was positioned in the center of the cell, the centroid of the mitotic cell and spindle apparatus were determined using ImageJ software. Spindle positioning was then determined by calculating how far the spindle centroid deviates from the metaphase cell centroid.

From the images of the z-stacks, we observed that the two mitotic centrosomes of control cells were focused on the same z-plane for most cells (Figure 3-58). This observation was support by our measurement of spindle angles. We found that the mean spindle angle (θ) of control cells was 6° (± 3.7°) (Figure 3-59), suggesting that the control metaphase spindle orientation is positioned relatively parallel to the substratum. In contrast, we observed that the mitotic centrosomes of cells treated with actin perturbing drugs were not focused on the same z-plane in most of the cells.
The mean spindle angles of cells treated with actin perturbing drugs were also significantly larger than those of the control cells (Jasplakinolide mean $\theta = 18.0^\circ \pm 8.6^\circ$; C3-Transferase mean $\theta = 18.2^\circ \pm 8.3^\circ$; Y27632 mean $\theta = 16.5^\circ \pm 9.0^\circ$; Cytochalasin B mean $\theta = 18.4^\circ \pm 8.3^\circ$) (Figure 3-59). These data suggest that the mitotic spindle apparatus of cells treated with drugs, which perturb the actin cytoskeleton dynamics, are not positioned parallel to the substratum.

We then proceeded to investigate if perturbing the actin cytoskeleton dynamics would result in spindle mis-positioning. HeLa cells were treated with the actin perturbing drugs as described earlier and immuno-stained with $\alpha$-Tubulin and Phalloidin to visualize the mitotic spindles and cell boundaries, respectively (Figure 3-60). From our fluorescence microscopy images, we observed that the mitotic spindle apparatus of control cells were positioned at the center of cells (Figure 3-60A) In contrast, we observed that the mitotic spindle apparatus of cells treated with actin perturbing drugs we positioned closer to the cell periphery (Figure 3-60A). We decided to calculate how far the mitotic spindle apparatus were positioned away from the cell center (Figure 3-57B). From the measurement of spindle position, we found that the mitotic spindle apparatus of control cells was positioned at an average distance of 0.88 μm (± 0.45 μm) from the cell center (Figure 3-60B). This deviation from the cell center was significantly higher in cells treated with actin perturbing drugs (Jasplakinolide mean $d = 2.41 \mu m \pm 0.76 \mu m$; C3-Transferase mean $d = 2.40 \mu m \pm 0.80 \mu m$, Y27623 mean $d = 2.79 \mu m \pm 0.97 \mu m$, Cytochalasin B mean $d = 2.42 \mu m \pm 0.97 \mu m$), suggesting that the mitotic spindle apparatus are not positioned at the center of the cells (Figure 3-60B).
Figure 3-58. Perturbing actin dynamics results in mitotic spindle mis-orientation. HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with the respective actin perturbing drugs. Treated cells were then immuno-stained with Pericentrin to visualize centrosome. Mitotic chromosomes were stained with DAPI. Metaphase cells were imaged for z-stack at 0.5 μm distance apart. Numbers above the images indicate the Z-plane of the respective image.
Figure 3-59. Perturbing actin dynamics results in increased mitotic spindle angle. HeLa cells were treated as described in Figure 3-56. The spindle angles of each drug treatment were then calculated as described in the Methods section. The mean spindle angle ($\theta$) for each condition were then calculated and plotted. Experiment was performed in triplicates; n = 120. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent 5-95 percentile; black dots represent outliers; red dots represent the mean spindle angle. **** represents $p \leq 0.0001$.

Taken together, our data suggest that disruption of actin cytoskeleton dynamics results in mitotic spindle mis-orientation relative to the substratum. In addition, actin cytoskeleton dynamics is important for the mitotic spindle apparatus to position at the center of the cells.
Figure 3-60. Treatment with actin perturbing drugs results in mitotic spindle apparatus mis-positioning. (A) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with the respective actin perturbing drug. Treated cells were then immuno-stained with α-Tubulin and Phalloidin to visualize mitotic spindle and cell boundary, respectively. Mitotic chromosomes were stained with DAPI. Representative images of normal and off-center mitotic spindles were processed with ImageJ and presented. Scale Bar = 10 µm. (B) HeLa cells were treated as described in (A) and the distance between the center of the cell and mitotic spindle was measured. The mean distance from cell center was then calculated and plotted. All statistical analysis was compared against DMSO treated cells. Experiment was performed in triplicates; n = 120. The error bars represent standard deviation. **** represents p ≤ 0.0001.
3.2.2 Depleting LIMK1 results in mitotic spindle orientation and positioning defects.

The experimental results obtained and reported in previous sections showed that treatment with C3-Transferase and Y27623 resulted in mitotic spindle mis-orientation and mis-positioning. Since C3-Transferase and Y27623 are specific inhibitors of Rho proteins and ROCK, these observations suggest that Rho-ROCK signaling could be involved in regulating mitotic spindle orientation and positioning (Uehata et al., 1997; Wilde and Aktories, 2001). LIMK1 is one of the many downstream effectors of Rho-ROCK signaling in regulating actin cytoskeleton dynamics (Ohashi et al., 2000).

Therefore, we proceeded to investigate if LIMK1 is involved in regulating spindle orientation and positioning. To achieve this aim, we transfected HeLa cells with either control or LIMK1 siRNA and then immuno-stained the transfected cells with Pericentrin to visualize the mitotic centrosome. Z-stacks of immuno-stained samples were obtained and the spindle angles were then calculated (Figure 3-61). We observed that for control cells, both the mitotic centrosomes were in focused on the same z-plane (Figure 3-61A). However, the centrosomes of LIMK1 siRNA treated cells were not in focused in the same z-plane (Figure 3-61A). Analysis of their spindle angle revealed that the mean spindle angle (θ) of LIMK1 depleted cells was significantly higher than that of the control cells \[ θ = 16.3^\circ \pm 8.5^\circ \text{ vs } θ = 6.1^\circ \pm 4.3^\circ \]; \( p \leq 0.0001 \) (Figure 3-61B), suggesting that the mitotic spindle apparatus in LIMK1 depleted cells are not oriented parallel to the substratum.

We then proceeded to investigate if LIMK1 depletion would result in mitotic spindle mis-positioning. HeLa cells were transfected with the respective siRNA and immuno-stained with α-Tubulin and Phalloidin to visualize the mitotic spindles and cell boundaries. In control siRNA treated cells, the mitotic spindle apparatus were observed to position at the center of the cells (Figure 3-62A). However, the mitotic spindle apparatus of LIMK1 depleted cells were often observed to be closer to the cell periphery (Figure 3-62A). Detailed analysis revealed that the mitotic spindle of LIMK1 siRNA treated cells deviated further away from the cell center compared to control cells \[ 2.87 \mu m \pm 0.92 \mu m \text{ vs } 0.64 \mu m \pm 0.40 \mu m \]; \( p \leq 0.0001 \) (Figure 3-
62B). This finding suggests that the mitotic spindle apparatus in LIMK1 depleted cells is not positioned properly at the cell center.

Taken together, our RNAi experiment suggests that LIMK1 is involved in regulating mitotic spindle apparatus orientation and positioning.

3.2.3 LIMK1 functions downstream Rho-ROCK pathways in regulating mitotic spindle orientation and positioning

After establishing that LIMK1 is important for mitotic spindle orientation and positioning, we next investigate if LIMK1 functions downstream of Rho-ROCK signaling in regulating spindle orientation. Upon activation, Rho in turn activates ROCK kinase, leading to the phosphorylation of LIMK1 on Threonine 508 (Thr508) (Ohashi et al., 2000). Phosphorylation of Thr508 residue by ROCK kinase results in the activation of LIMK1 (Ohashi et al., 2000). To investigate if LIMK1 functions downstream of Rho-ROCK pathways, we generated a phospho-dead (T508A) and phospho-mimic (T508E) LIMK1 mutants. These mutants were then introduced into either C3-Transferase (Rho inhibitor) or Y27623 (ROCK inhibitor) treated cells. Cells treated with DMSO and GST-FLAG empty vector served as negative control for comparison. The treated and transfected cells were then imaged for spindle angle calculation (Figure 3-63 and 3-64).
Figure 3-61. LIMK1 depletion results in spindle mis-orientation. (A) HeLa cells were seeded onto Fibronectin coated glass coverslips and transfected with either control or LIMK1 siRNA. Transfected cells were then immuno-stained with Pericentrin to visualize centrosome. Metaphase cells were imaged for z-stack at 0.5 μm distance apart. Numbers above the images indicate the Z-plane of the respective image. (B) HeLa cells were treated as described in (A) and the mitotic spindle angles (θ) were calculated. The mean spindle angle were then calculated and plotted. Experiment was performed in triplicates; n = 120. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent 5-95 percentile; black dots represent outliers; red dots represent mean. **** represents p ≤ 0.0001.
Figure 3-62. LIMK1 depletion results in spindle mis-positioning. (A) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with the respective actin perturbing drug. Treated cells were then immuno-stained with α-Tubulin and Phalloidin to visualize mitotic spindle and cell boundary, respectively. Mitotic chromosomes were stained with DAPI. Representative images for each treatment were processed with ImageJ and presented. Scale Bar = 10 µm. (B) HeLa cells were treated as described in (A) and the distance between the center of the cell and mitotic spindle is measured. The mean distance from cell center were then calculated and plotted. All statistical analysis was compared against DMSO treated cells. Experiment was performed in triplicates; n = 120. The error bars represent standard deviation. **** represents p ≤ 0.0001.

The mitotic centrosome of cells treated with DMSO and transfected with GST-FLAG were focused on the same z-plane and the mean spindle angle was 6.0° (± 3.7°) and
6.1° (± 4.3°) (Figure 3-63 – 3-65). However, spindle angle of cells treated with C3-Transferase and transfected with GST-FLAG were significantly higher compared to control cells [θ = 17.3° (± 7.6°) vs θ = 6.0° (± 3.7°); p ≤ 0.0001] (Figure 3-63 and 3-65A). Similar observations were obtained for cells treated with ROCK inhibitors and transfected with GST-FLAG [θ = 16.3° (± 8.5°) vs θ = 6.1° (± 4.3°); p ≤ 0.0001] (Figure 3-64 and 3-65B). When LIMK1-T508E was introduced into C3-Transferase or Y27623 treated cells, the spindle angle was significantly reduced compared to when cells were transfected with GST-FLAG (Figure 3-63 – 3-65). In contrast, introducing phospho-dead form of LIMK1 into either Rho or ROCK inhibited cells did not significantly change the spindle angle when compared to cells transfected with GST-FLAG (Figure 3-65). These observations suggested that Rho-ROCK mediated phosphorylation of LIMK1 is important for regulating spindle orientation.

In addition to mitotic spindle orientation, we also observed a smaller deviation from cell center when LIMK1-T508E was introduced into C3-Transferase or Y27623 treated cells, compared to when GST-FLAG were introduced (Figure 3-66A and B). In contrast, LIMK1-T508A was not able to rescue the mis-positioning of the mitotic spindles when introduced into Rho or ROCK inhibited cells. These data lead us to conclude that Rho-ROCK mediated phosphorylation of LIMK1 is important for proper mitotic spindle apparatus positioning.
Figure 3-63. LIMK1 functions downstream of Rho signaling in regulating spindle orientation. HeLa cells were seeded onto Fibronectin coated glass coverslips, and treated with C3 Transferase and transfected with the respective LIMK1 constructs. Treated cells were then immuno-stained with Pericentrin to visualize centrosome. Metaphase cells were imaged for z-stack at 0.5 μm distance apart. Numbers above the images indicate the Z-plane of the respective image.
Figure 3-64. LIMK1 functions downstream of ROCK signaling in regulating spindle orientation. HeLa cells were seeded onto Fibronectin coated glass coverslips, and treated with Y27623 and transfected with the respective LIMK1 constructs. Treated cells were then immuno-stained with Pericentrin to visualize centrosome. Metaphase cells were imaged for z-stack at 0.5 μm distance apart. Numbers above the images indicate the Z-plane of the respective image.
Figure 3-65. Phospho-mimic LIMK1 mutant is able to rescue the mitotic spindle orientation defects in Rho and ROCK inhibited cells. (A and B) HeLa cells were treated as described in Figure 3-61 and 3-62. The spindle angles were then measured for each condition. The mean spindle angles ($\theta$) were then calculated and plotted. Experiment was performed in triplicate; n = 120. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent 5-95 percentile; black dots represent outliers; red dots represent mean. **** represents $p \leq 0.0001$; ns represents $p \geq 0.05$
Figure 3-66. Phospho-mimic LIMK1 mutant is able to rescue the mitotic spindle positioning defects in Rho and ROCK inhibited cells. (A and B) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with the respective drugs and constructs. Cells were then immune-stained with α-Tubulin and Phalloidin to visualize mitotic spindle and cell boundary, respectively. The distance between the center of the cell and mitotic spindle was measured. The mean distance from cell center was then calculated and plotted. Experiment was performed in triplicates; n = 120. The error bars represent standard deviation. The error bars represent standard deviation. **** represents p \leq 0.0001, ns represents p \geq 0.05.

3.3.3 The kinase activity of LIMK1 is important for regulating spindle orientation and positioning

As ROCK-mediated phosphorylation positively regulates LIMK1 kinase activity, we hypothesized that LIMK1 kinase activity is important for maintaining spindle orientation and positioning (Ohashi et al., 2000). To investigate the importance of LIMK1 kinase activity, wild-type (LIMK1-WT), kinase-dead (LIMK1-D460A) and constitutively active (LIMK1-T508EE) LIMK1 constructs were introduced into LIMK1-depleted cells. Cells transfected with control siRNA and GST-FLAG empty vectors were used as negative control for comparison. Transfected cells were then imaged at different z-plane and the spindle angles were analyzed (Figure 3-67A). From our analysis, we observed that the mean spindle angles were not significantly reduced when kinase dead mutant were introduced into LIMK1-depleted cells, compared to cells co-transfected with LIMK1 siRNA and GST-FLAG (Figure 3-67B). In contrast, the mean spindle angles were significantly reduced when LIMK1-WT and LIMK1-T508EE were introduced into LIMK1-depleted cells, compared to cells co-transfected with LIMK1 siRNA and GST-FLAG (Figure 3-67B). These observations suggest that the kinase activity of LIMK1 is important for cells to orientate their mitotic spindle apparatus parallel to the substratum.

Next, we investigated if LIMK1 functions downstream of Rho-ROCK pathway in regulating mitotic spindle apparatus positioning by analyzing how far the apparatus deviates from the cell center. From our data, we observed that only wild-type and
LIMK1-T508EE were able to rescue the mitotic spindle positioning defects observed in C3 Transferase and Y27623 treated cells (Figure 3-68A).

To confirm the importance of LIMK1 kinase activity, we proceeded to treat cells with Compound 22 [a chemical shown to inhibit LIMK kinase activity (Harrison et al., 2009)] and repeated the spindle orientation and positioning analysis. Consistent with earlier experimental results, treatment with Compound 22 significantly increased the mean spindle angle compared to DMSO (control) treated cells $\theta = 18.1^\circ \pm 5.9^\circ$ vs $\theta = 4.6^\circ \pm 4.5^\circ$; $p \leq 0.0001$ (Figure 3-67C). In addition, we observed that the spindle apparatus were positioned away from the center of metaphase cells $3.01 \mu m \pm 0.84 \mu m$ vs $0.75 \mu m \pm 0.43 \mu m$; $p \leq 0.0001$ (Figure 3-68B). This data further emphasizes the importance of LIMK1 kinase activity in regulating spindle orientation and positioning.

3.3.3 Cofilin functions downstream of Rho-ROCK-LIMK1 pathway in regulating mitotic spindle orientation and positioning

We next proceeded to determine the possible LIMK1 substrate that is responsible for the regulation of spindle orientation and positioning. Cofilin is a downstream effector of LIMK1 and required for regulating actin cytoskeleton dynamics (Agnew et al., 1995; Arber et al., 1998). Since our earlier data suggest that actin cytoskeleton dynamics is important for proper mitotic spindle orientation and positioning, it is reasonable to hypothesize that Cofilin could be functioning downstream of LIMK1 in regulating mitotic spindle orientation and positioning. The activity of Cofilin is negatively regulated by LIMK1-mediated phosphorylation on residue Serine 3 (Ser3) (Agnew et al., 1995). To test our hypothesis, we generated phospho-dead (Cofilin-S3A) and phospho-mimic (Cofilin-S3E) Cofilin mutant, and introduced them into LIMK1 depleted cells. Transfected cells were then imaged at different z-plane for further analysis.
Figure 3-67. LIMK1 kinase activity is important for maintaining spindle orientation. (A) HeLa cells were seeded onto Fibronectin coated glass coverslips and transfected with the respective combination of siRNAs and LIMK1 constructs. Transfected cells were then immuno-stained with Pericentrin to visualize centrosome. Mitotic chromosomes were visualized with DAPI. Metaphase cells were imaged at z-stack, which was 0.5 μm apart. Numbers above the images indicate the z-plane that the spindle pole is focused. Scale bar = 10 μm. (B) HeLa cells were treated as described in (A) and the spindle angles were measured. The mean spindle angle (θ) were calculated and plotted. Experiment was performed in triplicates; n = 120. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent 5-95 percentile; black dots represent outliers; red dots represent mean. (C) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with either DMSO or Compound 22. Treated cells were then immuno-stained.
with Pericentrin to visualize centrosome. Metaphase cells were imaged at z-plane, which was 0.5 μm distance apart and the spindle angle were calculated. The mean spindle angle ($\theta$) were then calculated and plotted. Experiment was performed in triplicates; n = 120. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent 5-95 percentile; black dots represent outliers; red dots represent mean. **** represents $p \leq 0.0001$; *** represents $p \leq 0.001$; ns represents $p \geq 0.05$.

Figure 3-68. LIMK1 kinase activity is important for maintaining spindle positioning (A) HeLa cells were seeded onto Fibronectin coated glass coverslips and transfected with the respective siRNA and constructs. Cells were then immuno-stained with α-Tubulin and Phalloidin to visualize mitotic spindle and cell boundary, respectively. The distance between the center of the cell and mitotic spindle was measured. The mean distance from cell center was then calculated and plotted. Experiment was performed in triplicates; n = 120. The error bars represent standard deviation. (B) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with DMSO or Compound 22. Cells were then immuno-stained with α-Tubulin and Phalloidin to visualize mitotic spindle and cell boundary, respectively. The distance between the center of the cell and mitotic spindle was measured. The mean distance from cell center was then calculated and plotted. Experiment was performed in triplicates; n = 120. The error bars represent standard deviation. **** represents $p \leq 0.0001$, ns represents $p \geq 0.05$.

When Cofilin-S3E (inactive form of Cofilin) was introduced into LIMK1-depleted cells, the mean spindle angle ($\theta$) was significantly reduced compared to LIMK1 siRNA and
GST-FLAG co-transfected cells $[\theta = 7.2^\circ \pm 4.5^\circ]$ vs $\theta = 16.5^\circ \pm 9.0^\circ$, $p \leq 0.0001$] (Figure 3-69C). However, we did not observed any significant difference in mean spindle angle when Cofilin-S3A (active form of Cofilin) were introduced into LIMK1-depleted cells compared to LIMK1 siRNA and GST-FLAG co-transfected cells $[\theta = 18.0^\circ \pm 8.8^\circ]$ vs $\theta = 16.5^\circ \pm 9.0^\circ$, $p \geq 0.05$. When we analyzed the mitotic spindle positioning, we observed the similar trend. Mitotic spindle positioning defect observed in LIMK1 siRNA and GST-FLAG co-transfected cells was rescued when Cofilin-S3E mutant were introduced (Figure 3-70C). However, the mitotic spindle of LIMK1 siRNA and Cofilin-S3A co-transfected cells continued to deviate away from the cell center. These findings suggest that LIMK1-mediated phosphorylation of Cofilin at residue Ser3 is important for maintaining spindle orientation and positioning.

We next proceeded to investigate if Cofilin functions downstream of Rho-ROCK-LIMK1 pathway in regulating spindle orientation and positioning as LIMK1 was discovered to function downstream of Rho-ROCK signaling. The different Cofilin mutants were introduced into either Rho (C3-Transferase) or ROCK (Y27623) inhibitor treated cells (Figure 3-69A and B). When Cofilin-S3E was introduced into C3-Transferase or Y27623 treated cells, the mean mitotic spindle angle was significantly lower compared to cells transfected with empty vector. In addition, the mitotic spindle was positioned closer to the center of the cells when Cofilin-S3E was introduced into C3-Transferase or Y27623 treated cells, compared to cells transfected with empty vector (Figure 3-70B and C). In contrast, introducing Cofilin-S3A could neither significantly reduce the mean spindle angle nor rescue the mitotic spindle positioning defects observed in cells treated with either C3-Transferase or Y27623, compared to when empty vector was introduced. These data suggest that Cofilin functions downstream of Rho-ROCK-LIMK1 pathway to regulate spindle orientation.
Figure 3-69. Cofilin functions downstream of Rho-ROCK-LIMK1 pathway to regulate spindle orientation. (A and B) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with the respective drugs and constructs. Treated cells were then immuno-stained with Pericentrin to visualize centrosome. Metaphase cells were imaged at z-planes 0.5 μm apart. Mean spindle angle then were calculated and plotted. Experiment was performed in triplicates; n = 120. (C) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with the respective siRNA and constructs. Treated cells were then immuno-stained with Pericentrin to visualize centrosome. Metaphase cells were imaged at z-plane 0.5 μm apart. Mean spindle angle then were calculated and plotted. Experiment was performed in triplicates; n = 120. Box-and-whiskers diagram; box lines represent upper quartile, median and lower quartile; whiskers represent 5-95 percentile; black dots represent outliers; red dots represent mean. **** represents $p \leq 0.0001$; *** represents $p \leq 0.001$; * represents $p \leq 0.05$; ns represents $p \geq 0.05$. 
Figure 3-70. Cofilin functions downstream of Rho-ROCK-LIMK1 pathway to regulate spindle positioning. (A and B) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with the respective drugs and constructs. Treated cells were then immuno-stained with α-Tubulin and Phalloidin to visualize mitotic spindle and cell boundary. The distance between the center of the cell and mitotic spindle was measured. The mean distance from cell center was then calculated and plotted. Experiment was performed in triplicates; n = 120. The error bars represent standard deviation. (C) HeLa cells were seeded onto Fibronectin coated glass coverslips and treated with the respective siRNAs and constructs. Treated cells were then immuno-stained with α-Tubulin and Phalloidin to visualize mitotic spindle and cell boundary. The distance between the center of the cell and mitotic spindle was measured. The mean distance from cell center was then calculated and plotted. Experiment was performed in triplicates; n = 120. The error bars represent standard deviation. **** represents p ≤ 0.0001; ns represents p ≥ 0.05.
3.3.4 Disrupting Rho-ROCK-LIMK1 signaling disrupts and weaken mitotic astral microtubule

Astral microtubules are shown to be important for mitotic spindle orientation and positioning (Schultz and Onfelt, 2001; Tame et al., 2014; Woolner and Papalopulu, 2012). As we observed defects in mitotic spindle orientation and positioning in Rho-ROCK pathway inhibited cells, we hypothesized that the astral microtubule might be affected in these cells. To investigate the role of Rho-ROCK-LIMK1 signaling pathway, cells were treated with C3-Transferase, Y27623, or LIMK1 siRNA. Cells transfected with control siRNA and GST-FLAG served as control for comparison. Astral microtubule intensities of these cells were then measured and analyzed as described. We observed less astral microtubule radiating from the centrosome when cells were treated with C3-Transferase and Y27623, compared to DMSO treated cells (Figure 3-71). Astral microtubule intensity analysis confirms our microscopy observations (Figure 3-72A). We also observed significantly lower astral microtubule intensity in LIMK1 siRNA treated cells compared to control (Figure 3-71 and 3-72A). These data suggest that less astral microtubules are radiating from the mitotic centrosome when Rho, ROCK or LIMK1 is inhibited.

Next, we investigate if LIMK1 functions downstream of Rho-ROCK signaling in regulating astral microtubule dynamics. To achieve this aim, we introduced phospho-dead (T508A) and phospho-mimic (T508E) LIMK1 mutants into C3-Transferase or Y27623 treated cells. Cells transfected with GST-FLAG served as control for comparison. Astral microtubule intensities of these cells were then calculated and analyzed. From our astral microtubule data, we observed a significant decrease in astral microtubule intensity when empty vector was introduced into C3-Transferase and Y27623 treated cells (Figure 3-72B and C). The mean astral microtubule intensity was increased to a level close to control cells when LIMK1-T508E was introduced into C3-Transferase and Y27623 treated cells (Figure 3-72B and C). In contrast, the mean astral microtubule intensity was not significantly increased when LIMK1-T508A was introduced into C3-Transferase and Y27623 treated cells (Figure 3-72B and C). This observation suggests that LIMK1 functions downstream of Rho-ROCK signaling pathway to regulate astral microtubule dynamics. This data further
highlights the importance of Rho-ROCK-LIMK1 pathway in regulating astral microtubule dynamics.

Figure 3-71. Inhibiting Rho-ROCK-LIMK1 pathway weaken astral microtubule. HeLa cells were seeded onto acid-washed glass coverslips and treated with the respective chemical and siRNAs. Treated cells were then immuno-stained with α-Tubulin to visualize mitotic spindle apparatus. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X.
objective lens. Representative images of the respective treatment and phenotypes were processed with ImageJ and presented. Scale Bar = 10 µm.

Figure 3-72. LIMK1 functions downstream of Rho-ROCK signaling pathway to regulate astral microtubule. (A) HeLa cells were treated as described in Figure 3-70 and the astral microtubule intensities were measured. The mean astral microtubule intensities were then calculated and plotted. Experiment was performed in triplicates; n = 300. The error bars represent standard deviation. (B and C) HeLa cells were seeded onto acid-washed glass coverslips and treated with the respective chemical and constructs. Treated cells were then immuno-stained with α-Tubulin to visualize mitotic spindle apparatus and the astral microtubule intensities were measured. The mean astral microtubule intensities were then calculated and plotted. Experiment was performed in triplicates; n = 300. The error bars represent standard deviation. **** represents p ≤ 0.0001. n.s represents p ≥ 0.05. a.u. represents arbitrary unit.
3.3.5 Cofilin functions downstream of LIMK1 in regulating astral microtubule dynamics through its activity on actin cytoskeleton

Cofilin is a downstream effector of Rho-ROCK-LIMK1 pathway and has been shown to be important for several processes in mitosis (Amano et al., 2002; Kaji et al., 2008). Therefore, we decided to investigate if Cofilin functions directly in the Rho-ROCK-LIMK1 pathway to regulate astral microtubules. We introduced phospho-dead (Cofilin-S3A) and phospho-mimic (Cofilin-S3E) into LIMK1 siRNA treated cells. The astral microtubule intensities were then analyzed by immuno-fluorescence microscopy (Figure 3-73A). We observed that only Cofilin-S3E could rescue the astral microtubule defects observed in LIMK1-depleted cells (Figure 3-73A and 3-74A). Neither the empty vector nor the Cofilin-S3A mutant could restore the astral microtubule level back to control level (Figure 3-74A). The data suggests that Cofilin activity might be crucial for the regulation of astral microtubule stability.

To determine if Cofilin influences astral microtubule dynamics, we treated cells with actin de-polymerization drugs (Cytochalasin B and Latrunculin A) (Figure 3-73B). As active Cofilin causes actin de-polymerization, we hope to mimic the effect of over-active Cofilin by treating the cells with the above mention chemicals. Cells treated with DMSO served as control for comparison. We observed a significant decrease in astral microtubule intensity in Cytochalasin B and Latrunculin A treated cells, compared to cells treated with DMSO (Figure 3-74B). The data suggests that disrupting actin cytoskeleton dynamics affects astral microtubule stability, which lends support to our hypothesis that Cofilin affects astral microtubule dynamics through its activity on actin cytoskeleton.

Taken together, our data suggests that Cofilin is functioning downstream of LIMK1 to regulate astral microtubule stability. In addition, Cofilin might affect astral microtubule stability through its activity on actin cytoskeleton. Since LIMK1 functions downstream of Rho-ROCK pathway, it is reason to speculate that Cofilin functions directly in the Rho-ROCK-LIMK1 pathway to affect astral microtubule stability.
Figure 3-73. Cofilin regulates astral microtubule through its activity on actin cytoskeleton. (A). HeLa cells were seeded onto acid-washed glass coverslips and treated with the respective siRNAs.
and constructs. Treated cells were then immuno-stained with α-Tubulin to visualize mitotic spindle apparatus. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of the respective treatment and phenotypes were processed with ImageJ and presented. Scale Bar = 10 µm. (B) HeLa cells were seeded onto acid-washed glass coverslips and treated with the respective chemicals. Treated cells were then immuno-stained with α-Tubulin to visualize mitotic spindle apparatus. Mitotic chromosomes were stained with DAPI. Immuno-stained samples were then observed under 63X objective lens. Representative images of the respective treatment and phenotypes were processed with ImageJ and presented. Scale Bar = 10 µm.

Figure 3-74. Quantification of astral microtubule intensities. (A and B). HeLa cells were treated as described in Figure 3-72 and the astral microtubule intensities were measured. The mean astral microtubule intensities were then calculated and plotted. Experiment was performed in triplicates; n = 300. The error bars represent standard deviation. **** represents \( p \leq 0.0001 \). n.s represents \( p \geq 0.05 \). a.u. represents arbitrary unit.
3.3 Identification of novel M-phase substrates of LIMK1 using mass spectrometry

We reported in previous section that LIMK1 displayed a dynamic localization pattern during mitosis, suggesting that LIMK1 could perform multiple roles during M-phase. Therefore, we decided to investigate if there are additional novel substrates of LIMK1 that are important for regulating processes in M-phase. To achieve this aim, we decided to utilize mass spectrometry analysis to identify novel substrates of LIMK1.

3.3.1 A summary of the SILAC-MS data of LIMK inhibited cells.

Stable isotope labeling by amino acids in cell culture (SILAC) for mass spectrometry (MS) quantitative proteomics (SILAC-MS) has been utilized successfully to analyze phosphorylation dynamics during M-phase (Dulla et al., 2010). Therefore, we decided to adopt SILAC-MS to identify novel substrates of LIMK1 in M-phase. First, the cells were cultured in media containing either “Heavy” \([^{13}\text{C}_6-\text{Arginine (heavy)} \text{ and } ^{13}\text{C}_6-\text{Lysine (heavy)}]\) or “Light” \([^{12}\text{C}_6-\text{Arginine}, ^{12}\text{C}_6-\text{Lysine (light)}]\) amino acids. The heavy amino acids were incorporated into newly synthesized proteins during normal protein turnover and would cause a predictable mass shift (6 Dalton for \(^{13}\text{C}_6-\text{Arginine}\) and 8 Dalton for \(^{13}\text{C}_6-\text{Lysine}\)) in MS analysis (Figure 3-75). After culturing cells in heavy or light media for seven generations, the cells were synchronized to M-phase with nocodazole and treated with Compound 22 (inhibitor of LIMK). Cell lysates were collected for phospho-peptide/protein enrichments. In this experiment, we used Electrostatic repulsion hydrophilic interaction chromatography (ERLIC) and titanium oxide column to enrich the phospho-peptides (Alpert, 2008). Enriched samples were then sent for MS/MS analysis to determine the identity of the peptides/proteins and examine if these peptides were phosphorylated. By calculating the ratio between “heavy” and “light” MS peaks of a particular phospho-peptide, we could determine changes to the phosphorylation levels of a protein of interest.
Figure 3-75. Illustration of SILAC mass spectrometry experimental workflow. HeLa cells were cultured in media containing either “heavy” or “light” for seven generation. These cells were then synchronized with nocodazole for 16 hours before treating the synchronized “light” cells with 1 µM of Compound 22 and “heavy” cells with DMSO. The cell lysates for each treatment were then collected for phospho-protein/peptide enrichment and analyzed using mass spectrometry.
In total, we identified 1944 unique phospho-peptides out of the 4270 phospho-peptides identified in the MS analysis data (Figure 3-76A). In addition, we annotated 2223 unique phosphorylation sites in our SILAC-MS analysis. Out of the 2223 phosphorylation sites, we found 1695 phospho-Serine residues (76.3%), 472 phospho-Threonine residues (21.2%), and 56 phospho-Tyrosine residues (2.5%) (Figure 3-76B). This observation supports an earlier report, which suggests that phosphorylation mainly occurs on serine and threonine residues in mammalian cells (Olsen et al., 2006). In addition, close to 31% (682 phospho-sites) of the 2223 phospho-sites were novel sites when compared to PhosphoSite database (Hornbeck et al., 2004).

3.3.2 LIMK inhibition significantly changes the phosphorylation level of several proteins in M-phase

We proceeded to identify peptides with more than two-fold changes to their phosphorylation levels by analyzing the ratio between “light” (Compound 22 treated) and “heavy” (DMSO treated). The ratio was then expressed as log₂ function value. A log₂ value of (-1) or less indicates that the phosphorylation of a particular peptide/protein is down-regulated in LIMK inhibited cells, while a value of (1) or more indicates that the phosphorylation of a particular peptide/protein is up-regulated in LIMK inhibited cells. In total, we identified 6 peptides with more than two-fold decrease in their phosphorylation levels in LIMK inhibited cells compared to those of control cells (Figure 3-77 and Table 1). We also identified 27 peptides with more than two-fold increase in their phosphorylation level in LIMK inhibited cells compared to those of control cells (Figure 3-77 and Table 2).
Figure 3-76. A summary of the phosphopeptide analysis. (A) The total number of phosphopeptides, number of unique phospho-peptides, number of unique phospho-proteins and number of unique phospho-sites quantified by SILAC-MS based mass spectrometry were shown. The numeric values besides the bar charts represent the numbers counted for each category. (B) Phospho-sites annotation of unique phosphorylation sites identified in SILAC-MS based mass spectrometry. In total, 2223 unique phospho-sites were annotated according to the PhosphoSite databases. The number of novel phospho-sites (upper, light red) and known phospho-sites (lower, dark red) were quantified.

As we are looking at potential novel substrate of LIMK1 during mitosis, we focused our effort on peptides that are significantly down-regulated when LIMK is inhibited (Table 1). All six down-regulated proteins have been reported to play a role in M-phase (Cao et al., 2007; McGuinness et al., 2005; Mitsushima et al., 2009). For instance, PAK2 has been implicated to function downstream of Cdc42 to regulate
Spindle orientation (Mitsushima et al., 2009). In addition, inhibiting PAK2 activation and recruitment to the centrosome have been shown to delay G2/M-phase transition (May et al., 2014). Shugoshin-like 1 (SGOL1) is localized to the mitotic centromere during mitosis and prevents premature segregation of mitotic chromosomes (McGuinness et al., 2005). Interestingly, a short isoform of SGOL1 (isoform 3 of SGOL1) has been reported to be regulated by PLK1 and is involved in preventing pre-mature centrioles separation (Wang et al., 2008). Pre-mature separation of centrioles separation will result in the formation of multi-polar spindle during mitosis (Maiato and Logarinho, 2014). The proteins identified in the SILAC-MS experiment are good potential leads for future investigation to study possible roles of LIMK1 during M-phase.

In our previous section (section 3.1.17), we proposed that DYNC1LI1 and 2 could be potential substrates of LIMK1 based on the PhosTag PAGE analysis (Figure 3-56). Therefore, we proceeded to examine if both dynein light intermediate chains were identified in our list of phospho-proteins. To our surprise, we identified two phosphorylation sites (Ser366 and Ser369) on cytoplasmic dynein 1 light intermediate chain 2 (DYNC1LI2) in our phospho-peptide data that are significantly up-regulated instead of phospho-sites which are down-regulated upon inhibition of LIMK1. The discrepancy in phosphorylation sites between the SILAC-MS data and our hypothesis is not immediately clear. However, the difference in the duration of treatment/experimental times between Compound 22 treatment and siRNA transfection might contribute to the discrepancies.
Figure 3-77. Log$_2$ ratio distribution plot of the total unique phospho-peptide. The Light vs Heavy ratio of all the unique phospho-peptides were measured and calculated. The ratio was subsequently converted to Log$_2$ Ratio and plotted.

Table 1. Phospho-proteins which are significantly down-regulated in LIMK inhibited cells. Identified phospho-sites are highlighted in red and annotated in PhosphoSite database.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phospho-peptide</th>
<th>Phospho-site</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMNA</td>
<td>LSP$^S$PTS$^Q$r</td>
<td>S392</td>
</tr>
<tr>
<td>MKI67</td>
<td>AQSLVISPPAP$^S$$^P$r</td>
<td>S224</td>
</tr>
<tr>
<td>PAK2</td>
<td>GTEAPAVV$^I$EEEDDDEETAPPVIAP$^D$$^T$HTk</td>
<td>T169</td>
</tr>
<tr>
<td>PRPF4B</td>
<td>LcDFGSASHVADNDITPYLV$^s$r</td>
<td>S852</td>
</tr>
<tr>
<td>SGOL1</td>
<td>sFIAAPcQIITNTSTLLk</td>
<td>S38</td>
</tr>
</tbody>
</table>
Table 2. Phospho-proteins which are significantly up-regulated in LIMK inhibited cells.
Identified phospho-sites are highlighted in red and annotated in PhosphoSite database.
Phosphorylation sites not annotated in PhosphoSite database are identified as “(novel)”.  

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phospho-peptide</th>
<th>Phospho-site</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRM1</td>
<td>SQSAAVtPSSTTSiR</td>
<td>T217</td>
</tr>
<tr>
<td>AHNAK</td>
<td>TVIRLPSGsiGAAsPTGSAVDIR</td>
<td>S212, S216</td>
</tr>
<tr>
<td>DYNC1LI2</td>
<td>TGsPGsPGAGGViQSTAk</td>
<td>S366, S369</td>
</tr>
<tr>
<td>EIF4G2</td>
<td>tQTPPLGqTPQLGLK</td>
<td>T11</td>
</tr>
<tr>
<td>EIF4G2</td>
<td>TqtPPLGqTPQLGLk</td>
<td>T13</td>
</tr>
<tr>
<td>FLNB</td>
<td>IAGPGLGsGVR</td>
<td>S1433</td>
</tr>
<tr>
<td>HIST1H1E</td>
<td>KAsGPPVSELITK</td>
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<tr>
<td>KRT8</td>
<td>DGKLVsiESsDVLPK</td>
<td>S475, S478</td>
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<tr>
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<td>TTKIPEDGDYsYEIEEK</td>
<td>T1806, S1813</td>
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<td>S386</td>
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<td>T115</td>
</tr>
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<td>RsSPAaFINPPiGTVPALKiK</td>
<td>S126, T146 (novel)</td>
</tr>
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<td>RsSPAaFINPPiGTVPALKTK</td>
<td>S127, T140</td>
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<td>S127</td>
</tr>
<tr>
<td>Protein</td>
<td>Peptide Sequence</td>
<td>Reference(s)</td>
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<tr>
<td>-----------</td>
<td>----------------------------------</td>
<td>-----------------------</td>
</tr>
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<td>PTBP1</td>
<td>EGQEDQGLTKDYGNsPLHR</td>
<td>S433</td>
</tr>
<tr>
<td>SRA1</td>
<td>VAAPQDGsPRVPASEtSPGPPPMGPPPPSSK</td>
<td>S60, T68 (novel)</td>
</tr>
<tr>
<td>SRA1</td>
<td>VAAPQDGsPrVPASEtSPGPPPMGPPPPSSk</td>
<td>S60, S69</td>
</tr>
<tr>
<td>SRA1</td>
<td>VAAPQDGSPRVPAsEtSPGPPPMGPPPPSSK</td>
<td>S66, T68 (novel)</td>
</tr>
<tr>
<td>ZMYM3</td>
<td>SAPTAPtPPPPPPPAtPR</td>
<td>T819, T828</td>
</tr>
<tr>
<td>cDNA FLJ51308</td>
<td>SQAQQPQKEAALSS</td>
<td>S113</td>
</tr>
<tr>
<td>cDNA FLJ55368, highly similar to Epsin-1</td>
<td>GSLAEAVGsPPPAAtPTP PTRK</td>
<td>S414, T420, T422</td>
</tr>
<tr>
<td>SWI related protein</td>
<td>GPsPSPVGsPASVAQSR</td>
<td>S313, S319</td>
</tr>
</tbody>
</table>
Chapter 4  Discussion

Centrosome is the primary microtubule organizing center (MTOC) in mammalian cells. It helps to initiate microtubule polymerization and provides an anchorage point for microtubule filaments to radiate towards the cell periphery. These highly organized microtubules provide a platform for regulatory and motor proteins to regulate organelle positioning, vesicular transportation, microtubule cytoskeleton dynamics, and mitosis. Abnormal centrosomal function and number have been reported to be a contributing factor for tumor development (D'Assoro et al., 2002). Cells containing abnormal number of centrosomes have been observed in several cancer cell types (Chan, 2011). Therefore, it is of interest to study and understand the proteins and the factors that regulate the function and number of centrosome

Several proteins that can regulate the actin structures in the cells are reported to associate with the centrosome and regulate its function during M-phase (Chircop, 2014; Heng and Koh, 2010). LIM-kinase 1 (LIMK1) is involved in regulating actin cytoskeleton dynamics through phosphorylation of Cofilin (Agnew et al., 1995; Scott and Olson, 2007). Although previous studies are mainly focused on the role of LIMK1 in interphase actin cytoskeleton regulation, recent studies suggest that LIMK1 can play an important role in M-phase. CDK1, an important kinase that regulates several events in M-phase, has been shown to phosphorylate and activate LIMK1 during mitosis (Amano et al., 2002; Sumi et al., 2002). In addition, phosphorylated form of LIMK1 is shown to localize to the mitotic spindle apparatus during M-phase (Chakrabarti et al., 2007). Interestingly, over-expression of LIMK1 has been observed to result in abnormal spindle assembly and the formation of supernumerary centrosome during mitosis (Davila et al., 2007). These findings suggest that LIMK1 could potentially be involved in regulating the function of mitotic spindle apparatus. Although earlier findings have identified the role of LIMK1 in regulating spindle orientation and cytokinesis, the exact role of the kinase in mitosis is not be fully explored. In this study, we set out to investigate the role(s) of LIMK1 in regulating events and processes in M-phase.
In our current study, we observed that LIMK1 was localized to the mitotic centrosome and site of contractile ring formation during M-phase (Figure 3-1). This localization pattern suggests that LIMK1 might perform multiple roles during mitosis. From RNAi experiments, we observed that LIMK1-depleted metaphase cells formed multi-polar spindle and display mitotic centrosome defocusing defects, suggesting that the kinase is important for regulating mitotic centrosome integrity (Figure 3-4). We hypothesized that these mitotic defects could be due to reduced accumulation of proteins that are important for regulating centrosome maturation and integrity at mitotic spindle poles. This hypothesis was supported by our microscopy data, which showed lower fluorescence intensities of centrosomal proteins at the spindle poles when LIMK1 was depleted (Figure 3-15). The reduced accumulation of proteins important for maintaining centrosome integrity can also lead to pericentriolar material (PCM) fragmentation, which in turn resulted in multi-polar spindle formation or centrosome defocusing (Figure 3-10 and 3-11). All the above mentioned mitotic defects could be rescued when wild-type (LIMK1-WT) and constitutively active LIMK1 (LIMK1-T508EE) were introduced into LIMK1-depleted cells (Figure 3-18 and 3-22). These observations suggest that the kinase activity of LIMK1 is important for maintaining centrosome integrity. In addition, a novel LIMK1 substrate could potentially be functioning downstream of LIMK1 in regulating centrosome integrity.

We then proceeded to identify the downstream substrate/effector(s) of LIMK1 that was responsible for maintaining centrosome integrity by first introducing known substrates of LIMK1 into LIMK siRNA-treated cells to determine if they could rescue the resultant phenotypes. Although Cofilin and TPPP/p25 were two well-studied substrates of LIMK1, both proteins could not rescue the mitotic defects observed in LIMK1 depleted cells (Figure 3-28, 3-30 and 3-32) (Acevedo et al., 2007; Arber et al., 1998). This observations suggest that both Cofilin and TPPP/p25 do not function downstream of LIMK1 in regulating centrosome integrity. As we observed reduced accumulation of some PCM proteins in LIMK1 depleted cells, we hypothesized that LIMK1 could affect transportation of proteins to the centrosome. Therefore, we introduced dynein light intermediate chain 1 and 2 (DYNC1LI1 and DYNC1LI2) into LIMK1-depleted cells as both light intermediate chains are reported to define cargo specificity of the dynein motor complex (Purohit et al., 1999; Schmoranzer et al.,
2009). Indeed, introducing DYNC1LI1 and DYNC1LI2 could rescue the mitotic spindle and centrosomal protein accumulation defects observed in LIMK1 depleted cells (Figure 3-40 and 3-42). The data suggest that DYNC1LI1 and DYNC1LI2 could be downstream effectors of LIMK1. This hypothesis was further supported by the GST pull-down conducted, which showed that both dynein light intermediate chains interacted with the kinase domain of LIMK1 (Figure 3-52 and 3-53). The interaction between LIMK1 and DYNC1LIs was further supported by the endogenous immunoprecipitation assay in our study (Figure 3-55). In addition, the overall phosphorylation status of both dynein intermediate chains were affected in LIMK1 depleted cells, suggesting that DYNC1LI1 and DYNC1LI2 could be downstream substrates of LIMK1 (Figure 3-56).

Based on our experimental findings, we proposed a model to illustrate the potential role of LIMK1 in during mitosis (Figure 4-1). During G2/M-phase transition, LIMK1 is phosphorylated by an M-phase kinase, such as CDK1, leading to the activation of LIMK1. M-phase activated LIMK1 will then phosphorylate DYNC1LI1 and DYNC1LI2, and indirectly regulates the function of cytoplasmic dynein during mitosis. LIMK1-mediated phosphorylation of DYNC1LI1 and DYNC1LI2 might potentially regulate the interaction between dynein and PCM proteins, thus regulating the transportation of PCM proteins to the mitotic spindle poles. These PCM proteins would then help to maintain the structural integrity of the mitotic centrosome. Although we proposed that LIMK1-mediated phosphorylation could potentially affect DYNC1LIs’ interaction with their cargoes, LIMK1 phosphorylation might not be the only post-translational event needed for cargo interactions. This is because LIMK1 depletion did not completely abolish PCM protein localization (Figure 3-15), suggesting that PCM could still accumulate at the mitotic centrosome in LIMK1-depleted cells. In addition, over-expressing DYNC1LI1 or DYNC1LI2 alone rescued the defects observed in LIMK1-depleted cells (Figure 3-40 and 3-42), suggesting that DYNC1LIs protein levels or their availability are also contributing factors. Therefore, LIMK1-mediated phosphorylation could play a supportive role rather than functioning as a crucial regulator of DYNC1LI-cargo interactions. LIMK1-mediated phosphorylation, together with other post-translational modifications, could in turn enhance the accumulation of
PCM proteins onto the centrosome mitotic centrosome during centrosome maturation.

Lastly, we validated the role of LIMK1 in spindle orientation and positioning during mitosis (Figure 3-61 and 3-62). LIMK1 could be regulating both processes through its influence on cortical actin, which in turn affects astral microtubule stability (Figure 3-73 and 3-74).

4.1 LIMK1 displayed a dynamic localization pattern during M-phase.

LIM-kinase 1 (LIMK1) belongs to the LIM motif containing protein kinase (LIMK) family and regulates actin cytoskeleton dynamics through phosphorylation of Cofilin (Arber et al., 1998). Earlier studies have always focused on the role of LIMK1 on interphase actin cytoskeleton regulation. However, recent studies demonstrate that LIMK1 could have possible roles in M-phase. Phosphorylated LIMK1 has been found to co-localize with γ-tubulin at the mitotic centrosome (Chakrabarti et al., 2007). In this current study, we observed that LIMK1 displayed a dynamic localization pattern as the cell progressed through M-phase (Figure 3-1). From G2/M phase to metaphase, LIMK1 was observed to localize at the mitotic centrosome (Figure 3-1). Interestingly, several LIM domain containing proteins such as Paxillin and Ajuba have been found to co-localize with γ-Tubulin at the centrosome (Abe et al., 2006; Herreros et al., 2000). These findings suggest a possibility that the LIM domain may be an important domain to recruit proteins to the centrosome. LATS2-Ajuba complex has been shown to recruit γ-Tubulin to the centrosome during centrosome maturation and spindle apparatus formation (Abe et al., 2006). Interestingly, Ajuba is reported to be a positive regulator of AurkA, a protein known to regulate several functions of the mitotic centrosome (Hirota et al., 2003). In addition, LIMK1 is reported to phosphorylate and activate AurkA, a process speculated to be important for proper bipolar spindle assembly (Ritchey et al., 2012). These earlier findings, together with our localization studies, suggest that LIM domain containing protein
could be involved in recruiting and regulating proteins that are crucial for the proper functioning of the mitotic centrosome.

Figure 4-1. Proposed mechanism on how LIMK1 regulates centrosome maturation and integrity. During late G₂/M-phase, LIMK1 is phosphorylated by an M-phase kinase, leading to its activation. Activated LIMK1 will then phosphorylate DYNC1LI1 and DYNC1LI2, and indirectly regulates the function of cytoplasmic dynein during mitosis. LIMK1-mediated phosphorylation of DYNC1LI1 and DYNC1LI2 might potentially regulate the interaction between dynein and PCM proteins, thus regulating the transportation of PCM proteins to the mitotic spindle poles. These PCM proteins would then help to maintain the structural integrity of the mitotic centrosome.

The centrosome is believed to serve as an anchorage point for several signaling pathways and helps to integrate external and internal signaling events for cell cycle control (Lange, 2002). Several important kinases that regulate the cell cycle have
been reported to localize at the centrosome (Doxsey et al., 2005). Cyclin-B/CDK1 complex, which is important for regulating M-phase progression, begins to localize at the centrosome during prophase (Jackman et al., 2003). Serine/threonine-protein kinase Chk1 (CHK1), a protein activated during DNA damage response, has been shown to localize at the centrosome to prevent pre-mature activation of cyclin-B/CDK1 (Kramer et al., 2004). A recent study has demonstrated that siRNA-mediated depletion of some centrosomal components triggers the activation of p53-dependent G1-phase arrest (Mikule et al., 2007). These earlier reports strongly support the idea that centrosome could function as a “signaling hub” for cellular processes. LIMK1 is also thought to act as an integrator for signals from various upstream pathways as LIMK1 is shown to be regulated by several upstream kinases (Scott and Olson, 2007). Interestingly, AurkA is discovered to phosphorylate LIMK1 at residue 307, an event found to be essential for the activation phosphorylation at Thr508 (Ritchey et al., 2012). In a recent study, RhoA and PLK1 have been observed to work in synergy to activate of ROCKII, a protein found to localize at the centrosome (Lowery et al., 2007; Ma et al., 2006). It is reasonable to speculate that ROCKII could also regulate the function of LIMK1 at the centrosome as ROCKII is reported to be an upstream kinase of LIMK1 (Manetti, 2012). In addition, CDK1 has also been identified as a potential kinase involved in activating LIMK1 (Sumi et al., 2002). Since several evidences have shown that LIMK1 is localized at the mitotic centrosome and it could be phosphorylated by several kinases important for M-phase progression, we proposed that LIMK1 localized at the centrosome could serve to integrate the signals from various pathways. M-phase activated LIMK1 could potentially phosphorylate other downstream targets and regulate their function in mitosis.

Rho-GTPases were reported to function synergistically for the formation of the contractile ring, an acto-myosin structure important for physically separating the two newly formed nuclei during cytokinesis (Narumiya and Yasuda, 2006). RhoA, together with PLK1 and AurkB, forms the centralspindlin. Centralspindlin would then function as a scaffold allowing proteins that are important for regulating cytokinesis to be recruited to the site (Chircop, 2014). Therefore, LIMK1 could be recruited to the site of cytokinesis by one of those component found in the centralspindlin. It is likely
that LIMK1, together with Slingshot 1 (SSH1) and LATS1, helps to organize the actomyosin contractile ring that is important for furrow ingression during cytokinesis (Kaji et al., 2003; Yang et al., 2004b).

4.2 LIMK1 performs several roles during cell cycle

As discussed earlier, we observed a dynamic localization pattern of LIMK1 as the cells progress through M-phase (Figure 3-1). This observation suggests that LIMK1 could be involved in several events during mitosis. In the current study, we showed that LIMK1 depletion affects centrosome integrity, spindle orientation and positioning of the mitotic spindle apparatus. In addition, several earlier studies have demonstrated the importance of LIMK1 in the process of cell cycle progression, cytokinesis, and centrosome maturation (Davila et al., 2003; Davila et al., 2007; Ritchey et al., 2012). Taken together, these findings suggest that LIMK1 could be involved in several processes that would eventually result in smooth progression through the cell cycle.

Firstly, LIMK1 could regulate progression through interphase and G2/M-phase transition, as previous studies have demonstrated that over-expressing or depleting LIMK1 could affect both processes (Davila et al., 2003; Davila et al., 2007; Higuchi et al., 1996). LIMK1 could regulate the progression through interphase via its activity on Cofilin, which is involved in regulating actin cytoskeleton dynamics (Arber et al., 1998). Disrupting actin cytoskeleton has previously been shown to arrest cells at G1-phase and delay G2/M-phase transition (Huang and Ingber, 2002; Lee and Song, 2007). In addition, some studies have pointed to the possibility that cells could “sense” the integrity of actin cytoskeleton, which influence the progression through the various stages of cell cycle (Huang and Ingber, 2002; Lee and Song, 2007; Reshetnikova et al., 2000). Therefore, the activity of LIMK1-Cofilin on actin cytoskeleton dynamics could potentially affect cell cycle progression. Interestingly, earlier studies showed that p57Kip2, a protein known to regulate cell cycle progression, interact with and activate LIMK1 to influence actin cytoskeleton
dynamics (Vlachos and Joseph, 2009; Yokoo et al., 2003). This further demonstrates the importance of LIMK1-mediated regulation of actin cytoskeleton on cell cycle progression.

Centrosome undergoes maturation, a highly regulated process where centrosome increases its microtubule nucleating and organizing capacity, during late G$_2$-phase and G$_2$/M-phase transition (Bettencourt-Dias and Glover, 2007). In our study, LIMK1 was observed to localize at the centrosome during G$_2$/M-phase transition, suggesting that LIMK1 may play a role during centrosome maturation process (Figure 3-1). LIMK1 has been shown to phosphorylate and activate AurkA, a protein known to be involved in centrosome maturation (Ritchey et al., 2012). AurkA in turn participates in the recruitment of proteins that are essential for centrosome maturation (Barr and Gergely, 2007; Lens et al., 2010; Terada et al., 2003). AurkA has been shown to form a complex with Protein aurora borealis (Bora) and the Bora-AurkA complex is required for activating PLK1 (Bruinsma et al., 2014). Activated PLK1 would in turn phosphorylate Pericentrin, an important event required for the recruitment of AurkA, CEP192, γ-Tubulin, and PLK1 to centrosome during maturation (Lee and Rhee, 2011). Pericentrin and γ-Tubulin form a scaffold at the centrosome, allowing additional factors to be recruited to the centrosome for the maturation process (Buchman et al., 2010; Dictenberg et al., 1998; Fong et al., 2008; Zimmerman et al., 2004). The newly recruited γ-Tubulin then forms additional γ-TuRC at the centrosome, thus increasing the microtubule nucleation activity of the mitotic centrosome (Moritz et al., 1995). Besides activating AurkA, LIMK1 could also influence centrosome maturation by mediating the transportation of centrosomal proteins, such as γ-Tubulin, NuMA, Pericentrin, and PLK1. As discussed earlier, these proteins are all reported to contribute positively to the process of centrosome maturation. Our hypothesis is supported by the current study, which shows that LIMK1 depletion led to reduced accumulation of the above mentioned centrosomal protein at mitotic spindle pole (Figure 3-15). Therefore, LIMK1 could be involved in centrosome maturation via its activity on AurkA and through regulating the transport of centrosomal proteins.
Our experimental data suggest that it is possible that LIMK1 could regulate the integrity of the mitotic spindle poles by preventing abnormal fragmentation of pericentriolar material (PCM) (Figure 3-10 and 3-11). As discussed earlier, Pericentrin and γ-Tubulin are required for the recruitment of additional centrosomal proteins, such as PLK1, to the mitotic centrosome (Lee and Rhee, 2011). Once recruited to the centrosome, PLK1 phosphorylates and recruits centrosomal protein Kizuna (Kiz) to the centrosome (Oshimori et al., 2006). Kiz then acts as a “linking bridge” to bind other PCM proteins together, thus preventing mitotic spindle pole fragmentation (Oshimori et al., 2006). This will ensure the structural integrity of the mitotic spindle poles and maintain the bipolar status of the spindle apparatus. Besides PLK1, NuMA has also been shown to be important for maintaining the bipolar status of the mitotic spindle (Radulescu and Cleveland, 2010; Yang and Snyder, 1992). Immuno-depletion of NuMA from *Xenopus* egg extracts disrupts microtubule anchorage to the spindle pole and prevents the formation of a focused spindle pole (Merdes et al., 1996). These defective phenotypes observed in NuMA-depleted egg extracts have been linked to spindle pole fragmentation (Merdes et al., 1996). Similar defects are also observed when NuMA are immuno-depleted in mitotic mammalian cells (Gaglio et al., 1995). The reduced accumulation of NuMA observed in LIMK1 knockdown cells can explain the defocused and multi-polar spindles observed in these cells.

In our GST pull-down assay, immuno-precipitation assay and PhosTag SDS-PAGE analysis, we showed that DYNC1LI1 and DYNC1LI2 could be potential substrates of LIMK1 (Figure 3-52, 3-53, 3-55 and 3-56). In addition, the rescue experiments we performed suggested that both dynein light intermediate chains function downstream of LIMK1 in regulating centrosome integrity and the accumulation of centrosomal protein at mitotic spindle (Figure 3-40 and 3-42). Therefore, we proposed that LIMK1 regulates the integrity of the mitotic spindle poles via its action on cytoplasmic dynein light intermediate chains. The function of various dynein motor complex subunits is shown to be regulated by phosphorylation during mitosis. CDK1-mediated phosphorylation of dynein light intermediate chain 1 releases the dynein motor complex from membranous organelles and is required for DYNC1LI1 to localize at the mitotic spindle apparatus (Addinall et al., 2001; Dell et al., 2000). DYNC1LI1
would then participate in the removal of spindle assembly checkpoint (SAC) proteins from kinetochore, thus allowing the cells to progress into anaphase (Sivaram et al., 2009). PLK1-mediated phosphorylation of dynein intermediate chain is important for the localization of dynein onto the kinetochore complex on the chromosome (Arber et al., 1998). The cytoplasmic dynein motor complex at the kinetochores would in turn regulate microtubule-kinetochore attachment, mitotic chromosome motion, and SAC signaling (Howell et al., 2001; Varma et al., 2008; Yang et al., 2007).

Besides regulating cytoplasmic dynein localization, phosphorylation of dynein subunits and cargo proteins have been implicated in regulating cargo binding. Phosphorylation of dynein intermediate chains on residue Serine 84 (Ser84) promotes dynein interaction with dynactin complex (Vaughan et al., 2001). Since dynactin regulates the interaction between dynein and its cargo, the authors of this study suggest that phosphorylation of dynein intermediate chains on residue Ser84 could potentially regulate dynein-cargo interaction. Interestingly, changing the phosphorylation site on dynein intermediate chains changes its cargo specificity (Whyte et al., 2008). Dynein intermediate chain that is phosphorylated on residue threonine 89 (Thr89) binds preferably to Centromere/kinetochore protein zw10 homolog (ZW10) at the kinetochore (Whyte et al., 2008). In contrast, dephosphorylating Thr89 allows cytoplasmic dynein to dissociate from ZW10 and binds preferably to dynactin complex (Whyte et al., 2008). These observations highlight the importance of phosphorylation in regulating dynein-cargo binding. In the current study, we observed that LIMK1 depletion altered the phosphorylation profile of DYNC1LI1 and DYNC1LI2 (Figure 3-56). Since both dynein light intermediate chains were reported to mediate dynein-cargo interaction, we speculated that LIMK1-mediated phosphorylation of DYNC1LI1 and DYNC1LI2 could potentially regulate dynein-PCM protein interactions.

Proteins involved in proteolytic degradation have been observed to localize at the centrosome, suggesting that proteolytic degradation play a role in regulating centrosome function (Fabunmi et al., 2000; Wigley et al., 1999). Indeed, Skp1-cullin-F-box (SCF) ubiquitin ligase is found to be involved in regulating centrosome
duplication as inhibiting SCF ubiquitin ligase inhibits centriole duplication (Freed et al., 1999). SCF could regulate centrosome duplication by regulating the degradation of PLK4, as the kinase is found to contain a conserve motif that interacts with SCF ubiquitin ligase component (Cunha-Ferreira et al., 2009). Interestingly, inhibition of proteolytic degradation during mitosis by MG132 treatment has been shown to cause multi-polar spindle formation (Ehrhardt and Sluder, 2005). The authors of this study have proposed that excessive accumulation of centrosomal protein at the spindle poles result in the formation of a structurally unstable spindle pole. This instability in turn leads to centrosome fragmentation, resulting in the formation of multi-polar spindle. Although we observed multi-polar spindle in LIMK1-depleted cells, our data does not support the hypothesis that the multi-polar phenotype in our study was due to excessive accumulation of centrosomal protein. Instead in the current study, our data indicate that there were lower amounts of centrosomal proteins at the spindle poles (Figure 3-15).

4.2 LIMK1 does not regulate centrosome duplication

Our finding in this study revealed that LIMK1 depletion led to multi-polar spindle (Figure 3-4). Abnormal centrosome number has been linked to multi-polar spindle formation, which could eventually lead to genomic instability and cancer (Chan, 2011; Jiang et al., 2003). Centrosome over-duplication is one way in which cells obtain abnormal centrosome number (D'Assoro et al., 2002). Rho GTPase protein signaling has been implicated in regulating centrosome duplication. Over-expression of constitutively active RhoA and RhoC results in centrosome over-duplication while RNAi-mediated RhoA and RhoC knock-down suppresses centrosome over-duplication (Kanai et al., 2010). Activation of RhoA and RhoC further activates ROCK, allowing the kinase to form a complex with Nucleophosmin/B23 (NPM/B23) (Ma et al., 2006). ROCK complexes with NPM/B23 displays higher kinase activity and could be involved in phosphorylating proteins involved in centrosome duplication (Ma et al., 2006). As LIMK1 is a reported substrate of ROCK, it is reasonable to speculate that LIMK1 could be a substrate of ROCK-NPM/B23 complex (Ohashi et al., 2000). However, our findings in this study suggested that LIMK1 might not be a
downstream effector of ROCK-NPM/B23 or involved in regulating centrosome duplication. Firstly, from the centrosome duplication assay conducted, our data showed that the number of γ-Tubulin foci per cell was not significantly different in control and LIMK1 siRNA treated U2-OS (Figure 3-6). Secondly, the majority of the LIMK1-depleted cells with multi-polar spindle defects only contained two pairs of centriole positive foci, suggesting that the multi-polar spindle phenotype is not due to centrioles re-duplications or abnormal centriole fragmentation (Figure 3-10 and 3-11). Lastly, we did not observed significant increase in the number of cells with more than two pairs of centriole positive foci in LIMK1-depleted G2/M cells as compared to control cells (Figure 3-7). From the above experimental results and observations, we proposed that LIMK1 is not involved in the centrosome duplication pathway and the multi-polar spindle phenotype observed in LIMK1 siRNA treated cells is not due to centrosome over-duplication.

4.3 LIMK1 depletion does not affect cytokinesis

Acto-myosin contractile unit formation is an important event during cytokinesis and RhoA signaling has also been implicated in this processes (Chircop, 2014; Kamijo et al., 2006; Piekny et al., 2005). LIMK1 is one of the many downstream effectors of RhoA signaling in regulating cytokinesis as either over-expression or depletion of LIMK1 leads to cytokinesis defects (Amano et al., 2002; Yang et al., 2004b). In addition, over-expression of Cofilin, a downstream effector of LIMK1, also has a negative impact on the completion of the cytokinesis process (Kaji et al., 2003). As cytokinesis defects could potentially lead to abnormal centrosome number in subsequent cell division, we hypothesized that the multi-polar spindle observed in LIMK1-depleted cells could be due to cytokinesis failure. Surprisingly, our data did not suggest that LIMK1 depletion resulted in cytokinesis failure. From the FACS analysis, we did not observed any cells with abnormal DNA content in LIMK1 siRNA treated cells, suggesting that cytokinesis is not affected (Figure 3-8). In addition, LIMK1 siRNA treatment did not significantly increase the number of multi-nucleated cells or interphase cells with abnormal centrosome content, as compared to control
It is not immediately clear why our findings contradict earlier reports, which demonstrate the importance of LIMK1 in the process of cytokinesis (Amano et al., 2002; Yang et al., 2004b). However, earlier study has showed that transient expression of LIMK1 could prolong mitosis timing but cells could exit mitosis without cytokinesis defects (Davila et al., 2007). We hypothesized that LIMK2 could substitute the function of LIMK1 in regulating cytokinesis. LIMK2 is a closely related kinase of LIMK1 and both kinases are involved in several common signaling pathways (Manetti, 2012; Scott and Olson, 2007). Rho-ROCK signaling and Rac/Cdc42-PAK1 signaling is known to activate both LIMK1 and LIMK2 (Sumi et al., 2001a; Sumi et al., 1999). Both Cofilin and TPPP/p25 are shown to be substrates of LIMK1 and LIMK2 (Acevedo et al., 2007; Heng et al., 2012; Sumi et al., 1999). Therefore, it is possible that LIMK2 could substitute the function of LIMK1 in regulating acto-myosin dynamics during cytokinesis. However, several earlier studies have also highlighted some functional differences between LIMK1 and LIMK2 during mitosis (Heng et al., 2012; Sumi et al., 2006). For instance, LIMK1 is observed to localize to the centrosome and site of cytokinesis during mitosis (Sumi et al., 2006). In contrast, LIMK2 is observed to localize exclusively on spindle microtubules during M-phase (Sumi et al., 2006). In addition, an earlier study by our laboratory has revealed that depletion of LIMK2, but not LIMK1, affects TPPP/p25 localization to the mitotic spindles and astral microtubule dynamics (Heng et al., 2012). In the same study, the authors also show that depletion of LIMK2 does not lead to multi-polar spindle formation, suggesting that LIMK2 is not as essential as LIMK1 in regulating this feature (Heng et al., 2012). In the PhosTag SDS-PAGE analysis that we conducted, we observed that depletion of LIMK1, but not LIMK2, affected the phosphorylation profile of DYNC1LI1 and DYNC1LI2 (Figure 3-56). Results from the current study, together with earlier reports, suggest that both LIMK1 and LIMK2 could perform distinct function during mitosis. Therefore, further experiments are required to determine if LIMK2 could substitute the function of LIMK1 during cytokinesis. Live cell imaging microscopy can be utilized to examine and compare M-phase progression of LIMK1- and LIMK2-depleted cells. We can observe for...
cytokinesis defects in LIMK1 and LIMK2 siRNA treated cells. In addition, we can examine the DNA profiles, number of centrosome, and observe the number of multinucleated cells in LIMK2-depleted cells. Data from these experiments can potentially provide us with valuable insights into the functional similarity and difference of LIMK1 and LIMK2 during M-phase and cytokinesis.

4.4 Rho-ROCK functions upstream of LIMK1 in regulating mitotic spindle orientation and positioning

Proper mitotic spindle orientation and positioning have been implicated in several cellular processes, such as tissues organization and cell fate determination during development (Tang and Marshall, 2012). In many organs, such as lungs and blood vessels, the epithelial cells are organized into tubes that branch out into complex network. This organization is partly influenced by the orientation of the mitotic spindles. In general, the mitotic spindles of these epithelial cells are oriented with respect to the longitudinal axis of the epithelial tube. This would allow the tube to extend longitudinally and maintain a consistent diameter as the tube develops. Disrupting the orientation of the mitotic spindle in epithelial cells has been shown to cause an increase kidney tubule diameter and disrupt the organization of the kidney epithelium (Fischer et al., 2006). Besides kidney tubules, changes in the spindle orientation have also been shown to alter the organization of the airways in the lung (Tang et al., 2011). In addition to tissue organization, spindle orientation and positioning have also been implicated in determining cell fate during development. During embryonic development, asymmetric cell division is important in the generation of daughter cells with distinct distribution of cellular contents. This difference in cellular content would in turn influence the cell’s developmental path. Asymmetric cell division is greatly determined by the orientation and positioning of the mitotic spindle (Tang and Marshall, 2012). For instance, the *Drosophila* neuroblast orientates their mitotic spindle along the apical-basal axis to generate a self-renewing neuroblast and a daughter cell destined for neuronal cell differentiation (Chang et al., 2012; Doe, 2008). As the position of the spindle apparatus influences the orientation and positioning of the cleavage furrow, the positioning of the spindle apparatus could contribute to the asymmetric distribution of cellular components.
during embryonic development (Oliferenko et al., 2009). These findings highlight the importance of maintaining proper mitotic spindle orientation and positioning in development and tissue organization. Recently, several tumor suppressor signaling pathways, such as Hippo tumor suppressor pathway, PTEN-PI3K signaling pathway, and Wnt signaling pathway, have been shown to regulate spindle orientation and positioning (Lu and Johnston, 2013). These recent findings suggest that spindle orientation and positioning are not only important during embryonic development, but may potentially be a factor contributing to cancer development and progression.

Rho-GTPases proteins and their downstream signaling pathways have shown to regulate spindle orientation and positioning. Depletion of Cdc42 in Caco-2 cells disrupts spindle orientation and results in the defective apical surface positioning and tissue organization (Jaffe et al., 2008). This finding suggests that Cdc42 is involved in the process of spindle orientation. Cdc42 could be regulating spindle orientation via Cdc42-PAK2-βPIX and the phosphatidylinositol 3,4,5 triphosphate (PIP3) pathways (Mitsushima et al., 2009). Cdc42-PAK2-βPIX signaling regulates spindle orientation through its ability to regulate cortical actin network organization, which is required for proper astral microtubule-cortex attachment (Mitsushima et al., 2009). The interaction between astral microtubules and cortical actin attachment would in turn influence spindle orientation and positioning.

Several studies have implicated RhoA in the process of controlling spindle orientation. Over-expressing dominant-negative RhoA in chick neuro-epithelium cells causes an increase in the spindle orientation angle in mitotic cells and leads to disruption of the neuron tissue organization (Roszko et al., 2006). RhoA-ROCK signaling may cooperate with Moesin to maintain spindle orientation and positioning, as disrupting either RhoA-ROCK or Moesin negatively affects spindle orientation and positioning (Carreno et al., 2008; Heng et al., 2012; Kunda et al., 2008). It has been reported that RhoA-ROCK and Moesin help to organize the cortical actin network, thus providing a stable platform for maintaining spindle positioning and orientation (Fehon et al., 2010). Interestingly, SLK-dependent activation of ezrin/radixin/moesin (ERMs) proteins is required for proper localization of LGN–NuMA complex at the
cellular cortex (Machicoane et al., 2014). The authors of this study proposed that ERM proteins could organize the actin cytoskeleton network, allowing LGN-NuMA localization at the cellular cortex. The LGN-NuMA complex then recruits dynein to the cellular cortex for the maintenance of spindle orientation (Kiyomitsu and Cheeseman, 2012; Kotak et al., 2012). Interestingly, disrupting cortical F-actin organization with pertussis toxin had been shown to negatively affect the localization of Gαi, a protein required for the recruitment of LGN–NuMA complex to the cellular cortex, localization (Machicoane et al., 2014). This observation further highlights the importance of an organized and stable cortical actin network in maintaining proper spindle orientation and positioning. In the current study, we showed that disrupting actin dynamics increased the mean spindle angle and resulted in off-center positioning of the mitotic spindle apparatus, suggesting that actin cytoskeleton dynamics is important for maintaining proper spindle orientation and positioning (Figure 3-59 and 3-60) In addition, we showed that LIMK1-Cofilin regulates spindle orientation and positioning via their activity in regulating actin cytoskeleton dynamics (Figure 3-69 – 3-70 and 3-74). LIMK1-Cofilin could potentially maintain an organized and stable cortical actin network, thus allowing Gαi-LGN-NuMA complex to be anchored onto the mitotic cell cortex. Gαi-LGN-NuMA complex would then recruit cytoplasmic dynein to the cell cortex and generate the necessary “pulling” force for proper spindle orientation and positioning. In addition, Rho-ROCK signaling could function upstream of LIMK1-Cofilin (Figure 3-65, 3-66, 3-69 and 3-70) in organizing the cortical actin network for proper spindle orientation and positioning.

A stable and organized actin cytoskeleton network has also been implicated in regulating the stability of astral microtubule (Zhu et al., 2013). Treating cells with actin depolymerization drugs, such as Cytochalasin B and Latrunculin A, had been shown to negatively affect astral microtubule stability (Kaji et al., 2008; Machicoane et al., 2014). The same defect was also observed when we analyzed the astral microtubule intensity in cells treated with Cytochalasin B and Latrunculin A (Figure 3-73B and 3-74B). An earlier study demonstrates that LIMK1 depletion could potentially cause astral microtubule instability (Kaji et al., 2008). In the current study, we observed weaker astral microtubule fluorescence intensity in LIMK1-depleted
metaphase cells, suggesting that LIMK1 could affect astral microtubule stability (Figure 3-71 and 3-72A). Over-expression a Cofilin mutant (Cofilin-S3A; active form of Cofilin) that cannot be phosphorylated has been shown to disrupt cortical actin organization and results in astral microtubule instability (Kaji et al., 2008). This finding suggests that Cofilin could indirectly regulate the stability of astral microtubules. In the current study, we demonstrated that LIMK1-Cofilin might also regulate astral microtubule stability (Figure 3-73A and 3-74A). By taking all these into consideration, we speculate that LIMK1-Cofilin could regulate astral microtubule indirectly via their ability to regulate cortical actin dynamics.

4.5 SILAC-MS identified several potential M-phase substrate of LIMK1

In our attempt to identify novel substrate of LIMK1 during M-phase, we utilized SILAC base mass spectrometry analysis to study changes in the phospho-proteome profiles in LIMK inhibited cells. In our analysis, we identified six proteins which showed down-regulated phosphorylation by at least two-fold in Compound 22 treated cells (Table 1). These six proteins could be potential substrates of LIMK1 during M-phase. Interestingly, some of these proteins are reported to be active in M-phase. For example, the phosphorylation on residue Thr169 on PAK2 had been identified to be down-regulated in our SILAC-MS data. Although this phosphorylation site is identified in previous mass spectrometry data, the upstream kinase responsible for modifying this site has yet to be identified (Dephoure et al., 2008; Gnad et al., 2013). Phosphorylation on Thr169 could potentially be important for regulating the activity of PAK2. Once localized at the centrosome, PAK2 is proposed to regulate the activation of AurkA and cyclin-B/CDK1 complex at the centrosome (May et al., 2014). In addition, PAK2 is reported to function downstream of Cdc42 in regulating cortical actin dynamics and spindle orientation (Mitsushima et al., 2009). These reports suggest that PAK2 could play multiple roles during mitosis. Coincidentally, we also observed LIMK1 to localize at the centrosome during G2/M-phase (Figure 3-1). Therefore, it is reasonable to speculate that LIMK1 could be the upstream kinase responsible for phosphorylating PAK2 on Thr169. LIMK1-mediated phosphorylation of PAK2 could potentially lead to activation of AurkA and cyclin-B/CDK1. Since PAK2
is an upstream kinase of LIMK1 during Interphase, LIMK1-mediated phosphorylation of PAK2 could potentially allow both kinases to form a positive feedback loop during M-phase (Misra et al., 2005).

Shugoshin-like 1 (SGOL1) is another protein we identified in our mass spectrometry study to show down-regulated phosphorylation in LIMK inhibited cells. SGOL1 is localized at the mitotic centromere of the chromosome and is recruited to the centromere by the Mitotic checkpoint serine/threonine-protein kinase BUB1 (Bub1) (Kitajima et al., 2005; McGuinness et al., 2005). In addition, Bub1 also recruits protein phosphatase 2A to counter-act the PLK1-mediated phosphorylation and removal of SGOL1 from the centromere (Tang et al., 2006). This regulation maintains SGOL1 at the centromere and prevents pre-mature chromosome segregation. In addition, SGOL1 could also be involved in regulating kinetochore-microtubule attachment as depletion of SGOL1 has been shown to increase the number of monotelic microtubule attachment at the mitotic kinetochores (Fu et al., 2007). Abnormal kinetochore-microtubule attachment can potentially cause uncontrolled scattering of the mitotic chromosomes, leading to prolonged mitosis (Yasuda et al., 2004). Interestingly, prolonged mitosis has been linked to the formation of multi-polar spindle due to centrosome cohesion fatigue (Alieva and Vorobjev, 1991; Brinkley and Rao, 1973; Ehrhardt and Sluder, 2005). In the current phospho-proteomic study, we identified Ser38 on SGOL1 as the potential phosphorylation site modified by LIMK1. Since phosphorylation has the potential to regulate the localization of SGOL1, it is possible that LIMK1-mediated phosphorylation of SGOL1 on Ser38 could regulate its localization onto the centromere. In addition, we also observed that the chromosomes of LIMK1-depleted cells do not congregate properly at the metaphase plate, suggesting a defect in kinetochore-microtubule attachment (Figure 3-4). Therefore, examining the localization and recruitment of the key kinetochore proteins, such as PLK1, AurkB, CENP-E and CREST, in LIMK1-depleted cells can potentially yield useful information about the integrity of the kinetochore-microtubule attachment (Sanhaji et al., 2011). If LIMK1 depletion is indeed found to negatively affect centromere integrity, we could then examine the role of Ser38 phosphorylation in regulating centromere structure and cohesion.
Recently, isoform 3 of SGOL1 (sSGOL1) has been demonstrated to be a substrate of PLK1 and PLK1-mediated phosphorylation of sSGOL1 is important for its recruitment to the centrosome (Wang et al., 2008). At the centrosome, sSGOL1 prevents pre-mature fragmentation of mother and daughter centrioles, thus preventing the formation of multi-polar spindles. In the current study, we observed multi-polar spindle in LIMK1-depleted cells. However, our findings did not suggest that the multi-polar phenotype was due to pre-mature fragmentation of centrioles during M-phase (Figure 3-10 and 3-11). Majority (about 83%) of LIMK1-depleted mitotic cells contained only two pairs of centrioles. Only a very small percentage of cells (about 17%) of LIMK1-depleted mitotic cells contained more than two pairs of centrioles. These observations suggest to us that pre-mature centriole fragmentation is not the leading cause of multi-polar spindle in LIMK1-depleted cells. Since our data suggest a possible defect in kinetochore-microtubule attachment, it is still worthwhile to observe the time taken for LIMK1-depleted cells to complete mitosis. This is because prolonged mitosis has been demonstrated to increase the chance of centrosome cohesion fatigue and multi-polar spindle formation (Daum et al., 2011; Stevens et al., 2011).

As discussed earlier, our phospho-proteomics data suggest that the phosphorylation of DYNC1LI2 is up-regulated in Compound 22 treated cells compared to control cells (Table 2). This data seems to contradict our PhosTag SDS-PAGE analysis, which suggested that LIMK1 could be an upstream kinase of DYNC1LI2 (Figure 3-56B). In addition, we also observed differences in the phenotype displayed in LIMK1 siRNA treated and Compound 22 treated cells. The reason for such discrepancies is not immediately clear, but the difference in activity of Compound 22 towards LIMK1 and LIMK2 could explain the differences in the data obtained. When Compound 22 is developed, the activity of the compound against LIMK1 and LIMK2 are tested (Harrison et al., 2009). The authors discover that Compound 22 is marginally more active against LIMK2 compare to LIMK1 (Harrison et al., 2009). Therefore, it is likely that Compound 22 inhibits LIMK2 with higher efficacy than LIMK1 during our short chemical treatment of 1 hour. This could potentially produce a phenotype that is closer to LIMK2 depletion compare to LIMK1 depletion. Recently, our laboratory has reported that LIMK2-depletion results in centrosome defocusing and does not lead to
a significant increase in multi-polar spindle compared to control and LIMK1 depleted cells (Heng et al., 2012). In addition, LIMK2 depletion is also reported to result in spindle mis-orientation. These phenotypes are similar to our observation in cells that were treated with Compound 22. Therefore, it is reasonable to speculate that Compound 22 could potentially be marginally more effective against LIMK2 than LIMK1, resulting in the discrepancies in our observations.

4.6 Future works

Although we had eliminated the possibility that the multi-polar spindle defects was not due to cytokinesis defects and abnormal centrosome duplication, we have not fully explored all possible factors that could result in multi-polar spindle. For instance, prolonging metaphase-to-anaphase transition has been shown to result in centrosome fragmentation and multi-polar formation (Daum et al., 2011). Interestingly, LIMK1 over-expression has been shown to prolong the time taken to complete M-phase, without causing any cytokinesis defects (Davila et al., 2007). In addition, RNAi-mediated knock-down of DYNC1LI1 has been shown to arrest cells at metaphase and delays the removal of SAC proteins away from the kinetochores of properly aligned chromosomes (Sivaram et al., 2009), resulting in delayed metaphase-to-anaphase transition. Therefore, it is important to explore the overall time taken for LIMK1-depleted cells to progress through M-phase and metaphase-to-anaphase transition. This could potentially provide us with the information needed to eliminate the possibility that the formation of multi-polar cells in LIMK1-depleted cells is due to metaphase arrest.

In our attempt in identifying the upstream regulator of LIMK1 for the maintenance of centrosome integrity, we explored the possibility of Rho-ROCK pathway as the activator of LIMK1. However, findings in the current study showed that inhibiting Rho and ROCK did not fully replicated the defects observed in LIMK1-depleted cells, suggesting that Rho and ROCK might not function upstream of LIMK1 in regulating centrosome integrity. Firstly, inhibition of Rho and ROCK led to centrosome
defocusing defects, but did not lead to a significant increase in the number of cells exhibiting multi-polar spindle (Figure 3-33). An earlier study by our laboratory has also shown that inhibiting Rho or ROCK results in centrosome defocusing but not multi-polar spindle formation (Heng et al., 2012). Secondly, inhibiting Rho or ROCK did not result in reduced centrosomal protein accumulation at the mitotic spindle pole (Figure 3-37), suggesting that Rho and ROCK do not regulate PCM protein transportation. The above two observations lead us to speculate that Rho-ROCK signaling does not function upstream of LIMK1 in regulating centrosome integrity.

CDK1 is known to phosphorylate LIMK1, possibly on residue other than Thr508, leading to its hyper-activation (Amano et al., 2002; Sumi et al., 2002). PAK1 is an upstream activator of LIMK1 during interphase and PAK1-LIMK1-Cofilin signaling has been implicated in tumor cell migration (Edwards et al., 1999; Jang et al., 2012). Interestingly, PAK1 has been implicated in the phosphorylation of AurkA, which is reported to be an activator of LIMK1 during mitosis (Ritchey et al., 2012; Zhao et al., 2005). Therefore, it is possible that PAK1-mediated phosphorylation of AurkA could lead to the activation of LIMK1. LIMK1 would in turn phosphorylate DYNC1LIs to regulate the transportation of PCM proteins to the mitotic centrosome. Previous mass spectrometry works had identified several unique M-phase phosphorylation sites on LIMK1 (Dephoure et al., 2008). These unique phosphorylation sites could be involved in regulating LIMK1 function during M-phase. It would be useful to create these phospho-mimic LIMK1 mutant constructs to investigate the roles of these phosphorylation sites in regulating centrosome integrity. These constructs could be introduced into LIMK1-depleted cells and observe for any mitotic defects. Mutants that can rescue the defects observed in LIMK1-depleted cells may provide clues to identify phosphorylation sites that are important for regulating LIMK1 activity in maintaining centrosome integrity. After identifying the possible residue(s), we can then proceed to identify the upstream kinases responsible for phosphorylating LIMK1.

Our PhosTag SDS-PAGE analysis suggests that DYNC1LI1 and DYNC1LI2 could be potential substrates of LIMK1. However, it is still unclear whether LIMK1 can directly phosphorylate both dynein light intermediate chains and which residue(s) on DYNC1LI1 and DYNC1LI2 are phosphorylated. We propose to use in-vitro kinase assay to determine if DYNC1LI1 and DYNC1LI2 are direct substrates of LIMK1. To
narrow down our search for potential residue phosphorylated by LIMK1, we utilized PhosphoNet software to predict potential phosphorylation site(s) and the potential kinase involved. We were able to shortlist 4 potential residues on DYNC1LI1 (Thr231 and Thr389) and DYNC1LI2 (Thr69 and Thr441) that are potentially phosphorylated by LIMK1. Phospho-dead mutant (mutating Thr to Ala) of these sites will be constructed and utilized for in-vitro kinase assay. Besides performing the in-vitro kinase assay, we can also determine if these predicted sites are important for regulating the function of DYNC1LIs. We will introduce the phospho-dead and phospho-mimic mutants of DYNC1LI1 and DYNC1LI2 into LIMK1-depleted cells and observe if these mutants can rescue the mitotic spindle defects observed in LIMK1-depleted cells. In addition, we also plan to examine the localization pattern of these mutants to determine if these phosphorylation sites are crucial for regulating dynein light intermediate chain localization onto the mitotic spindle apparatus.

Although we found that DYNC1LI1 and DYNC1LI2 could be potential substrates of LIMK1, the function of LIMK1-mediated phosphorylation on both dynein light intermediate chains is still unknown. Since phosphorylation of dynein motor subunits has been shown to regulate cargo-motor interactions, we speculate that LIMK1-mediated phosphorylation of DYNC1LI1 and DYNC1LI2 could affect binding to PCM proteins (Addinall et al., 2001; Vaughan et al., 2001; Whyte et al., 2008). Although we proposed that LIMK1-mediated phosphorylation could potentially affect DYNC1LIs interaction with their cargoes, LIMK1 phosphorylation might not be the only post-translational event needed for cargo interactions. LIMK1 depletion did not completely abolish PCM protein localization (Figure 3-15), suggesting that PCM could still accumulate at the mitotic centrosome in LIMK1-depleted cells. In addition, over-expressing DYNC1LI1 and DYNC1LI2 alone rescued the defects observed in LIMK1-depleted cells (Figure 3-40 and 3-42). Therefore, the role of LIMK1-mediated phosphorylation could be a supportive role rather than functioning as a crucial regulator of DYNC1LIs-cargoes interactions. LIMK1-mediated phosphorylation, together with other post-translational modifications, could in turn enhance the accumulation of PCM proteins onto the mitotic centrosome during centrosome maturation. Therefore, there is a need to generate the phospho-dead and phospho-mimic forms of DYNC1LI1 and DYNC1LI2 for immuno-precipitation assay. We could

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then determine if these phosphorylation sites could affect DYNC1LI1 and DYNC1LI2 interaction with their known binding partners, such as Pericentrin, γ-Tubulin and Par3 (Purohit et al., 1999; Schmoranzer et al., 2009; Young et al., 2000). The findings will help to determine if the phosphorylation status of DYNC1LI is important for cargo interaction.
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