COILED-COIL DOMAIN-CONTAINING PROTEIN 69 (CCDC69) ACTS AS A SCAFFOLD
AND A MICROTUBULE-DESTABILIZING FACTOR TO REGULATE CENTRAL
SPINDLE ASSEMBLY

by

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Abstract

Proper regulation of mitosis and cytokinesis is fundamentally important for all living organisms. During anaphase, antiparallel microtubules are bundled between the separating chromosomes, forming the central spindle (also called the spindle midzone), and the myosin contractile ring is assembled at the equatorial cortex. Regulators of central spindle formation and myosin contractile ring assembly are mostly restricted to the interdigitated microtubules of central spindles and they can be collectively called midzone components. It is thought that characteristic microtubule configurations during mitosis and cytokinesis are dictated by the coordinated action of microtubule-stabilizing and -destabilizing factors. Although extensive investigations have focused on understanding the roles of microtubule-bundling/stabilizing factors in controlling central spindle formation, efforts have been lacking in aiming to understand how microtubule-destabilizing factors regulate the assembly of central spindles.

This dissertation describes the role of a novel microtubule-destabilizing factor termed CCDC69 (coiled-coil domain-containing protein 69) in controlling the assembly of central spindles and the recruitment of midzone components. Endogenous CCDC69 was localized to the nucleus during interphase and to the central spindle during anaphase. Exogenous expression of CCDC69 in HeLa cells destabilized microtubules and disrupted the formation of bipolar mitotic spindles. RNA interference (RNAi)-mediated knockdown of CCDC69 led to the formation of aberrant central spindles and interfered with the localization of midzone components such as aurora B kinase, protein regulator of cytokinesis 1 (PRC1), MgcRacGAP/HsCYK-4, and polo-like kinase 1 (Plk1) at the central spindle. CCDC69 knockdown also decreased equatorial RhoA staining, indicating that CCDC69 deficiency can impair equatorial RhoA activation and ultimately lead to cytokinesis defects. Four coiled-coil domains were found in CCDC69 and the C terminal coiled-coil domain was required for interaction with aurora B. Disruption of aurora B function in HeLa cells by treatment with a small chemical inhibitor led to the mislocalization of CCDC69 at the central spindle. Further, vitro kinase assay showed that Plk1 could phosphorylate CCDC69. Taken together, we propose that CCDC69 acts as a scaffold and a microtubule-destabilizing factor to control the recruitment of midzone components and the assembly of central spindles.

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Dedication

To my wonderful parents Mala and Debasis Pal, and to the loving memory of my grandmother Sushama Pal

CHAPTER 1 - INTRODUCTION

An overview of cell cycle

The animal cell cycle is a complex, ubiquitous process that involves diverse events and regulatory steps culminating in producing two identical daughter cells from a mother cell. Cell cycle is involved in cell proliferation, normal growth of organism and disease conditions such as cancer. During developmental stages, most of the cells divide actively. But in a mature human body, most of the cells are quiescent; only a few specialized cells such as hematopoietic cells or gut epithelial cells proliferate actively. Upon receiving growth signal, quiescent cells (except the terminally differentiated cells) can enter the cell cycle and undergo division. Cancer cells often have mutations in the signal transduction pathways that regulate the cell cycle resulting in unrestrained cell proliferation (Norbury and Nurse 1992; Schafer 1998; Malumbres and Barbacid 2001).

Regulation of the cell cycle

The cell cycle consists of a synthesis or S phase, where the genomic material is duplicated and a division phase called mitosis or M phase where the genetic material is equally divided into two daughter cells. At the end of M phase, the cells can either stop dividing and become quiescent by entering G0 (gap zero) phase; or undergo another round of division. Entering a new cycle requires a cell to initially go through a preparatory gap phase called G1. In G1, the cell synthesizes all the proteins required for genomic material duplication in S phase. Another gap phase named G2 that lies between S and M phases perform regulatory functions to ensure the suitability of the cell to enter M phase. G1, S and G2 together are known as interphase. Mitosis consists of the following stages: prophase, metaphase, anaphase, telophase and cytokinesis. Mitosis encompasses the events of segregation of chromosomes and other cellular materials in equal halves. To maintain the genomic integrity of the organism, the cell cycle events need to be carried out properly. Thus the cell has developed a precise regulatory mechanism for the cell cycle which can sense errors in the cell cycle, and correct the errors in

order to maintain the genetic integrity of the organism in the daughter cells. The regulation mechanism has two main components: 1) cell cycle checkpoints that prevent cells from entering the next phase until all functions of the current phase have been performed and the cell is ready for the next phase, and 2) molecular players that include several protein kinases whose timely activation leads to phosphorylation of target proteins needed to move the cycle forward (Malumbres and Barbacid 2001).

Cell cycle checkpoints

The cell cycle checkpoints protect cells from acquiring irreversible genetic damages by inflicting a precise regulatory mechanism. Absence of mitogen/growth signal triggers cell cycle arrest at mid G1 phase, ensuring that proliferation occurs only in presence of external signal. This arrest is governed by a checkpoint named G1 (restriction) checkpoint. G1 checkpoint is one of the important regulatory steps in proper cell cycle progression as it induces INK4 family of CDK inhibitors to inactivate CDK4/6 and prohibits cell cycle progression in absence of mitogens.

In presence of UV ray- or gamma ray-induced damaged DNA, the DNA damage checkpoint halts the cell cycle at G1/S and G2/M transitions. The checkpoint activates transcription factor p53 to transcribe p21, an inhibitor of cyclin-CDK1, -CDK2, -CDK4 and -CDK6 complexes. A DNA damage repair signaling cascade is initiated that repairs the DNA break and, in presence of irreparable DNA lesions, p53 induces apoptosis of the cell.

Presence of unreplicated genetic material arrests the cycle at S phase.

The spindle checkpoint is activated upon improper alignment of chromosomes on the mitotic spindle during metaphase. A multiprotein complex termed as mitotic checkpoint complex containing Mad2 and Bub1 proteins is activated at chromosome kinetochores that inhibit anaphase promoting complex thus preventing mitotic progression. This checkpoint allows sufficient time for the cell to bring about proper attachment of chromosomes to the mitotic spindle (Schafer 1998; Vermeulen, Van Bockstaele et al. 2003; Musacchio and Salmon 2007).

Molecular regulators of cell cycle

The key molecular players of the cell cycle are cyclin-CDK complexes. A cyclin-CDK complex consists of a regulatory subunit cyclin and its cognate partner cyclin dependent kinase (CDK). CDKs, a class of serine threonine kinases are activated at different time points in a cell cycle. Activating phosphorylation as well as binding to cyclin activates these kinases. There are other kinases namely polo like kinase 1 (Plk1), aurora family kinase, NIMA kinase, mitosis checkpoint kinases like Bub1 which also play a significant role in proper cell cycle progression (Nigg 2001).

Quiescent cells or cells upon completion of a cycle prepare for another round of division in presence of mitogens. Proteins synthesized in the G1 phase include transcription factor E2F, cyclin D, CDK4, CDK6 etc. Presence of antimitogen signals cause INK4 family of inhibitors to bind and inactivate CDK4 and CDK6. Newly formed E2F is inhibited by retinoblastoma (Rb) protein. At mid G1, cyclin D-CDK4/CDK6 can initially phosphorylate Rb. E2F, bound to this partially phosphorylated Rb, transcribes cyclin E and CDK2. The active cyclin E-CDK2 complex phosphorylates Rb to release active E2F. E2F then stimulates synthesis of proteins necessary for G1/S transition and DNA replication such as DNA polymerase (Meraldi, Lukas et al. 1999; Kalma, Marash et al. 2001). Rb is kept phosphorylated and inactive till the end of M phase by different CDKs. Completion of mitosis triggers PP1 phosphatase to dephosphorylate Rb, restoring its growth suppressive role (Schafer 1998; Malumbres and Barbacid 2001).
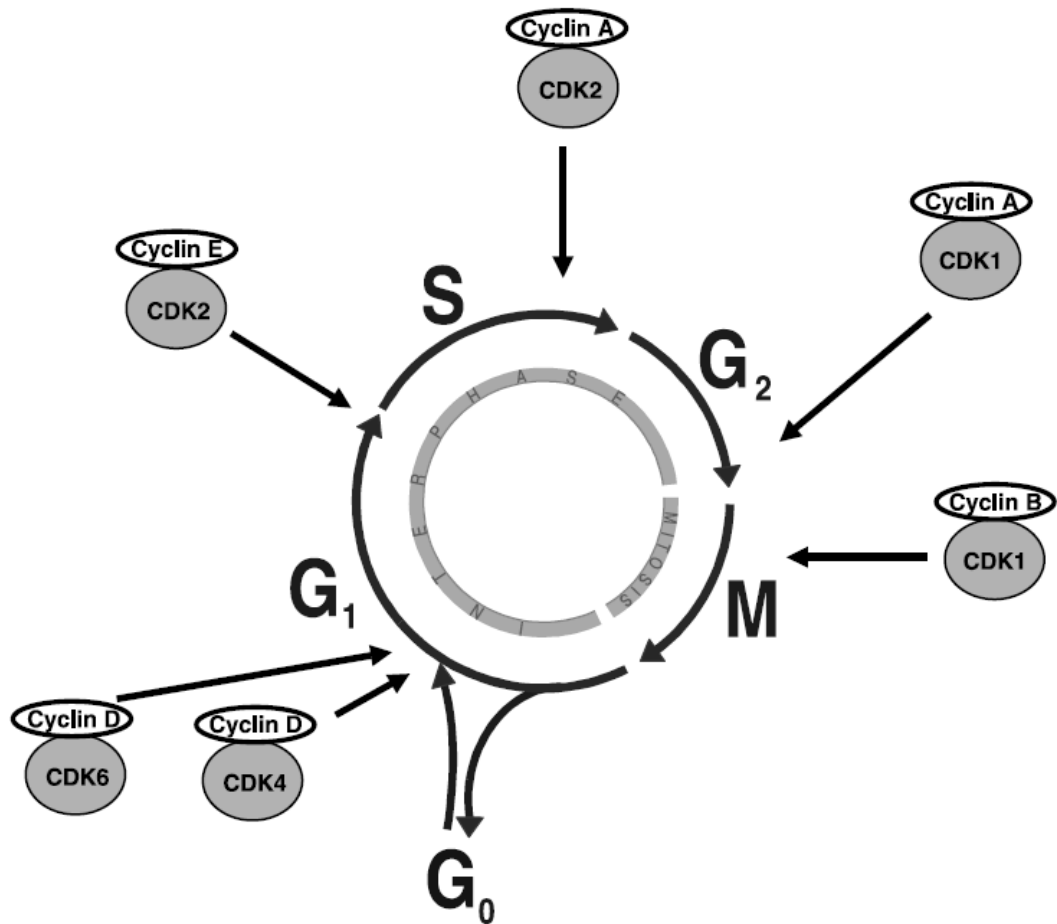
Figure 1-1. A schematic diagram of the model for regulation of eukaryotic cell cycle showing the involvement of different phase-specific cyclins and CDKs in the cell cycle modulation. Adapted with permission from John Wiley and Sons, *Cell Proliferation*, 2003, 36, 131–149.

Cyclin A is synthesized as the cells proceed through S phase and the cyclinA-CDK2 complex initiates DNA replication. Towards the end of S phase, an increase in the cyclinB expression level is observed, which reaches its peak in G2 and M phase. The principal CDK of G2 and M phase is CDK1; Wee1 kinase phosphorylates CDK1 thereby inactivating the kinase. Cdc25, a phosphatase capable of removing the inactivating phosphates from CDK1 is phosphorylated by DNA structure checkpoint kinases Chk1/Chk2 to keep it inactive until the cell is ready to enter M phase (Nigg 1995). At the end of G2, Plk1 phosphorylates Wee1, rendering it inactive. On the contrary, phosphorylation by Plk1 activates Cdc25 phosphatase and cyclinB. Removal of the inhibitory phosphates from CDK1 by Cdc25 is followed by formation of active

cyclinA-CDK1 and cyclinB-CDK1 complexes (Jackman, Lindon et al. 2003; Barr, Sillje et al. 2004). These cyclin-CDK complexes, in addition with other mitotic kinases initiate the mitosis by inducing chromosome condensation and nuclear envelope breakdown (Nigg 2001). This event is followed by mitotic spindle assembly and alignment of condensed chromosomes at metaphase plate. Proper attachment of chromosomes to kinetochore microtubules of mitotic spindles is followed by activation of anaphase promoting complex (APC^{CDC20}) by M phase cyclin-CDK and Plk1. APC^{CDC20} initiates ubiquitin mediated degradation of anaphase inhibitor securin. Proteolysis of securin releases separase, a protease that subsequently cleaves a component of the multiprotein complex condensin. Condensin holds the sister chromatids together and its breakdown separates sister chromatids and triggers the onset of anaphase. In late anaphase, APC^{CDC20} mediated destruction of cyclin A and cyclin B occur resulting in decrease in activity of CDK1 (Irniger 2002). This drop in mitotic cyclin-CDK level allows chromosome condensation, nuclear envelope formation and prepares the cell for cytokinesis (Kramer, Scheuringer et al. 2000; Vermeulen, Van Bockstaele et al. 2003).

Coiled-coil domain

The α-helical coiled-coil is a structural motif found in numerous proteins (Lupas 1996). This ubiquitous motif occurs in 5-10% of translated proteins and often functions as a protein oligomerization motif (Wolf, Kim et al. 1997; Arndt, Pelletier et al. 2002; Moutevelis and Woolfson 2009). Usually two to five α-helices wrap around each other to form a left handed superhelical or coiled-coil structure. Each helix in a coiled-coil has been found to have a repeat of seven amino acids. This repeat is generally denoted as a-b-c-d-e-f-g in the helix (Figure 1-2). Amino acids at position a and d are usually nonpolar amino acids that lie at the interface of two helices. Residues e and g are polar residues that remain exposed to the solvent (Mason and Arndt 2004). The 'peptide velcro' hypothesis proposed by O'Shea and colleagues outlines the structural elements required for the formation of a coiled-coil motif. According to this hypothesis, residues a and d should be occupied by hydrophobic amino acids, such as leucine, isoleucine and valine. This would result in hydrophobic and van der Walls interactions between the dimerizing helices, thus stabilizing the coiled-coil structure. The hypothesis also states that positions e and g should have charged amino acids such as arginine or aspartate. This would

allow residue g of one heptad to form ionic bond with the oppositely charged residue e' of a heptad from the neighboring helix (Arndt, Pelletier et al. 2002).
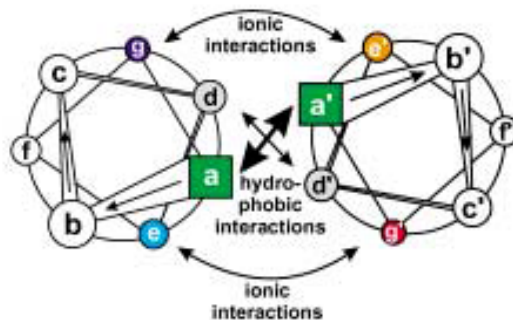
Figure 1-2. A helical wheel diagram of a dimeric coiled-coil domain. The diagram views down the axis of α−helices in an N to C terminus fashion. Jody M. Mason and Katja M, Coiled Coil Domains: Stability, Specificity, and Biological Implications, *ChemBioChem*, 2004, 5, 170 -176, Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

Several computational programs have been developed which can predict the probability of the formation of a coiled-coil domain in a given peptide sequence. The program COILS (http://www.ch.embnet.org/software/COILS_form.html) compares the given peptide sequence to a database of coiled-coil proteins and predicts the probability of the peptide forming a coiled-coil motif (Lupas, Van Dyke et al. 1991). COILS was used to predict the probability of formation of coiled-coil domains in human CCDC69 (Figure 2-3 A).

Coiled-coil domains can have a wide range of functions. In yeast transcription factor GCN4, coiled-coil domain forms the leucine zipper that drives the dimerization of GCN4 (O'Shea, Klemm et al. 1991). This domain is also found in other transcription factors such as oncoprotein Fos and Jun (Glover and Harrison 1995). In laminin that forms a part of the extracellular matrix, coiled-coil domain acts as a scaffold (Hunter, Schulthess et al. 1992; Kammerer, Schulthess et al. 1999). Coiled-coil domains in cohesin subunits SMC1 and SMC3 are implicated in sister chromatid cohesion (Gruber, Haering et al. 2003), whereas in motor protein kinesin, this domain acts as a linker between the motor and the tail domain of kinesin (de Cuevas, Tao et al. 1992).

Microtubule structure and function

The shape and structure of the cell is organized and maintained by a cellular component called cytoskeleton. It provides the cell with the mechanical strength to bear environmental stress. Microtubule, an important component of the cytoskeleton is found in a wide range of prokaryotic as well as eukaryotic cells (Olmsted and Borisy 1973). Microtubule is the integral structural component of prokaryotic cilia and flagella (Allen 1968). In eukaryotes, the function of microtubule includes positioning the nucleus and organelles, maintaining cell shape, intracellular transport of materials such as organelles, chromosomes and cellular motility to mention a few (Luykx 1970).

Microtubule Nucleation and Elongation

Microtubule is composed of a linear arrangement of $\alpha\beta$−tubulin heterodimers. Each α and β monomer has a molecular mass of about 50kDa and share 50% of primary sequence identity (Burns 1991; Desai and Mitchison 1997). The formation of microtubule occurs in two steps: nucleation and elongation. Unlike α− and β− tubulin that constitute the microtubule polymer, γ-tubulin is reported to mediate the nucleation of microtubule polymerization (Shu and Joshi 1995). It is believed that a centrosomal multiprotein complex is responsible for the microtubule nucleation in the cell. This multiprotein complex, known as γ-tubulin ring complex (γ−TuRC) is composed of γ-tubulin and gamma complex proteins (Luders and Stearns 2007). Since γ−TuRC lies in the centrosome, the arrangement is often named as microtubule organizing center (MTOC). A single centrosome can contain several copies of γ−TuRC.γ-tubulins can generate long lived spiral protofilament and extend a short stretch of the protofilament which acts as a seed for polymerization. $\alpha\beta$ tubulin heterodimers can then attach to the seed. The newly added $\alpha\beta$ heterodimers bind each other longitudinally and form lateral connections with the γ-tubulin. Though α-tubulin can bind GTP in a non-exchangeable manner, β-tubulin is reported to bind GTP in an exchangeable manner (Spiegelman, Penningroth et al. 1977). During polymerization, although α-tubulin retains its bound GTP, β−tubulin bound GTP is hydrolyzed

into GDP (David-Pfeuty, Erickson et al. 1977). While polymerized in microtubule, β−tubulin can not release the bound GDP; upon depolymerization, it can exchange the GDP for GTP and the αβ heterodimer can afterwards take part in the next round of microtubule polymerization. Addition of αβ tubulin heterodimers to γ-tubulin seed gradually increases the length of the microtubule protofilament. Once the next protofilament reaches a critical length of 12±2 dimers, the microtubule wall grows spontaneously in length and breadth and eventually closes the tube laterally (Erickson and Stoffler 1996; Pampaloni and Florin 2008). Figure 1-3 depicts this model of microtubule nucleation. The minus ends of microtubule protofilaments are attached to the γ-TuRC while protofilament plus ends grow outwards from the γ−TuRC to the cell periphery.
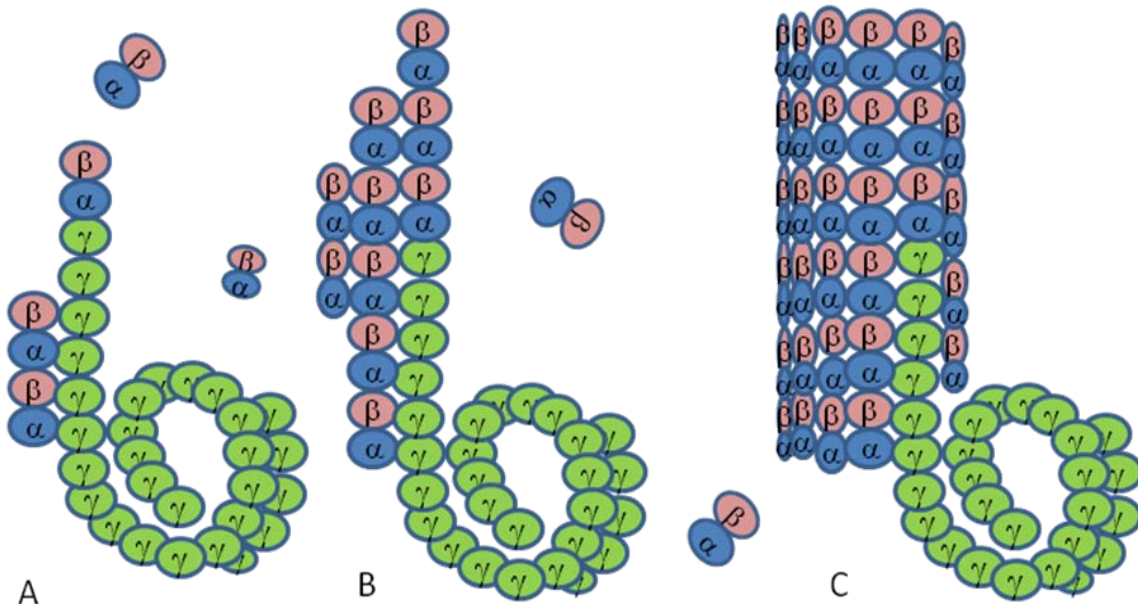
Figure 1-3. A model of microtubule nucleation from γ-Tubulin Ring Complex. A) α− and β− tubulin dimers add to the spiral protofilament ring formed by γ−tubulins. B) and C) shows the gradual growth of a microtubule wall. © Erikson and Stoffler, 1996. *J. Cell Biol.* 135:5–8.

During microtubule elongation tubulin αβ heterodimers are arranged head-to-tail to form 8nm wide linear protofilaments which then join laterally forming a hollow cylindrical microtubule with a diameter of 25nm. Microtubules assembled from centrosome *in vitro*

generally have 13 protofilaments (Evans, Mitchison et al. 1985). In the theoretical microtubule lattice protofilaments laterally interact in α to β manner. The more accepted microtubule lattice structure is where the protofilaments interact through α to α and β to β arrangement (Desai and Mitchison 1997). The GTP bound αβ-tubulin heterodimers can add to the end of a microtubule and elongate the same. The end of a microtubule which contains an exposed GTP bound α–tubulin is protected from depolymerization and thus is called minus end or slow growing end. The other end of microtubule, where GDP bound β–tubulin is exposed is the fast growing or plus end of microtubule. Thus, microtubule exhibits structural polarity in cells.

Dynamic properties of microtubule

Microtubule has two different dynamic properties: dynamic instability and treadmilling (or flux) (Mitchison and Kirschner 1984; Margolis and Wilson 1998). Dynamic instability refers to the phenomenon where an individual microtubule end can switch randomly and abruptly between growing and shrinking phases. Dynamic instability can be characterized by four parameters: frequency of catastrophe (a switch from a growing phase to a shrinking phase), frequency of rescue (shrinking to growing phase transition), rate of microtubule polymerization and that of depolymerization (Desai and Mitchison 1997). Treadmilling or flux refers to a steady state of microtubule resulting from a net growth at the plus end and a net shortening at the minus end, leading to a continuous flux of αβ-tubulin dimers from one microtubule end to the other (Margolis and Wilson 1998). Studies have also shown that microtubule dynamics is regulated by the coordinated action between microtubule-stabilizing factors such as microtubule-associated proteins (MAPs) and microtubule-destabilizing factors such as kinesin-13, Stathmin/Op18, and katanin (Drewes, Ebneth et al. 1998; Andersen 2000) .

Interphase microtubule

During interphase, the centrosome and thus the MTOC lie near the nucleus and define the center of the interphase cells. The plus end of microtubule grows towards the cell cortex in an astral manner whereas the minus ends are focused towards the centrosome. The primary functions of interphase microtubules include 1) development and maintenance of cellular

structure 2) intracellular transport of cargos 3) cell locomotion and 4) sensory transduction (Olmsted and Borisy 1973). Intracellular cargo, such as vesicles and cell organelles are trafficked over microtubule tracks with aid from microtubule associated motor proteins such as kinesin and dynein. Kinesins convey the cargo towards microtubule plus end while dyneins are directed towards microtubule minus end (Schroer 2004; Hancock 2008). Microtubule is also implicated in aiding the cell in locomotion by forming cilia, flagella and sperm tails – the integral structure for different cell movement machinery (Olmsted and Borisy 1973). The microtubules in tissue cultures have been reported to be involved in cell locomotion by extending the pseudopodia from these cells (Follett and Goldman 1970).

To prepare the interphase microtubules for mitosis, the centrosomes undergo structural reorganization, also referred to as maturation. More γTuRCs are recruited to the MTOC along with increase in microtubule nucleation activity (Khodjakov and Rieder 1999). The proteins that have been reported to regulate centrosome maturation are Plk1 (Lane and Nigg 1996), aurora A kinase (Berdnik and Knoblich 2002) and protein phosphatase 4 (Sumiyoshi, Sugimoto et al. 2002).

Assembly of mitotic spindles

The absence of proper chromosome segregation during mitosis results in tumorigenesis, while improper segregation during meiosis causes birth defects (Compton 2000). Chromosome segregation during mitosis and meiosis is carried out with the aid of a complex superstructure called the mitotic spindle. During prometaphase of mitosis two centrosomes containing the MTOC form spindle poles and the microtubules emanate from the poles to form a symmetric bipolar mitotic spindle. Microtubules originating from the centrosomes have their minus ends focused in the centrosomal region, while the plus ends emanate towards the cell cortex or chromosomes (Heidemann and McIntosh 1980).

There are three types of microtubules in the mitotic spindle: kinetochore, interpolar, and astral microtubules (Figure 1-4). Several kinetochore microtubules can attach to kinetochore, a multiprotein structure on the chromosome. The kinetochore is composed of structural proteins CENP-A,-B,-C, motor protein CENP-E, inner centromere protein INCENP, microtubule destabilizing enzyme MCAK and checkpoint signaling proteins Mad2, Bub1 (Rieder and Salmon

1998). Proper connection of chromosomes to kinetochore microtubules establish a bipolar attachment orienting the sister chromatids towards opposite poles and segregating the genetic material equally into two daughter cells. Moreover, the kinetochore proteins decrease the destabilization of the microtubules bound to it ensuring that the microtubules do not detach from the chromosomes during the segregation (Cassimeris, Rieder et al. 1990; Compton 2000). Astral microtubules originate from the centrosome and extend towards the cell cortex. Astral microtubules position the mitotic spindle and have a role in ascertaining the location of cleavage furrow (Hyman 1989). Interpolar microtubules extend from the centrosome and grow towards the center of the cell, where the microtubules tend to form antiparallel interactions with their counterparts emanating from the opposite centrosome (Mastronarde, McDonald et al. 1993). The unique fusiform shape of the mitotic spindle is achieved by cross linking interpolar microtubules at the middle of the mitotic spindle and focusing the microtubules' minus ends at the spindle poles (Glotzer 2009).
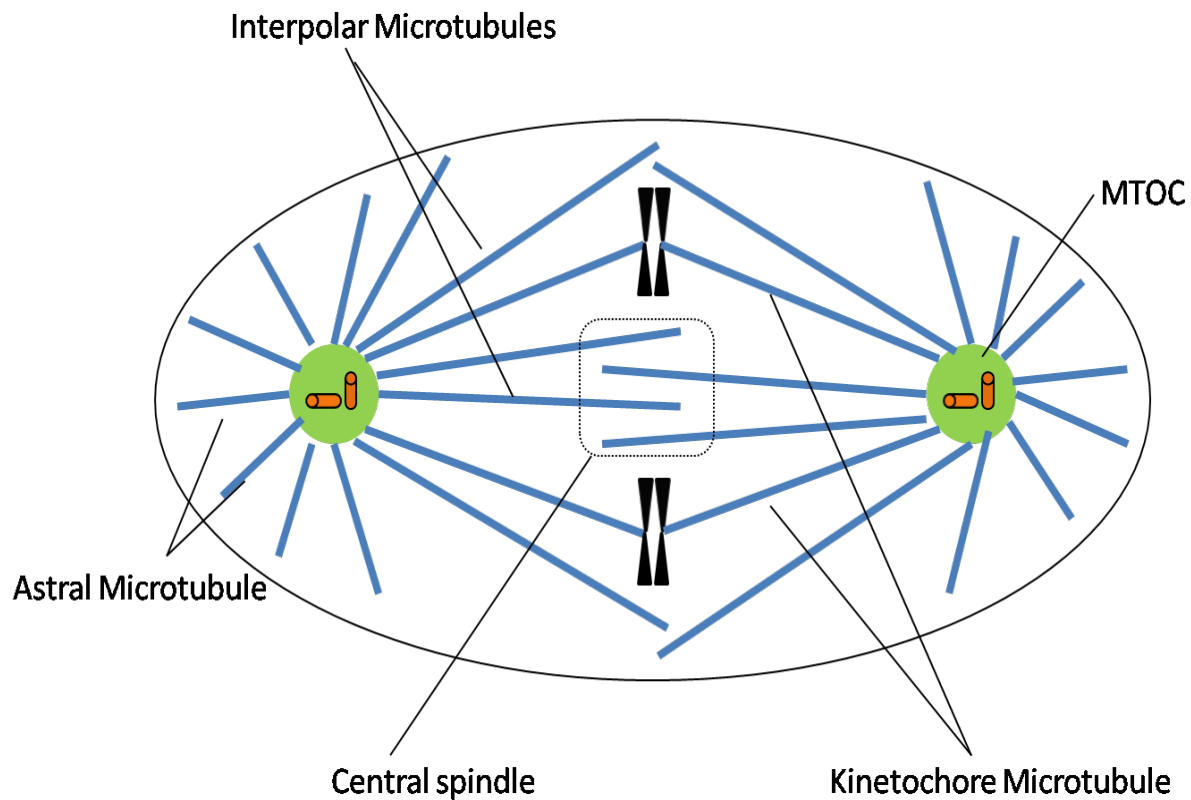
Figure 1-4. Different types of microtubules present in a cell during mitosis.

Central Spindles

Anaphase onset induces dramatic spindle reorganization which is characterized by elongation of astral microtubules and shortening of kinetochore microtubules, the latter causing poleward movement of chromosomes. This reorganization is also distinguished by relocalization of cytoplasmic proteins to the central spindle, for initiating central spindle assembly and antiparallel microtubule bundling. Antiparallel microtubules are assembled between the separating chromosomes and this region between the two poles is called the spindle midzone. The antiparallel microtubules at the spindle midzone are referred to as midzone microtubules. The center of the spindle midzone consisting of overlapping microtubule plus ends is referred to as the central spindle. Central spindle microtubule minus ends gradually lose their connection with mitotic spindle poles (Glotzer 2009).
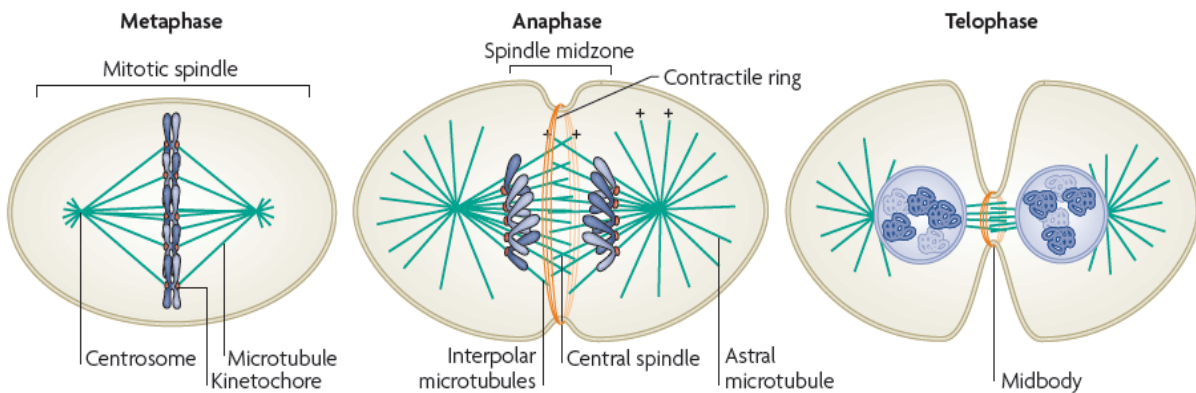
Figure 1-5. A schematic diagram representing the distribution of microtubule and chromosomes during different stages of cell division. Adapted with permission from *Nature Reviews Molecular Cell Biology* 10, 9-20.

Studies have shown that the microtubules at central spindle are highly dynamic and unstable during late anaphase, and lose their dynamicity in the telophase (Shelden and Wadsworth 1990). But the lengths of the interdigitated microtubules in the central spindle are quite uniform. This could be interpreted as that the bundling and polymerization of microtubules at the central spindle is counterbalanced by some microtubule destabilizing factors, so that the

central spindle does not overgrow. I will now, discuss here about the microtubule stabilizing and destabilizing factors, the gap in our knowledge and how our work can aid in filling the gap.

Regulators of central spindle assembly

It has been demonstrated that protein regulator of cytokinesis 1 (PRC1) (Jiang, Jimenez et al. 1998), the centralspindlin complex (containing HsCYK-4/MgcRacGAP and mitotic kinesin-like protein 1 (Mklp1)) (Toure, Dorseuil et al. 1998), and the chromosome passenger complex (CPC, containing aurora B, INCENP, borealin and survivin) (Adams, Carmena et al. 2001; Gassmann, Carvalho et al. 2004) serve as the core regulators of central spindle assembly. These proteins are concentrated as a narrow band at the central spindle, where the plus ends of antiparallel microtubules overlap, forming a dense region that often masks tubulin epitopes (Saxton and McIntosh 1987). There are additional proteins identified which also capacitate central spindle assembly. These factors include mitotic kinesis-like protein 2 (MKlp2) (Gruneberg, Neef et al. 2004), M phase phosphoprotein 1 (MPP1) (Abaza, Soleilhac et al. 2003) and MAST/orbit (Maiato, Sampaio et al. 2002). These regulators of central spindle assembly are mostly restricted to the interdigitated microtubules of central spindles and they are collectively called midzone components.

PRC1

Protein regulator of cytokinesis 1 (PRC1) serves as one of the important regulators of central spindle assembly (Jiang, Jimenez et al. 1998). Phosphorylation by CDK1 during early mitosis decreases microtubule binding efficiency of PRC1 (Mollinari, Kleman et al. 2002). At the onset of anaphase, PRC1 is activated following the inactivation of CDK1. A microtubule motor called KIF4A transports the PRC1 to the plus ends of interdigitated microtubules at the central spindle. This localization of PRC1 limits its activity to the small region of central spindle where PRC1 dimers bind directly to the microtubule and initiate bundling of antiparallel microtubules at the plus end (Jiang, Jimenez et al. 1998; Zhu and Jiang 2005). PRC1 has also been reported to recruit centralspindlin and CPC components to the central spindle (Ban, Irino et

al. 2004). PRC1 binds to Plk1 during anaphase and this interaction is required for proper cytokinesis (Neef, Gruneberg et al. 2007).

Centralspindlin

The centralspindlin is a heterotetrameric complex that contains a dimer of Mklp1 (a kinesin-6 motor protein) and a dimer of MgcRacGAP/HsCYK-4 (a GTPase-activating protein or GAP) (Pavicic-Kaltenbrunner, Mishima et al. 2007). It has been reported to promote microtubule bundling through its binding to the central spindle microtubule (Mishima, Kaitna et al. 2002). The components of centralspindlin are dependent on each other for their activity (Toure, Dorseuil et al. 1998; Jantsch-Plunger, Gonczy et al. 2000; Mishima, Kaitna et al. 2002).

CPC

The chromosome passenger complex is a multiprotein complex of aurora B kinase, INCENP, borealin and surviving (Adams, Carmena et al. 2001; Gassmann, Carvalho et al. 2004). It derives the name from the fact that, at the onset of mitosis, CPC proteins move from the chromosome arm and localize to the inner centrosome; then later at anaphase, CPC relocalize to the central spindle, as if the chromosomes have delivered them to the central spindle (Bucciarelli, Giansanti et al. 2003), though recent studies show that CPC localization to central spindle is independent of chromosomes (Vader, Kauw et al. 2006).

Aurora B, a serine threonine kinase is the catalytic subunit of the CPC. Live cell imaging studies have shown that aurora B localizes to the chromosomal centromeres through early anaphase. This is followed by aurora B relocalization to the central spindle, where initially aurora B is dispersed at the midzone microtubules between the separating chromosomes, and later concentrates as a sharp band at the equatorial plane of the central spindle. During cytokinesis aurora B localizes to the midbody structure (Murata-Hori, Tatsuka et al. 2002). Figure 1-6 shows the localization of aurora B-GFP at different time points during mitosis.
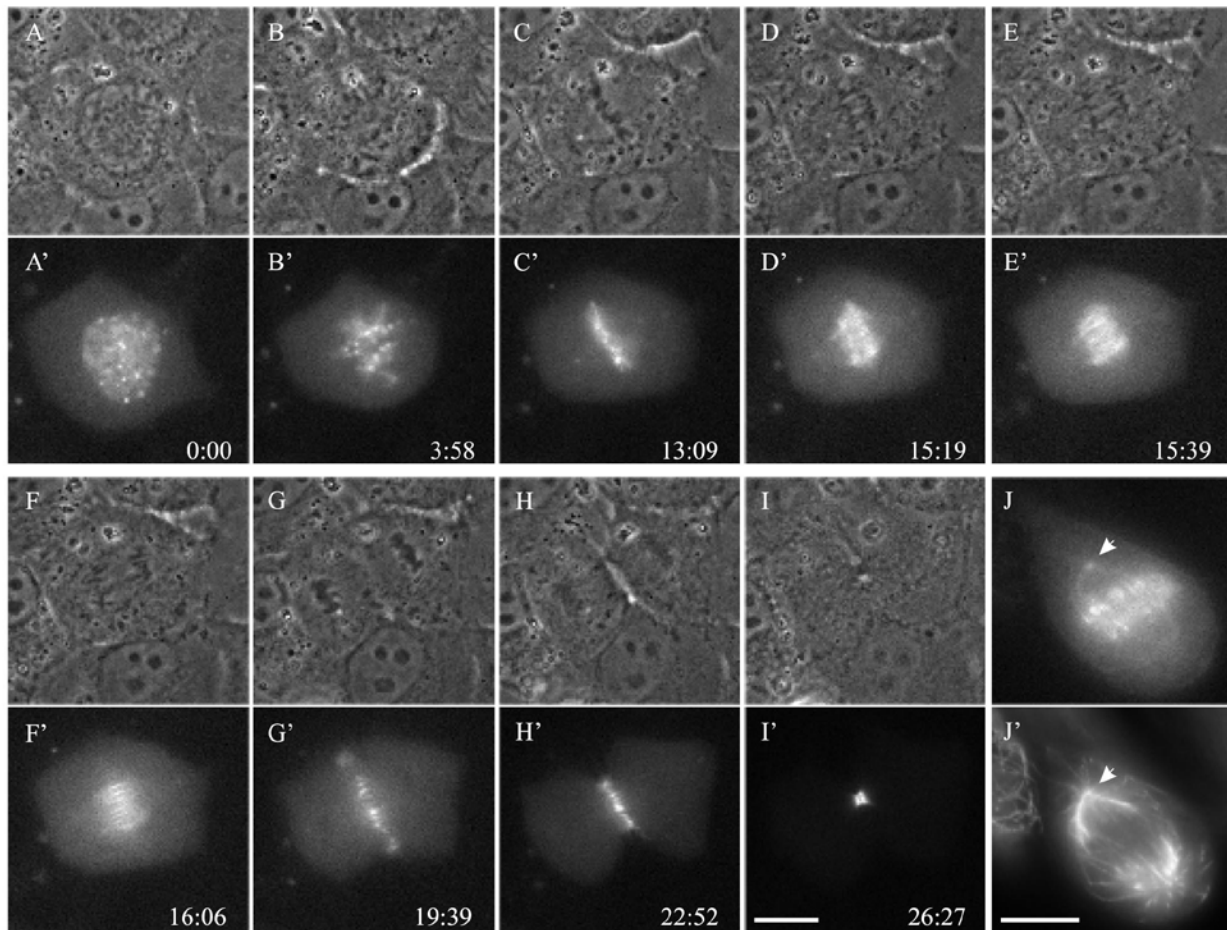
Figure 1-6. Aurora B dynamics in living mitotic NRK cells expressing GFP-Aurora B. Panels A-J represent the phase contrast microscopy image of the live cell; panels A'-J' represent the respective fluorescence microscopic images. Adapted with permission from American Society for Cell Biology, *Molecular Biology of the Cell*, 13, 1099–1108.

Aurora B redistribution from centrosome to central spindle during anaphase is regulated by CDK1 deactivation and assembly of midzone microtubules. In contrast, once localized at central spindle, aurora B kinase activity stabilizes its own distribution (Murata-Hori, Tatsuka et al. 2002). During mitosis aurora B can phosphorylate several proteins, including histone H3 which trigger chromosome condensation (Katayama, Brinkley et al. 2003). Aurora B also phosphorylates midzone components like INCENP (Bishop and Schumacher 2002) and centralspindlin (Guse, Mishima et al. 2005) which bring about proper central spindle formation and cytokinesis.

INCENP is considered as the structural component of the CPC, as it consists of a tubulin binding domain (Wheatley, Carvalho et al. 2001). Phosphorylation by aurora B activates INCENP (Bishop and Schumacher 2002). This is followed by INCENP binding and activation of aurora B and redistribution of aurora B to the central spindle (Romano, Guse et al. 2003; Sessa, Mapelli et al. 2005). It is also reported that Mklp2 localizes INCENP-aurora B complex to the central spindle during anaphase (Gruneberg, Neef et al. 2004). Therefore, INCENP and Mklp2 may redundantly or cooperatively contribute to the recruitment of the CPC complex to the central spindle. Survivin, another member of the CPC has been identified to mediate centromere and midbody docking of aurora B as well as to function in mitotic progression and cytokinesis (Adams, Carmena et al. 2001; Vader, Kauw et al. 2006). Borealin has been reported to promote binding of surviving to INCENP (Vader, Kauw et al. 2006).

Microtubule destabilizing factors in central spindle assembly

There is a growing family of proteins that promote microtubule destabilization. Examples of such proteins are kinesin-13 family proteins and stathmin/Op18. These microtubule destabilizing factors modulate microtubule dynamics during mitosis and are regarded as key regulators of central spindle assembly (Wordeman 2005). A thorough discussion of these proteins is included in the following section.

Kinesin-13 family proteins

Kinesin-13 family contains three ATPase proteins- Kif2a, Kif2b and Kif2c/MCAK. The kinesin-13 family proteins have a characteristic neck like domain that can bend to attach to the helical end of microtubule and enhance microtubule depolymerization (Ogawa, Nitta et al. 2004). It has also been observed that aurora B kinase can phosphorylate this microtubule binding domain of kinesin-13 family proteins to inactivate their depolymerization activity (Andrews, Ovechkina et al. 2004; Lan, Zhang et al. 2004). This indeed is an important regulatory step in maintaining proper central spindle structure.

Kinesin-13 family proteins are localized in the nucleus during the interphase. During mitosis Kif2a primarily localizes to the centrosomes while Kif2c/MCAK localizes to

chromosomal centromeres. Kif2b localize to central spindle and midbody during anaphase and telophase respectively (Rogers, Rogers et al. 2004). Consistent with their localization, Kif2a is responsible for destabilization of microtubule minus ends at mitotic spindle poles (Gaetz and Kapoor 2004), Kif2b accounts for microtubule destabilization at the central spindle and Kif2c confer depolymerization to microtubules at kinetochores. Depletion of Kif2a results in increase in mitotic spindle length (Gaetz and Kapoor 2004) and decrease in poleward chromosome movement (Rogers, Rogers et al. 2004). Cytokinesis defects were observed in cells lacking Kif2b (Manning, Ganem et al. 2007). This finding indicates that Kif2b may act as a microtubule-destabilizing factor to regulate microtubule dynamics at the central spindle, thus contributing to the regulation of cytokinesis.

Stathmin/Op18

Stathmin/Op18 is distributed in the cytoplasm of the interphase cells and localizes to mitotic spindles (but not central spindles) during mitosis (Gavet, Ozon et al. 1998). Stathmin can sequester tubulin dimers and reduce the concentration of free tubulin for microtubule polymerization. Since the rate of microtubule catastrophe depends on the availability of free tubulin, microtubule catastrophe increases at the plus end in presence of stathmin, under physiological condition (Belmont and Mitchison 1996; Curmi, Andersen et al. 1997). Exogenous expression of wild-type stathmin in transfected cells increases depolymerization of both interphase and mitotic microtubules (Gavet, Ozon et al. 1998). However, an earlier study has shown that stathmin is not essential for mitotic spindle assembly (Andersen, Ashford et al. 1997). According to Küntziger et. al., microtubule binds some kinases that can phosphorylate and inactivate stathmin near the central spindle (Kuntziger, Gavet et al. 2001). Proliferation signals have been observed to dictate certain kinases like CDK1, CDK2, MAP kinase, PKA to phosphorylate stathmin at particular conserved serine residues (Sobel 1991; Larsson, Melander et al. 1995). The phosphorylated stathmin weakly binds to tubulin dimers and does not promote microtubule catastrophe at the central spindle (Di Paolo, Antonsson et al. 1997; Holmfeldt, Larsson et al. 2001).

Interplay between central spindle protein components

It has been indicated that binding of midzone components to overlapping antiparallel microtubules is critical for their recruitment to the central spindle (Wheatley, Carvalho et al. 2001; Vader, Kauw et al. 2006). Investigations have demonstrated interdependence amongst the various protein components of the central spindle for their localization and assembly, thus suggesting presence of a networking amongst them (Jantsch-Plunger, Gonczy et al. 2000). Aurora B kinase phosphorylates INCENP and centralspindlin component Mklp1 and MgcRacGAP. This phosphorylation trigger their localization to the central spindle and completion of cytokinesis (Bishop and Schumacher 2002). Moreover, Mklp2 is critical for the translocation of INCENP-aurora B complex to the central spindle during anaphase (Gruneberg, Neef et al. 2004). Survivin mediate centromere and midbody docking of aurora B (Vader, Kauw et al. 2006). Plk1 can phosphorylate Mklp2, MgcRacGAP and PRC1; both PRC1 and Mklp2 are required for Plk1 localization to the central spindle (Neef, Preisinger et al. 2003). Hence, it is likely that the proper combination and arrangement of the midzone components are crucial for central spindle structure. However, the regulatory mechanism beyond the localization of midzone components particularly at the interdigitated microtubules of central spindle is not well understood. It is likely that the midzone components are interconnected and stabilized by scaffolds at the central spindle.

Model for central spindle assembly

A model on central spindle assembly has been proposed recently. According to this model, upon anaphase onset, growths of interpolar and astral microtubules induce elongation of the spindle. Meanwhile CDK1 inactivation activates the midzone components at central spindle. Following activation, PRC1 causes antiparallel microtubule bundling at the plus end. Centralspindlin subunit MgcRacGAP, which is concentrated at the central spindle, has preference for binding to and bundling antiparallel microtubules, and is stabilized by CPC. Thus the presence of PRC1, centralspindlin and CPC stabilizes the central spindle structure. The over bundling at the central spindle is perhaps prohibited by the presence of microtubule destabilizing proteins (Glotzer 2009).

Central spindle assembly and chromosome movement

It is believed that the chromosomal movement during mitosis is aided by the forces generated within the mitotic and central spindle. During late metaphase and early anaphase (also called anaphase A or chromosome-to-pole motion), sister chromatids move towards the opposite poles of mitotic spindles (Mitchison and Salmon 1992). This movement is primarily achieved by shortening of the kinetochore microtubules through their depolymerization either at the plus ends (called "Pac-Man" model) (Sharp and Rogers 2004) or at the minus ends (called "poleward flux" model) (Wadsworth 1993). The sliding force for poleward flux is generated by phosphorylated homotetrameric kinesin 5 motor protein EG5 (also known as KIF11). Eg5 pushes anti-parallel microtubules apart and promotes mitotic spindle pole separation (Mitchison 2005).

During late anaphase (also called anaphase B or pole-to-pole motion), the elongation and antiparallel sliding of overlapping microtubules at the central spindle provide a major force driving the mitotic spindle poles to move apart. One of these forces is generated by the poleward flux in kinetochore microtubules, where microtubule subunits are polymerized at the microtubule plus ends attached to kinetochores and depolymerizing from poleward microtubule minus ends at a constant rate (Saxton and McIntosh 1987; Mitchison 1989). Saxton and McIntosh also provided evidence suggesting that antiparallel microtubules can slide and produce forces within the central spindle (Saxton and McIntosh 1987). Thus the treadmilling phenomenon functions in generating the force in the central spindle for the chromatid movement to the mitotic spindle poles. The proteins that are thought to be the primary motors for antiparallel sliding during anaphase B are various plus-end-directed kinesins, such as CENP E, Kif 4A and MKLP1 that target to overlap microtubules at anaphase onset (Mitchison 2005).

Cytokinesis in eukaryotic cells

The cytokinesis is the final step in cell cycle division and leads to formation of two identical daughter cells. Cytokinesis onsets right after sister chromatid separation and consist of a few cortical rearrangements. The major steps of cytokinesis include 1) position of cleavage plane/cytokinesis furrow specification, 2) contractile ring assembly, 3) contractile ring

contraction and furrow ingression and 4) midbody formation, abscission and cytokinesis completion (Glotzer 2001; Bringmann 2005).

Cleavage plane determination

The chromosome segregation dictated by the positioning of mitotic spindles is spatially correlated to the cleavage plane determination during cytokinesis. The position of the plane of division is determined after chromosome segregation, between the segregated chromosomes, to ensure that each daughter cells receives all the cytoplasmic and nuclear materials (Guertin, Trautmann et al. 2002). It has been shown that central spindle disruption during early anaphase leads to cytokinetic defect, suggesting that transient signals from early anaphase central spindle influence cytokinesis (Cao and Wang 1996). A more recent study has shown that the mitotic spindle emits two consecutive signals which are required for positioning the furrow: the first signal comes from microtubule asters and is followed by a second signal from the central spindle (Bringmann 2005).

Contractile ring assembly

After the cleavage plane is established, a contractile ring/cleavage furrow is formed at the cleavage plane. The contractile ring made up of a ring of proteins position beneath the cortex and envelops the cell equator. The ring is composed of actin and myosin II. Activation of the RhoA GTPase is the key regulatory mechanism of this ring assembly (Yuce, Piekny et al. 2005). Plk1 plays a central role in recruiting a Rho specific guanine nucleotide exchange factor (RhoGEF) Ect2 to the central spindle (Sumara, Vorlaufer et al. 2002). Ect2 regulates the molecular switching of RhoA GTPase between active GTP bound and inactive GDP bound states, leading to the activation of RhoA and the assembly of the myosin contractile ring (Burkard, Randall et al. 2007). Activated RhoA associates with kinases such as Rho kinase (ROCK) and citron kinase. These kinases phosphorylate the regulatory light chain of myosin which is reported to be essential for regulation of cytokinesis (Satterwhite and Pollard 1992). The ROCK also increases actin filament assembly and bundling.

Contractile ring contraction, furrow ingression and cytokinesis completion

Localization of polymerized actin and myosin II at the furrow generates a mechanical force that drives the actin filament sliding to shrink the contractile ring and cause furrow ingression (Glotzer 2001). Cleavage furrow ingression is followed by formation of midbody, a narrow neck that connects the two cells. Midbody contains bundled microtubules from central spindle, and an array of proteins that include Plk1, aurora B, INCENP, centralspindlin proteins and others. The midbody is then resolved and two daughter cells are formed.

Polo like kinase 1

Polo like kinases, a family of serine threonine kinase co-operate with CDKs and orchestrate the cell cycle (Barr, Sillje et al. 2004). They were named after *Drosophila* Polo gene, which was the first member of this family to be identified. Plks are found in eukaryotes and mammalian Plk1 has many roles during mitosis and cytokinesis (Nigg 2001). The C terminal of Plk1 contains a domain called polo box domain (PBD) and the N terminal contains the serine threonine kinase domain. The protein folds back and the PBD autoinhibits the kinase domain. At mitosis onset, some upstream activating kinases such as Ste20-like kinase (SLK) and aurora A phosphorylate Plk1 in the kinase domain (Jang, Ma et al. 2002); this phosphorylation releases the autoinhibition in Plk1. The PBD can bind to substrates which have a motif (S/T-P) This motif on the Plk1 substrate is known as Plk1 docking site. The Ser/Thr residue in the docking site is initially phosphorylated or 'primed' by a priming kinase such as MAPK, CDKs or Plk1 itself. The Plk1 binds to the phosphorylated docking sites on substrate proteins via PBD and this interaction is believed to be responsible for Plk1 localization to diverse structures during mitosis. The Plk1 phosphorylation motif on the substrate protein is (E/D/Q)-X-(S/T)-Ø, where X denotes any amino acid and Ø represents hydrophobic amino acid (Qian, Erikson et al. 1998; Ellinger-Ziegelbauer, Karasuyama et al. 2000; Macurek, Lindqvist et al. 2008).

Studies have shown that Plk1 localizes to centrosome in the prophase, kinetochore and mitotic spindle pole in prometaphase and metaphase, central spindle during anaphase and telophase and finally midbody during cytokinesis (Elia, Cantley et al. 2003; Neef, Preisinger et al. 2003). Figure 1-7 depicts a schematic representation of Plk1 localization in the cell.
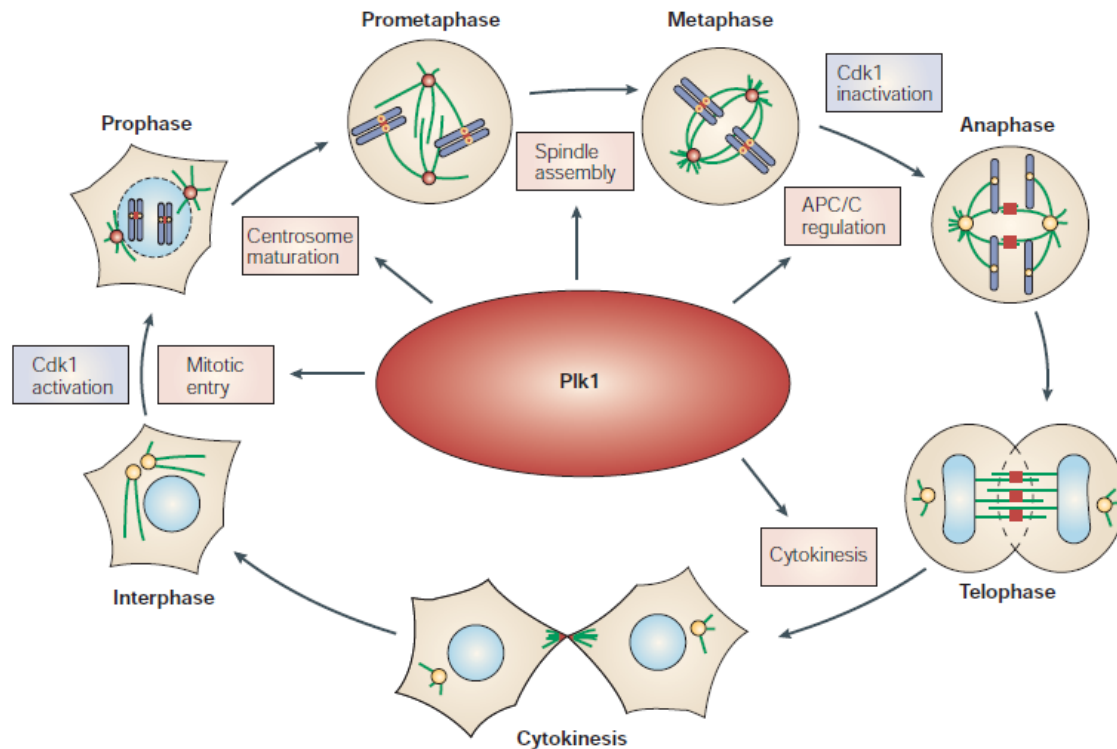
Figure 1-7. A schematic representation of mammalian cell cycle depicting Plk1 localization (shown in red) in the cell during various phases of the cycle. Adapted with permission from *Nature Reviews Molecular Cell Biology*, 5, 429-440.

Consistent with its intracellular distribution, Plk1 regulates multiple events during cell cycle progression. This enzyme is involved in G2/M transition of the cell cycle. Plk1 recruits γ-Tubulin to the centrosome, which is necessary for centrosome maturation and bipolar mitotic spindle formation (Barr, Sillje et al. 2004). During prophase, Plk1 phosphorylates cohesin subunit scc1 and thus removes the bulk of cohesin from the chromosomes to enhance proper chromosome condensation (Lane and Nigg 1996). Moreover Plk1 also phosphorylates Scc1 adjacent to the separase cleavage site to stimulate sister chromatid separation (Sumara, Gimenez-Abian et al. 2004). At the onset of anaphase, Plk1 activates APC (Kramer, Scheuringer et al. 2000). Plk1 also phosphorylates Mklp2 and NudC (a component of dyenin complex), that trigger Plk1 localization to the central spindle. Absence of phosphorylation of NudC by Plk1 causes cytokinesis defect (Sumara, Vorlaufer et al. 2002). Plk1 recruits RhoGEF Ect2 to the

central spindle, leading to the activation of RhoA and the assembly of the myosin contractile ring (Sumara, Vorlaufer et al. 2002).

Since the rate of microtubule catastrophe increases during mitosis (Moshe, Boulaire et al. 2004), and meanwhile stathmin is primarily inactivated, there must be other proteins that compensate for the activity of stathmin. It is also possible that, since stathmin is localized in the cytoplasm during interphase (Zhou, Aumais et al. 2003), it acts primarily during interphase, not in mitosis. Unlike stathmin, coiled-coil domain containing 69 (CCDC69) is sequestered in the nucleus during interphase, specifically localized to the overlapping interpolar microtubules between the separating chromosomes during metaphase-to-anaphase transition and to the interdigitated microtubules of the central spindle during anaphase. We have found that when exogenously expressed, CCDC69 can destabilize microtubules in interphase as well as in mitotic cells. We have also found out that CCDC69 can bind to midzone components such as aurora B kinase, and Plk1 can phosphorylate CCDC69. Depletion of CCDC69 interferes with the localization of both aurora B kinase and Plk1, which suggests that CCDC69 may coordinate with other midzone components such as Mklp2 to regulate the localization of Plk1 and aurora B to the central spindle. Thus we propose that CCDC69 is a novel microtubule destabilizing protein present at the central spindle and functional during mitosis as well as act as an adaptor or scaffold protein to coordinate the arrangement and alignment of midzone components at the central spindle.

CHAPTER 2 - COILED-COIL DOMAIN-CONTAINING PROTEIN 69 (CCDC69) ACTS AS A SCAFFOLD AND A MICROTUBULE-DESTABILIZING FACTOR TO REGULATE THE RECRUITMENT OF MIDZONE COMPONENTS AND THE ASSEMBLY OF CENTRAL SPINDLES

Introduction

Antiparallel overlapping microtubules are bundled between the separating chromosomes during anaphase, forming the central spindle (or the spindle midzone) and leading to the assembly of the myosin contractile ring (Wheatley, Carvalho et al. 2001). Regulators of central spindle formation and myosin contractile ring assembly are mostly restricted to the interdigitated microtubules of central spindles and they can be collectively called midzone components. Numerous regulators have been implicated in promoting antiparallel microtubule bundling at the central spindle. In particular, the chromosome passenger complex (CPC), the centralspindlin complex, and protein regulator of cytokinesis 1 (PRC1) are considered to be the core regulators of central spindle assembly (Glotzer 2009).

The CPC contains aurora B, survivin, INCENP (inner centromere protein), and borealin (Cooke, Heck et al. 1987; Moreno, Bagnat et al. 1999; Jang, Ma et al. 2002; Gassmann, Carvalho et al. 2004; Sampath, Ohi et al. 2004; Jeyaprakash, Klein et al. 2007). During metaphase-to-anaphase, aurora B is translocated along with survivin, INCENP, and borealin from centromeres to the central spindle. Perturbing the function of survivin, INCENP or borealin leads to the mislocalization of aurora B (Vagnarelli and Earnshaw 2004). The centralspindlin complex contains MgcRacGAP/HsCYK-4 (a GTPase-activating protein) and mitotic kinesin-like protein 1 (Mklp1) (Sellitto and Kuriyama 1988; Nislow, Lombillo et al. 1992; Toure, Dorseuil et al.

1998; Mishima, Kaitna et al. 2002). PRC1 can interact with kinesin family member 4A (Kif4A, a microtubule-based molecular motor), forming a PRC1-Kif4A complex (Jiang, Jimenez et al. 1998; Mollinari, Kleman et al. 2002; Kurasawa, Earnshaw et al. 2004; Zhu and Jiang 2005). A line of evidence shows that the CPC, the centralspindlin complex, and PRC1, promote the assembly and bundling of antiparallel microtubules at the central spindle. The formation of central spindles ultimately leads to the recruitments of cytokinesis regulators such as polo-like kinase 1 (Plk1). Plk1 belongs to a class of serine/threonine kinases (Barr, Sillje et al. 2004). It has been shown that Plk1 can phosphorylate and recruit critical cytokinesis regulators such as RhoGEFs to the central spindle (Niiya, Tatsumoto et al. 2006; Burkard, Randall et al. 2007; Petronczki, Glotzer et al. 2007; Asiedu, Wu et al. 2008; Wolfe, Takaki et al. 2009), leading to the activation of RhoA at the cleavage furrow and the assembly of the myosin contractile ring. Therefore, the localization of Plk1 to the central spindle is a critical step during mitosis and cytokinesis.

Proper localizations of midzone components at the central spindle are important for central spindle assembly (Glotzer 2009). It has been indicated that binding of midzone components to overlapping antiparallel microtubules is critical for their recruitment to the central spindle (Wheatley, Carvalho et al. 2001; Vader, Kauw et al. 2006; Glotzer 2009). Yet, localizations of midzone components to the central spindle are often interdependent. For instance, depletion PRC1 or Kif4A interferes with the localization of the centralspindlin complex and the CPC at the central spindle (Kurasawa, Earnshaw et al. 2004; Verni, Somma et al. 2004). In addition, disruption of centralspindlin function decreases the localization of the CPC (Jantsch-Plunger, Gonczy et al. 2000). In turn, the CPC is required for the stable localization of the centralspindlin complex (Severson, Hamill et al. 2000; Mishima, Kaitna et al. 2002). Therefore, it is likely that different midzone components are also interconnected and stabilized by scaffolds at the central spindle, thus contributing to the regulation of midzone component assembly at the central spindle. However, just how scaffolds are implicated in the assembly of midzone components at the central spindle is poorly understood.

Microtubules at central spindles are relatively more stable as compared with those at mitotic spindles. However, it has been shown that microtubules at central spindles are also highly

dynamic, especially during late anaphase (Shelden and Wadsworth 1990). Microtubules polymerization is primarily regulated by the coordinated action of microtubule-stabilizing factors such as microtubule-associated proteins (MAPs) and microtubule-destabilizing factors such as kinesin-13, stathmin, and katanin (Drewes, Ebneth et al. 1998; Houseweart and Cleveland 1999; Andersen 2000; Hunter and Wordeman 2000; Quarmby 2000; Kline-Smith and Walczak 2004). In particular, kinesin-13 family proteins can promote microtubule depolymerization and are key regulators of microtubule dynamics during mitosis (Hunter, Caplow et al. 2003; Kline-Smith and Walczak 2004; Wordeman 2005). The kinesin-13 family including Kif2a, Kif2b, and MCAK/Kif2c are localized to the central spindle and midbody during anaphase and telophase (Wordeman and Mitchison 1995; Ganem and Compton 2004; Manning, Ganem et al. 2007). However, it is still not clear how microtubule-destabilizing factors function at the central spindle to balance the microtubule-bundling activity of midzone components such as the CPC, the centralspindlin complex, and PRC1. We found that coiled-coil domain containing protein 69 (CCDC69) can destabilize microtubules in transfected HeLa cells. CCDC69 localizes to the central spindle and physically interacts with aurora B. Depletion of CCDC69 delocalizes aurora B (a component of the CPC), MgcRacGAP (a component of the centralspindlin complex), and PRC1. Our results suggest that CCDC69 may act as a microtubule-destabilizing factor and a scaffold to control central spindle assembly as well as to recruit midzone components to the central spindle.

Materials and Methods

Plasmids and cell culture

The human CCDC69 cDNA was amplified from HeLa cell total RNA by RT-PCR with the following primer pair: 5'-<u>CTCGAG</u>ATGGGCTGCAGACACAGCAGG-3' (forward primer; the underlined nucleotide sequence is the recognition site for XhoI) and 5'-<u>GGATCC</u> CTATGTGGCGAGGAAAGA-3' (reverse primer; the underlined nucleotide sequence is the recognition site for BamHI). The human CCDC69 cDNA was subcloned into pEGFP-C3 and pCS3+MT vectors to generate pEGFP-CCDC69 and pCS3-CCDC69. This work was done previously by Dr. D Wu and Akiko Haruta. Different truncated versions of CCDC69 were also subcloned into pEGFP-C3 or pGEX-6p to generate plasmids encoding GFP- or GST-tagged polypeptides. HeLa cells were purchased from Clontech (Mountain View, CA). U2OS and breast cancer cell line MDA-MB-231 were purchased from ATCC (Manassas, VA). MDA-MB-231 cells were grown in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum. HeLa, and U2OS cells were cultured in DMEM supplemented with 10% fetal bovine serum. Transfection was done with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). siRNAs specific for the human CCDC69 gene were purchased from Invitrogen (#1: GAG UCC AUU CUG AGC CGA AAC UAU A; #2: GCA CCA GAA GGA UAU AAC CAG AAU U).

Northern blot analysis

The human poly(A$^+$) RNA Northern Blot (purchased from OriGene Technologies, Inc.) contains poly(A$^+$) RNA samples from 12 major human tissues (brain, duodenum, esophagus, pancreas, PBL, prostate, salivary gland, testis, thymus, thyroid, urinary bladder, uterus). The full-length human CCDC69 cDNA was used as a probe. The probe was labeled with [α-32P]dCTP (PerkinElmer Life and Analytical Sciences) using the DECAprime™ II Kit (Applied Biosystems/Ambion). The hybridization was carried out in the ULTRAhyb® Ultrasensitive Hybridization Buffer (Applied Biosystems/Ambion) according to the manufacturer's instructions. The membrane was exposed to x-ray films overnight at -75ºC. After storage at -

20ºC for 2 months, the blot was re-probed using human actin cDNA as a probe. This work was done by Dr. Di Wu.

Protein expression and in vitro translation

The bacterial expression system was used to express GST-tagged polypeptides. BL21 bacterial cells expressing GST-fused polypeptides were homogenized by sonication and lysed in PBS containing 1% Triton X-100 for 1 h at 4°C. GST-tagged polypeptides were purified using glutathione-agarose beads. After elution with 100 mM Tris-HCl (pH 7.5) and 5 mM glutathione, the GST-polypeptides were dialyzed against 50mM Tris-HCl (pH 7.5), 50 mM NaCl. In vitro translated Myc-tagged Plk1 or aurora B protein was synthesized using the TNT SP6 quick coupled transcription/translation system (Promega) according to the manufacturer's instructions.

Immunoprecipitation and GST pull-down assays

Immunoprecipitation and GST pull-down assays were carried out as described previously (Asiedu, Wu et al. 2008). After lysis in RIPA (radioimmune precipitation assay) lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.25% deoxycholate, 1% NP-40, 1mM EDTA, 1mM PMSF, 1mM Na_3VO_4, 1mM NaF with Protease inhibitor mixture) for 10 min on ice, the transfected cell lysates were centrifuged for 10 min at 16000xg at 4°C to remove cell debris. The supernatants were collected and precleared with protein A/G agarose beads. Precleared cell lysate was then incubated with agarose-conjugated anti-Myc antibody overnight at 4°C. After anti-Myc-agarose beads were washed 4 times with RIPA lysis buffer, the bound proteins were eluted with SDS-PAGE loading buffer. For GST pull-down experiments, in vitro translated Myc-tagged protein or cell lysates from transfected cells were incubated with the immobilized GST-fused polypeptides overnight at 4°C. After washing 4 times with binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.05% Triton-X-100, 10% glycerol, 0.2 mM EDTA and 1 mM DTT), the bound proteins were eluted into SDS-PAGE loading buffer.

Immunoblotting

Cell lysates, immunoprecipitates and GST pull-down proteins were separated by 7% or 4%-12% SDS-PAGE gels, transferred to Immobilon-P transfer membranes (Millipore), blocked in 5% non-fat milk and incubated with primary antibodies as indicated. The following primary antibodies were used: mouse anti-Myc (9E10, 1:1000; Santa Cruz Biotechnology), rabbit anti-GFP (1:1000; Santa Cruz Biotechnology), rabbit anti-β-tubulin (1:2000; Santa Cruz Biotechnology), and rabbit anti-CCDC69 (1:500; rabbit anti-CCDC69 antibody was raised using the following peptide as antigen: CKPPKKKRQEPEPEQPPRPE; This peptide corresponds to amino acid residues 10-30 in human CCDC69). After washing three times, the blots were incubated with horseradish peroxide-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology) for 1 h at 23°C and visualized by SuperSignal West Pico Luminol/Enhancer solution (Pierce Biotechnology).

Immunofluorescence staining

HeLa cells were transfected with plasmids (GFP-tagged CCDC69 full-length or fragments) or siRNAs (control or CCDC69 siRNAs). 24 h (plasmids) or 72 h (siRNAs) after transfection, the transfected cells were fixed with 4% paraformaldehyde to stain microtubules, CCDC69, aurora B, INCENP, MgcRacGAP, PRC1, lamin A+C, and phosphorylated histone 3 (p-H3). For RhoA staining, cells were fixed with 10% TCA for 10 min on ice. For Plk1 and CCDC69 co-staining, cells were fixed with methanol/acetone. To disrupt Plk1 or aurora B activation, HeLa cells were treated with vehicle (DMSO; Sigma), GW 843682X (1 μM; Tocris Bioscience) or ZM 447439 (10 μM; Tocris Bioscience) for 25 min and then fixed with 4% paraformaldehyde. The following primary antibodies were used for immunofluorescence staining: mouse monoclonal to aurora B (1:1000; Abcam); goat polyclonal to RACGAP1/MgcRacGAP (1:100; Abcam); mouse monoclonal to INCENP (1:500; Abcam); goat polyclonal to PRC1 (1:100; Santa Cruz Biotechnology); mouse monoclonal to RhoA (1:100; Santa Cruz Biotechnology); mouse monoclonal to β-tubulin (1:1000; Sigma); rabbit polyclonal to phospho-histone H3 (1:100; Millipore); mouse monoclonal to Plk1 (1:200; Millipore); mouse monoclonal to lamin A+C (1:100; Abcam). The secondary antibodies Alexa Fluor 594 donkey

anti-mouse IgG (1:500), Alexa Fluor 350 donkey anti-mouse IgG (1:500), Alexa Fluor 594 donkey anti-goat IgG (1:500), Alexa Fluor 488 donkey anti-goat IgG (1:500), Alexa Fluor 594 donkey anti-rabbit IgG (1:500), and Alexa Fluor 488 donkey anti-rabbit IgG (1:500) were purchased from Invitrogen. The nuclei were visualized by DAPI (Sigma, St. Louis, MO). Images were taken using a Leica DMI 6000 B microscope (Leica, Deerfield, IL) and processed by blind deconvolution.

In vitro kinase assays

For in vitro kinase assays, 5 µg of purified GST-tagged full length or truncation mutants of CCDC69 were incubated in 1X kinase assay buffer (5mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1mM EGTA, 0.4 mM EDTA, 5mM MgCl2, 0.05 mM DTT, 200 µM ATP) with 2.5 µg of His-tagged Plk1 (Cell Signaling) and 5 µCi [γ32] ATP. The total volume for the reactions is 50ul. The reaction mixtures were incubated at $30^{\circ}C$ for 30 minutes, stopped by adding 2X SDS-PAGE loading buffer and boiling at 100°C for 10 minutes, resolved on a 10% SDS-PAGE, dried with the Gel Dryer Vacuum system (Fisher Scientific), and subjected to autoradiography.

Results

Expression of coiled-coil domain containing protein 69 (CCDC69)

Human CCDC69 (NM_015621) contains 296 amino acid residues with an expected molecular mass of ~42 kDa. Mouse (NM_177471) and *Xenopus* (BC124990) orthologs of CCDC69 are also found in the database (Appendix A). Northern blot analysis with full-length human CCDC69 cDNA as a probe showed that CCDC69 mRNA is highly expressed in duodenum, esophagus, pancreas, prostate, salivary gland, thymus, and urinary bladder (Figure 2-1A). The size of CCDC69 mRNA in the blot is ~3.5 kb, in agreement with that for the deposited CCDC69 mRNA in the NCBI database (NM_015621; 3416 bp in length). EST profile in the NCBI database also shows that CCDC69 is widely expressed in various tissues and organs (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist =Hs.655336). Further, immunoblot analysis with an antibody specific for human CCDC69 showed that CCDC69 is expressed in various cancer cell lines such as HeLa, U2OS and MDA-MB-231 (see Figure 2-1B). The northern blot was done by Dr. Di Wu.

Exogenous expression of CCDC69 destabilizes microtubules

We originally identified CCDC69 as a potential downstream target for the homeodomain transcription factor Pitx2a using a tetracycline-inducible expression system (Wei and Adelstein 2002; Wei 2005). DNA microarrays and RT-PCR showed that exogenous expression of Pitx2a in HeLa cells increased the mRNA levels of CCDC69 (data not shown). To examine the cellular function of CCDC69, a plasmid encoding GFP-CCDC69 was transfected into HeLa cells. 24 h after transfection, transfected HeLa cells were fixed and processed for immunofluorescence to stain microtubules and the nucleus. During interphase, GFP-CCDC69 was predominantly localized in the nucleus (Figure 2-2A). Yet, exogenous expression of GFP-CCDC69 decreased microtubule staining in the cytoplasm (n=145/200 cells; arrowhead in Figure 2-2Bb), indicating that a small amount of exogenously expressed GFP-CCDC69 in the cytoplasm was responsible for such microtubule-destabilizing activity. Consistent with this speculation, in cells expressing high levels of GFP-CCDC69, a significant amount of GFP-CCDC69 remained in the cytoplasm

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(arrow in Figure 2-2Be) and dramatically destabilized microtubules (n=105/105 cells; arrowhead in Figure 2-2Bf). As expected, bipolar mitotic spindles are assembled in untransfected cells during mitosis (n=120/120 cells; arrowheads in Figure 2-2Cb). However, mitotic cells expressing GFP-CCDC69 failed to form bipolar mitotic spindles (n=95/102; arrowhead in Figure 2-2Cf). To confirm that cells in panels e-h were at mitotic phase, immunofluorescence staining was done using antibodies specific for β-tubulin and the mitotic marker phosphorylated histone 3 (p-H3). As shown in Figure 2-2D, metaphase chromosomes of an untransfected cell were positively stained for p-H3 (arrowhead in panel c) and the bipolar mitotic spindles are nicely formed (arrowheads in panel b). However, chromosomes of a transfected cell were positively stained for p-H3 (arrowhead in panel g) but bipolar mitotic spindles of the cell failed to form (arrowhead in panel f). These results indicate that cells in Figures 2-2C and D are at mitotic phase. However, we did not observe cell death in interphase cells expressing GFP-CCDC69, suggesting that decreases in microtubule stability are not secondary to cell death. It should be noted that expression of GFP alone in HeLa cells had no impact on microtubule polymerization or mitotic spindle formation (data not shown).

The C-terminal half of CCDC69 is required for the microtubule-destabilizing activity

The COIL program (Lupas, Van Dyke et al. 1991) predicts that human CCDC69 contains 4 coiled-coil regions (amino acids 49-109, 122-159, 172-233, 237-269; see Figure 2-3A). Coiled-coil domains are also predicted in mouse and *xenopus* CCDC69. To identify the regions that are critical for the microtubule-destabilizing activity of CCDC69, we generated several truncated fragments of human CCDC69 (Figure 2-3B). Plasmids encoding CCDC69 fragments were transfected into HeLa cells. 24 h after transfection, the transfected cells were processed for immunofluorescence to visualize GFP-tagged CCDC69 fragments and microtubules. Unlike the full-length CCDC69 that was predominantly localized to the nucleus (Figure 2-2A), the CCDC69 fragment 49-296 (containing all coiled-coil regions) showed diffuse distribution in the cytoplasm and it dramatically destabilized microtubules (arrowheads in Figure 2-3Cb). CCDC69 fragments 172-296 and 1-233 are predominantly localized to the nucleus and also showed microtubule-destabilizing activity (arrowheads in Figures 2-3Ce and 2-3Ck). In contrast, CCDC69 fragment 1-159 showed diffuse distribution in the cytoplasm but it had no obvious

impact on microtubule polymerization (arrowhead in Figure 2-3Ch). These results indicate that amino acids 160-296 (containing coiled-coil regions172-233 and 237-269) are critical for the microtubule-destabilizing activity of CCDC69.

Localization of endogenous CCDC69 during cell cycle progression

Immunoblot analysis with the CCDC69 peptide antibody detects a single band with a molecular mass of ~42 kDa (Figure 2-4A; lane 1). To further confirm the specificity of the CCDC69 peptide antibody, HeLa cells transfected with CCDC69 siRNAs were subjected to immunoblot analysis with the CCDC69 peptide antibody. Treatment of HeLa cells with CCDC69 siRNAs decreased the expression levels of the 42-kDa protein (Figure 2-4A; compare lane 1 with lanes 2 and 3). Further, the CCDC69 peptide antibody also recognized GFP-CCDC69 from transfected HeLa cell lysate (Figure 2-4B). These results suggest that the CCDC69 peptide antibody can specifically recognize human CCDC69 proteins. We then carried out immunofluorescence staining to examine the subcellular distributions of endogenous CCDC69 proteins during cell cycle progression. HeLa cells were fixed with paraformaldehyde and subjected to immunofluorescence staining with antibodies specific for CCDC69 and β-tubulin. CCDC69 showed nuclear staining in interphase HeLa cells (Figure 2-4C). During metaphase, weak staining of CCDC69 was found along mitotic microtubules (arrow in Figure 2-4Da). However, CCDC69 was not concentrated at the spindle poles (arrowheads in Figures 2-4Da and 2-4Dd). During early anaphase, CCDC69 was localized along overlapping interpolar microtubules between the separating chromosomes (arrowheads in Figures 2-4De and 2-4Dh). During late anaphase, CCDC69 formed a focused band at the center of central spindles (arrowheads in Figures 2-4Di and 2-4Dl). Finally, CCDC69 was concentrated at the midbody during telophase (arrowheads in Figures 2-4Dm and 2-4Dp). Thus, our findings indicate that CCDC69 is predominantly localized to antiparallel overlapping microtubules of the central spindle during anaphase. It should be noted that immunoblot analysis with the CCDC69 peptide antibody showed that CCDC69 protein levels remained constant during cell cycle progression (data not shown).

Depletion of CCDC69 leads to the formation of aberrant central spindles

Exogenous expression of GFP-CCDC69 dramatically destabilized microtubules (Figures 2-2 and 2-3). In addition, endogenous CCDC69 is concentrated to the central spindle during anaphase (Figure 2-4). Therefore, we asked whether CCDC69 depletion has an impact on central spindle formation. HeLa cells depleted of CCDC69 by RNAi were subjected to immunofluorescence staining with antibodies specific for CCDC69 and β-tubulin. In some of the anaphase cells depleted of CCDC69, we observed disorganized microtubule bundling at central spindles (n=11/23 cells; Figure 2-5c-d). In these CCDC69-depleted cells, microtubule bundling was increased near the spindle poles (arrowheads in panels c and d) but not at the center of central spindles. We also observed that, in those CCDC69-depleted cells with organized central spindles, microtubule bundling was decreased at the central spindle but increased at the spindle poles (n=12/23; arrowhead in panel e; compare panel a with panel e). We also observed that midbodies in CCDC69-depleted cells were not well formed (n=4/4; arrowhead in panel f). The arrow in panel f indicated that CCDC69 was not depleted in a CCDC69 siRNA-transfected telophase cell whose midbody was nicely formed. These results suggest that CCDC69 is involved in regulating the integrity of central spindles. It should be noted that CCDC69 was not completely depleted by RNAi-mediated knockdown and CCDC69 proteins were still present in CCDC69 siRNA-treated cells (arrows in panels c-e). We were not able to carry out RNAi-rescue experiments because exogenous expression of CCDC69 disrupted mitotic spindle formation (Figure 2-2), leading to cell death.

Depletion of CCDC69 delocalizes RhoA at the cleavage furrow

The efficiency of CCDC69-depletion by RNAi is not high (see Figure 2-2A; ~55-65% knockdown) and cells with low CCDC69 levels may still be able to complete cytokinesis. Time-lapse microscopy does not allow the identification of cells whose CCDC69 is significantly decreased following siRNA-transfection. Thus, it is difficult to use live cell-imaging analysis to examine cytokinesis defects following transfection with CCDC69 siRNAs. In contrast, immunofluorescence staining of fixed transfected cells allows the identification of CCDC69-depleted cells. It is well established that activation of RhoA at the cleavage furrow is a key step

during cytokinesis (Yuce, Piekny et al. 2005; Kamijo, Ohara et al. 2006). Therefore, we examined whether depletion of CCDC69 had an impact on RhoA staining at the cleavage furrow. HeLa cells transfected with control or CCDC69 siRNAs were fixed with TCA and then subjected to immunofluorescence staining with antibodies specific for CCDC69 and RhoA. As shown in Figure 2-6, RhoA staining was not concentrated at the equatorial cortex in HeLa cells depleted of CCDC69 (n=12 cells). Since cortical RhoA staining at the cleavage furrow has been used to measure RhoA activation during cytokinesis and since deficiency in equatorial RhoA activation leads to defective cytokinesis (Yonemura, Hirao-Minakuchi et al. 2004; Yuce, Piekny et al. 2005; Nishimura and Yonemura 2006), our results suggest that depletion of CCDC69 decreased RhoA activation at the cleavage furrow, resulting in cytokinesis defects. This work was done by Dr. Di Wu.

CCDC69 contributes to the concentration of aurora B at the central spindle

The chromosome passenger complex (CPC) contains aurora B, survivin, INCENP, and borealin (Cooke, Heck et al. 1987; Moreno, Bagnat et al. 1999; Jang, Ma et al. 2002; Gassmann, Carvalho et al. 2004; Sampath, Ohi et al. 2004; Jeyaprakash, Klein et al. 2007). It has been shown that the CPC plays a central role in regulating central spindle formation (Glotzer 2009). Depletion of CCDC69 resulted in the formation of aberrant central spindles, raising the possibility that CCDC69 may be implicated in controlling the localization of the CPC complex. To examine whether CCDC69 is colocalized with CPC components during mitosis and cytokinesis, HeLa cells were fixed and processed for immunofluorescence staining with antibodies specific for aurora B and CCDC69. As shown in Figure 2-7A, CCDC69 and aurora B were not colocalized at centromeres during metaphase or at the midbody during telophase. Nonetheless, CCDC69 appeared to be colocalized with aurora B at the central spindle during anaphase (Figure 2-7A). Thus, we then asked whether CCDC69 could bind to CPC components. As shown in Figure 2-7C, lane 3, GST pull-down assays showed that GST-CCDC69 could pull down the in vitro translated Myc-aurora B, indicating that CCDC69 can physically interact with aurora B. To determine the regions in CCDC69 that are responsible for interaction with aurora B, we generated several truncated versions of CCDC69 (Figure 2-7B). GST pull-down assays showed that GST-tagged CCDC69 fragments 49-296 and 172-296, but not fragments 1-233, 1-

159, and 1-127, could precipitate Myc-aurora B (Figures 2-7C and 2-7D), suggesting that amino acids 234-296 are required for interaction with aurora B. In addition, amino acids 234-296 contain one of the coiled-coil domains in CCDC69 (Figure 2-3A). Therefore, our results indicate that CCDC69 may interact with aurora B through its coiled-coil domain at the C-terminus. Since aurora B does not contain any coiled-coil domain, it would be interesting to investigate the CCDC69 binding domain of aurora B.

We then asked whether depletion of CCDC69 has an impact on the localization of the CPC complex. To this end, HeLa cells transfected with control or CCDC69 siRNAs were subjected to immunofluorescence staining for CCDC69 and CPC components (aurora B or INCENP). Depletion of CCDC69 did not interfere with the translocation of aurora B or INCENP from centromeres to the central spindle (Figures 2-8A and 2-8B). However, both aurora B and INCENP staining appeared as broad bands at the central spindle (Figures 2-8A and 2-8B), indicating that CCDC69 is involved in concentrating aurora B and INCENP at the central spindle. Work for Figure 2-8B was done by Dr. Di Wu.

CCDC69 is required for the localization of PRC1 and MgcRacGAP at the central spindle

In addition to the CPC complex, PRC1 and the centralspindlin complex also play a pivotal role in regulating the assembly of central spindles (Jiang, Jimenez et al. 1998; Jantsch-Plunger, Gonczy et al. 2000; Mollinari, Kleman et al. 2002; Pavicic-Kaltenbrunner, Mishima et al. 2007). Both PRC1 and the centralspindlin complex can stimulate microtubule bundling at the central spindle (Jantsch-Plunger, Gonczy et al. 2000; Mollinari, Kleman et al. 2002). Thus, we asked whether depletion of CCDC69 interfered with the localization of PRC1 and the centralspindlin complex at the central spindle. As shown in Figure 2-8C, depletion of CCDC69 in HeLa cells disrupted the localization of PRC1 to the central spindle. The centralspindlin complex is a heterotetrameric complex that contains a dimer of Mklp1 (a kinesin-6 motor protein) and a dimer of MgcRacGAP/HsCYK-4 (a GTPase-activating protein GAP) (Mishima, Kaitna et al. 2002; Pavicic-Kaltenbrunner, Mishima et al. 2007). Notably, Mklp1 and MgcRacGAP localize to the center of the central spindle as a complex, i.e. Mklp1 or MgcRacGAP alone does not localize to the central spindle (Mishima, Kaitna et al. 2002). Figure

2- 8D showed that depletion of CCDC69 also disrupted the localization of MgcRacGAP at the central spindle. Thus, our results indicate that CCDC69 is required for the localization of PRC1 and the centralspindlin complex at the central spindle. However, in vitro GST pull-down assays showed that GST-CCDC69 was not able to pull down Myc-tagged PRC1 or MgcRacGAP (data not shown). This work was done by Dr. Di Wu.

CCDC69 is required for the localization of Plk1 to the central spindle

Polo-like kinase 1 (Plk1) is a critical mitotic kinase that plays a central role in recruiting RhoGEFs to the central spindle, leading to the activation of RhoA and the assembly of the actomyosin contractile ring (Burkard, Randall et al. 2007; Petronczki, Glotzer et al. 2007; Asiedu, Wu et al. 2008; Wolfe, Takaki et al. 2009). We found that depletion of CCDC69 interfered with the localization of RhoA at the cleavage furrow (Figure 2-6). Although it has been shown that PRC1 and Mklp2 are critical for the localization of Plk1 to the central spindle (Neef, Preisinger et al. 2003; Neef, Gruneberg et al. 2007), it is suggested that other mechanisms may be involved as well (Wolfe, Takaki et al. 2009). Thus, we asked whether CCDC69 had a role in localizing Plk1 to the central spindle. Immunofluorescence staining with antibodies specific for CCDC69 and Plk1 showed that CCDC69 and Plk1 were colocalized to the central spindle and midbody (Figure 2-9Ah,l,p), but not at the spindle poles (Figure 2-9A). To determine whether CCDC69 is required for the localization of Plk1 to the central spindle, HeLa cells transfected with control or CCDC69 siRNA were subjected to immunofluorescence staining for CCDC69 and Plk1. As shown in Figure 2-9B, depletion of CCDC69 interfered with the localization of Plk1 to the central spindle. It has been shown that Plk1 can phosphorylate midzone components such as PRC1, Mklp2, and MgcRacGAP (Neef, Preisinger et al. 2003; Neef, Gruneberg et al. 2007; Wolfe, Takaki et al. 2009). Thus, we performed an in vitro kinase assay to determine whether CCDC69 also served as a substrate of Plk1. As shown in Figure 2-9C, GST-tagged full-length CCDC69 could be phosphorylated by Plk1. However, although aurora B interacts with CCDC69 (Figure 2-7), it could not phosphorylate CCDC69 (Figure 2-9C; lane 3).

Inhibition of aurora B but not Plk1 disrupts the localization of CCDC69 to the central spindle

It has been shown that localization of midzone components to the central spindle is often interdependent. For instance, Mklp2 is critical for the localization of CPC components INCENP and aurora B to the central spindle (Gruneberg, Neef et al. 2004), whereas aurora B can phosphorylate centralspindlin components (Mklp1 and MgcRacGAP) and recruit them to the central spindle (Guse, Mishima et al. 2005; Neef, Klein et al. 2006). Thus, we asked whether inhibition of aurora B or Plk1 had an impact on CCDC69 localization at the central spindle. HeLa cells treated with small chemical inhibitors of aurora B or Plk1 were subjected to immunofluorescence staining for CCDC69 and β-tubulin. Because of critical functional roles of aurora B and Plk1 during earlier mitosis, exposure of HeLa cells to these small chemical inhibitors does not allow them to advance to anaphase. However, we should still be able to identify cells that had already advanced to anaphase when they were exposed to the inhibitors for a short period of time (~25 min). Consistent with previous reports (Hauf, Cole et al. 2003), we found that inhibition of aurora B compromised the integrity of central spindles (Figure 2-10; arrowhead in panel g) and caused defects in chromosome segregation (Figure 2-10; arrowhead in panel h). Treatment of HeLa cells with an aurora B inhibitor disrupted the localization CCDC69 to the central spindle (Figure 2-10; compare panels a-e with panels f-j). HeLa cells treated with a Plk1 inhibitor also showed defects in chromosome segregation (arrowhead in Figure 2-10m). However, inhibition of Plk1 activation had little impact on central spindle formation (Figure 2-10; arrowhead panel l) and did not interfere with CCDC69 localization at the central spindle (Figure 2-10; compare panels a-e with panels k-o).

Discussion

In this study, we have demonstrated that the coiled-coil protein CCDC69 can destabilize microtubules. Knockdown of CCDC69 by RNAi leads to the formation of aberrant central spindles and interferes with the localization of midzone components such as aurora B, PRC1, MgcRacGAP, and Plk1. CCDC69 is a substrate for Plk1 and also physically interacts with aurora B through coiled-coil domain at the C-terminus of CCDC69. Inhibition of aurora B leads to the mislocalization of CCDC69 at the central spindle. Our results suggest that CCDC69 may acts as microtubule-destabilizing factor and a scaffold to regulate the assembly of central spindles and the recruitment of midzone components to the central spindle.

Microtubule-destabilizing activity of CCDC69

Endogenous CCDC69 is exclusively localized in the nucleus (Figure 2-4C). Consistently, GFP-CCDC69 is also predominantly localized to the nucleus (Figure 2-2A). However, exogenous expression of CCDC69 decreased microtubule staining in transfected interphase cells. We believe that some of the exogenously expressed GFP-CCDC69 remained in the cytoplasm, thus impacting microtubule stability. There are several findings that support this speculation. First, HeLa cells expressing high levels of GFP-CCDC69 showed a stronger GFP signal in the cytoplasm and, accordingly, microtubule staining in those cells is much weaker than in those expressing low levels of GFP-CCDC69 (Figure 2-2B). Second, a CCDC69 fragment 49-296 was predominantly localized to the cytoplasm and exhibited greater capability of destabilizing microtubules as compared with full-length CCDC69 (Compare Figure 2-2Bb with Figure 2-3Cb). Third, exogenous expression of GFP-CCDC69 dramatically destabilized microtubules during mitosis and disrupted the formation of bipolar mitotic spindles (Figures 2-2C and 2-2D), suggesting that the breakdown of the nuclear envelope during mitosis and the release of GFP-CCDC69 from the nucleus may increase the impact of GFP-CCDC69 on microtubule polymerization. Consistent with these observations, we have never observed an anaphase cell expressing GFP-CCDC69 (data not shown). Thus, our results indicate that direct exposure of CCDC69 to microtubules is likely required for microtubule destabilization. Our results also indicate that, under physiological conditions, CCDC69 is sequestered in the nucleus

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during interphase and therefore prevented from destabilizing interphase microtubules. It would be interesting to know how the microtubule-destabilizing activity of CCDC69 is regulated once CCDC69 is released from the nucleus during mitosis.

Although exogenous expression of CCDC69 dramatically decreased microtubule staining in transfected HeLa cells (Figure 2-2), an in vitro microtubule turbidity assay (A350nm) revealed that addition of purified GST-CCDC69 into preformed microtubules did not affect microtubule stability (data not shown), suggesting that CCDC69 may indirectly impact microtubule stability. The roles of microtubule-destabilizing factors (i.e. kinesin-13 proteins and stathmin/Op18) and the microtubule-severing protein katanin in regulating microtubule dynamics have been well established (McNally and Vale 1993; Belmont and Mitchison 1996; Walczak, Mitchison et al. 1996). In particular, kinesin-13 proteins are localized to the nucleus during interphase and then translocated to both mitotic spindles and central spindles (Wordeman and Mitchison 1995; Ganem and Compton 2004; Manning, Ganem et al. 2007). Exogenous expression of wild-type stathmin in transfected cells decreases microtubule staining during interphase and mitosis (Gavet, Ozon et al. 1998). Stathmin is distributed in the cytoplasm of the interphase cells and localizes to the mitotic spindle during mitosis (Gavet, Ozon et al. 1998). Therefore, it is possible that CCDC69 decreases microtubule stability via increasing the activity of microtubule-destabilizing factors such as kinesin-13 proteins and stathmin. On the other hand, a large number of microtubule-stabilizing/bundling factors including numerous midzone components promote microtubule polymerization (Kline-Smith and Walczak 2004; Glotzer 2009). Thus, it is also possible that CCDC69 decreases microtubule stability through repressing the activity of microtubule-stabilizing factors.

Regulation of central spindle formation by CCDC69

HeLa cells expressing GFP-CCDC69 failed to form bipolar mitotic spindles and were not able to advance to anaphase (Figures 2-2C and 2-2D; data not shown). Thus, it is not clear whether exogenous expression of CCDC69 has an impact on antiparallel microtubule bundling at the central spindle during anaphase. Nonetheless, our results showed that RNAi-mediated knockdown of CDC69 led to the formation of abnormal central spindles with increased

microtubule bundling at or near the spindle poles (Figure 2-5). These results indicate that the microtubule-destabilizing activity of CCDC69 may be involved in regulating microtubule bundling at the central spindle. However, over-whelming microtubule bundling was not observed at the central spindle of CCDC69-deficient cells. One possibility is that depletion of CCDC69 at the central spindle also delocalizes microtubule-bundling factors such as PRC1 and MgcRacGAP, i.e. depletion of CCDC69 leads to a decrease in both microtubule-destabilizing and -stabilizing activities at the central spindle. Although it appears that this is a futile cycle that does not lead to net changes in the balance between the activities of microtubule-stabilizing and -destabilizing factors, it may be an effective way to maintain optimal microtubule dynamics at the central spindle under physiological conditions. Also, it is well established that aurora B, PRC1, and MgcRacGAP play a central role in regulating central spindle assembly, thus raising the possibility that the formation of abnormal central spindles following CCDC69-depletion is due to the mislocalization of aurora B, PRC1, or MgcRacGAP. However, excessive microtubule bundling at the spindle poles in CCDC69-depleted cells suggests that central spindle abnormalities are, at least in part, due to a decrease in microtubule-destabilizing activity of CCDC69. It is not clear at present how CCDC69 depletion increased microtubule bundling at the spindle poles.

Regulation of the assembly of midzone components at the central spindle by CCDC69

Correct localization of midzone components during anaphase is essential for central spindle formation and the assembly of the myosin contractile ring. Our results showed that RNAi-mediated knockdown of CCDC69 led to the mislocalization of midzone components such as aurora B, PRC1, INCENP, MgcRacGAP, and Plk1. It has been shown that the translocation of aurora B from centromeres to the central spindle requires the coordinated action of survivin, INCENP or borealin (Vagnarelli and Earnshaw 2004; Vader, Kauw et al. 2006; Jeyaprakash, Klein et al. 2007). In particular, INCENP can target and activate aurora B via acting as a scaffold (Wheatley, Carvalho et al. 2001; Bishop and Schumacher 2002; Mishima, Kaitna et al. 2002; Honda, Korner et al. 2003). We found that, in CCDC69-depleted cells, aurora B was successfully translocated from centromeres to the central spindle, but was not concentrated as a narrow band at the central spindle (Figure 2-8). Consistently, CCDC69 and aurora B were colocalized at the

central spindle but not at the centromeres (Figure 2-7A). These results suggest that CCDC69 has a distinctive role in localizing aurora B at the central spindle. It would be interesting to know whether CCDC69 can regulate aurora B activation at the central spindle. CCDC69 is predicted to be coiled-coil protein and it can physically interact with aurora B, suggesting that CCDC69 may promote the assembly of aurora B at the central spindle by acting as a scaffold. However, we found that CCDC69 does not interact with survivin or borealin (data not shown). It is not clear whether CCDC69 can bind to INCENP.

There are at least two possibilities regarding how CCDC69 depletion impacts the localization of midzone components. One possibility is that CCDC69 acts as scaffold at the central spindle to regulate the assembly of midzone components. For instance, CCDC69 binds to aurora B and is implicated in concentrating aurora B to the central spindle. It is of note that aurora B can phosphorylate centralspindlin components (Mklp1 and MgcRacGAP) and recruit them to the central spindle (Guse, Mishima et al. 2005; Neef, Klein et al. 2006). Thus, mislocalization of MgcRacGAP at the central spindle following CCDC69 knockdown can be secondary to the mislocalization of aurora B. Another possibility is that CCDC69 acts as a microtubule-destabilizing factor to regulate microtubule polymerization at the central spindle, thus contributing to the regulation of central spindle formation. Our results show that CCDC69 depletion leads to abnormalities in central spindle assembly (Figure 2-5). Thus, the effect of CCDC69 depletion on the mislocalization of midzone components can result from abnormalities in central spindle formation.

Regulation of Plk1 localization at the central spindle by CCDC69

Plk1 can phosphorylate the midzone components PRC1 and MKlp2. Binding of Plk1 to PRC1 and Mklp2 promotes the timely recruitment of Plk1 to the central spindle during anaphase (Neef, Preisinger et al. 2003; Neef, Gruneberg et al. 2007). In turn, Plk1 recruits RhoGEFs to the central spindle, leading to the activation of RhoA and the assembly of the myosin contractile ring (Niiya, Tatsumoto et al. 2006; Burkard, Randall et al. 2007; Petronczki, Glotzer et al. 2007; Asiedu, Wu et al. 2008). In particular, a recent study shows that phosphorylation of MgcRacGAP by Plk1 promotes the MgcARcGAP-Ect2 interaction and subsequently the

recruitment of Ect2 to the central spindle (Wolfe, Takaki et al. 2009). The study also indicates that, in addition to PRC1 and Mklp2, other mechanisms may also contribute to the regulation of Plk1 recruitment to the central spindle during anaphase (Wolfe, Takaki et al. 2009). Our results indicate that CCDC69 knockdown interfered with the localization of Plk1 to the central spindle (Figure 2-9B). Consistent with these observations, CCDC69 and Plk1 colocalized to the central spindle (Figure 2-9A). Further, our results also showed that CCDC69 depletion decreased equatorial RhoA staining (Figure 2-6). Therefore, it is likely that CCDC69 contributes to the recruitment of Plk1 to the central spindle. However, we cannot rule out the possibility that mislocalization of Plk1 following CCDC69 knockdown is secondary to central spindle defects.

We have found that CCDC69 plays a critical role in controlling the localization of midzone components including aurora B at the central spindle. In turn, aurora B activation is required for the localization of CCDC69 to the central spindle. It has been shown that the localization and assembly of midzone components at the central spindle are, to a certain extent, interdependent (Jantsch-Plunger, Gonczy et al. 2000; Severson, Hamill et al. 2000; Mishima, Kaitna et al. 2002; Kurasawa, Earnshaw et al. 2004; Verni, Somma et al. 2004). These observations are consistent with the concept that various midzone components and/or complexes are functionally and structurally connected through adaptors or scaffolds. Our results indicate that CCDC69 may be one such scaffold that provides physical interconnections among midzone components. Also, it is conceivable that antiparallel microtubule bundling at the central spindle is controlled by the coordinated action of microtubule-stabilizing/bundling and -destabilizing factors. Thus, a future direction will be to understand how the microtubule-destabilizing activity of CCDC69 is implicated in the regulation of central spindle formation.
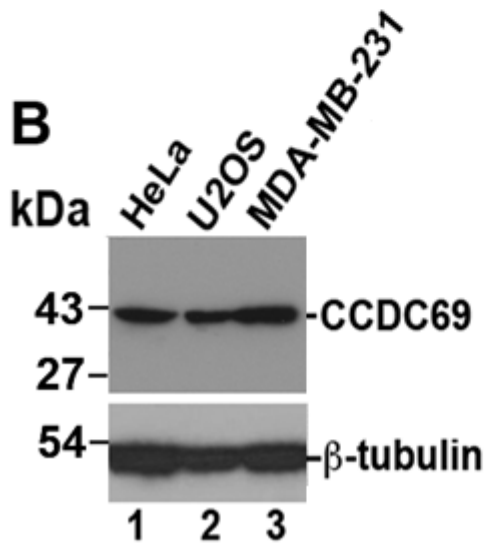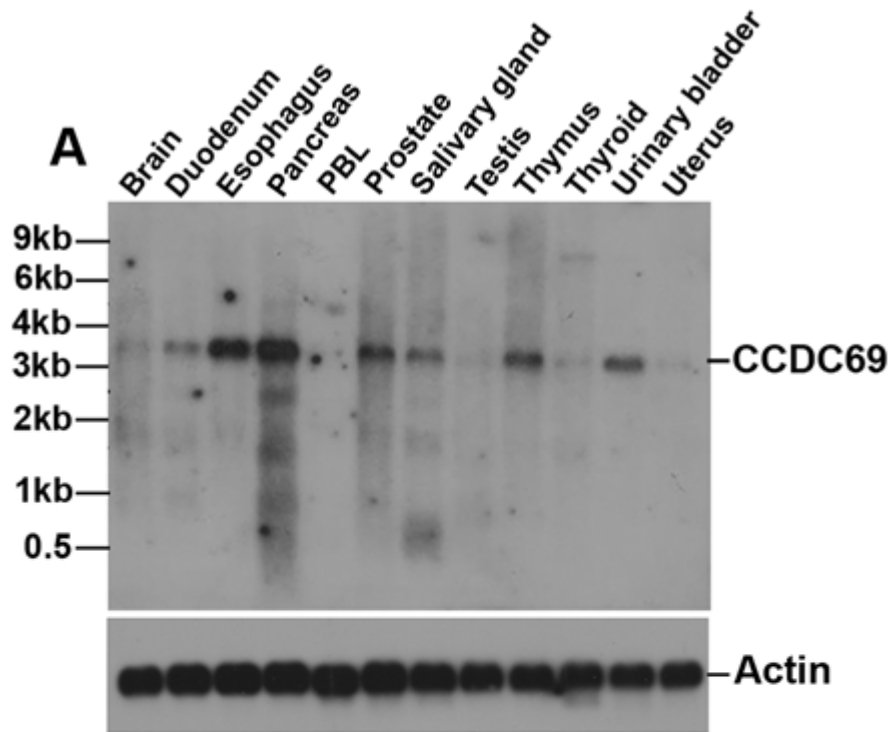
Figure 2-1. Expression of CCDC69. A) Expression of CCDC69 mRNA in human tissues. A human poly(A$^+$) RNA Northern Blot was probed with P^{32}-labeled human CCDC69 (upper panel; exposed overnight) or actin (lower panel; exposed for 2 h) cDNAs. Work done by Dr. Di Wu. B) Expression of CCDC69 proteins in human cancer cell lines. The whole cell lysates were subjected to immunoblot analysis with antibodies specific for CCDC69 (upper panel) and β-tubulin (lower panel).
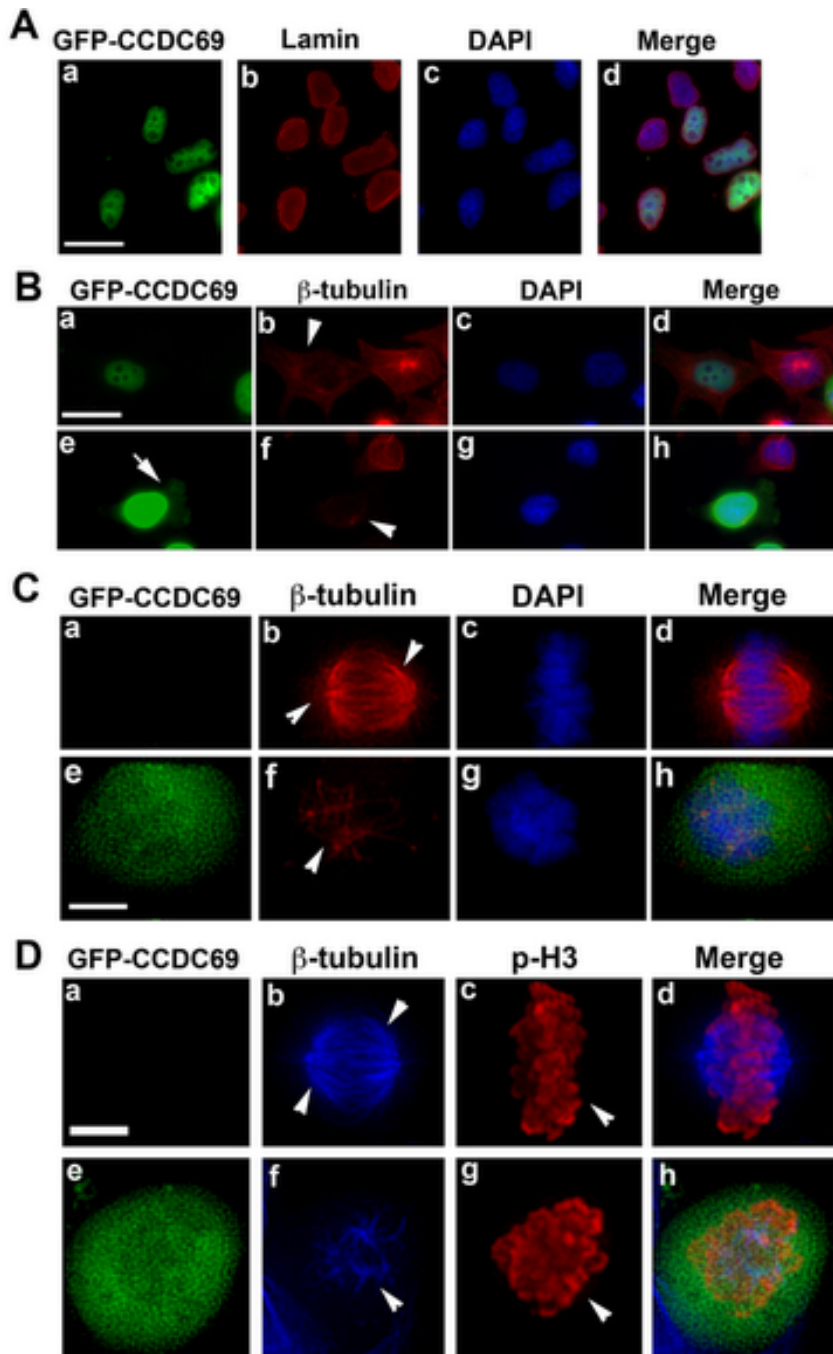
Figure 2-2. Exogenous expression of GFP-CCDC69 destabilizes microtubules. HeLa cells were transfected with a plasmid encoding GFP-CCDC69. 24 h after transfection, the transfected cells were fixed with paraformaldehyde and subjected to immunofluorescence staining. A) The transfected HeLa cells were stained with anti-lamin and DAPI. B-C) The transfected HeLa cells were stained with anti-β-tubulin and DAPI. The observations were

made for n=145/200 cells for Bb, 105/105 cells for Bf, 120/120 cells for Cb and 95/102 cells for Cf. Note that all images in B were collected using the same exposure time. D) The transfected HeLa cells were stained with anti-β-tubulin and anti-phosphorylated histone 3 (p-H3). Bar, 20 μm (A-B) or 5 μm (C-D).
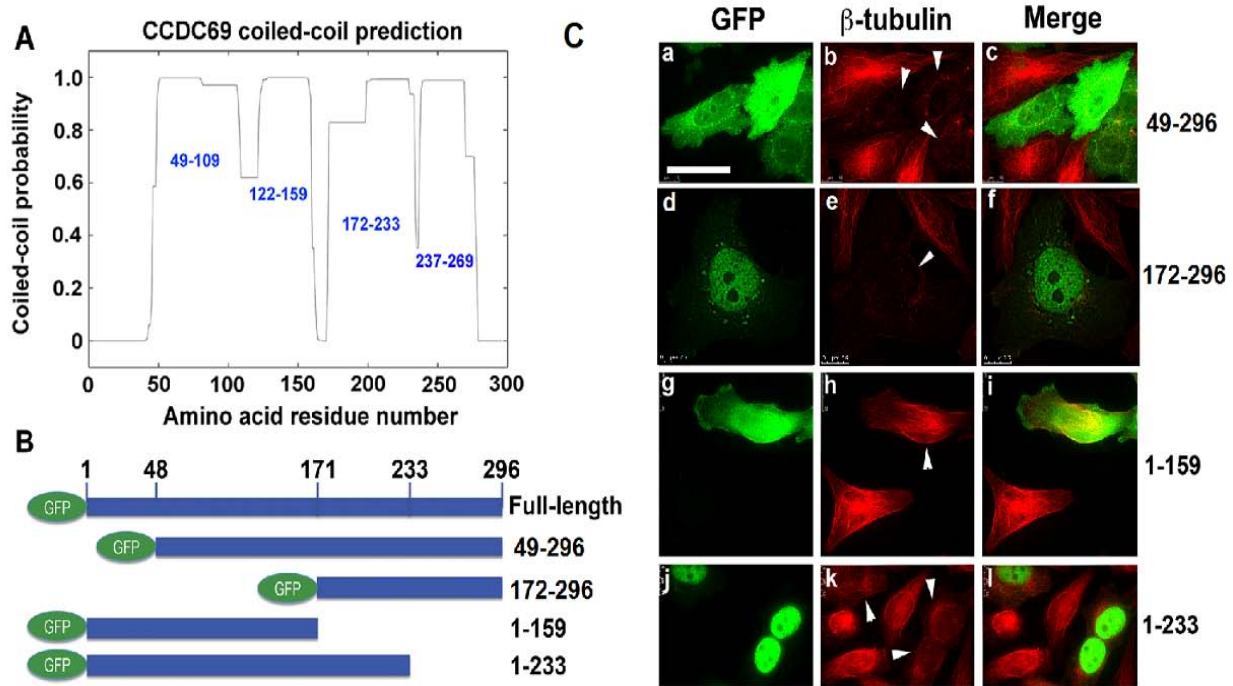
Figure 2-3. The C-terminal half of CCDC69 is required for microtubule-destabilizing activity. A) The COILS program predicts that human CCDC69 contains four coiled-coil domains (amino acids 49-109, 122-159, 172-233, and 237-269). B) Schematic diagram of CCDC69 fragments. C) HeLa cells were transfected with plasmids encoding GFP-tagged CCDC69 fragments 49-296 (a-c), 172-296 (d-f), 1-159 (g-i), 1-233 (j-i). The transfected cells were fixed with paraformaldehyde and subjected to immunofluorescence staining with anti-β-tubulin. Bar, 20 μm.
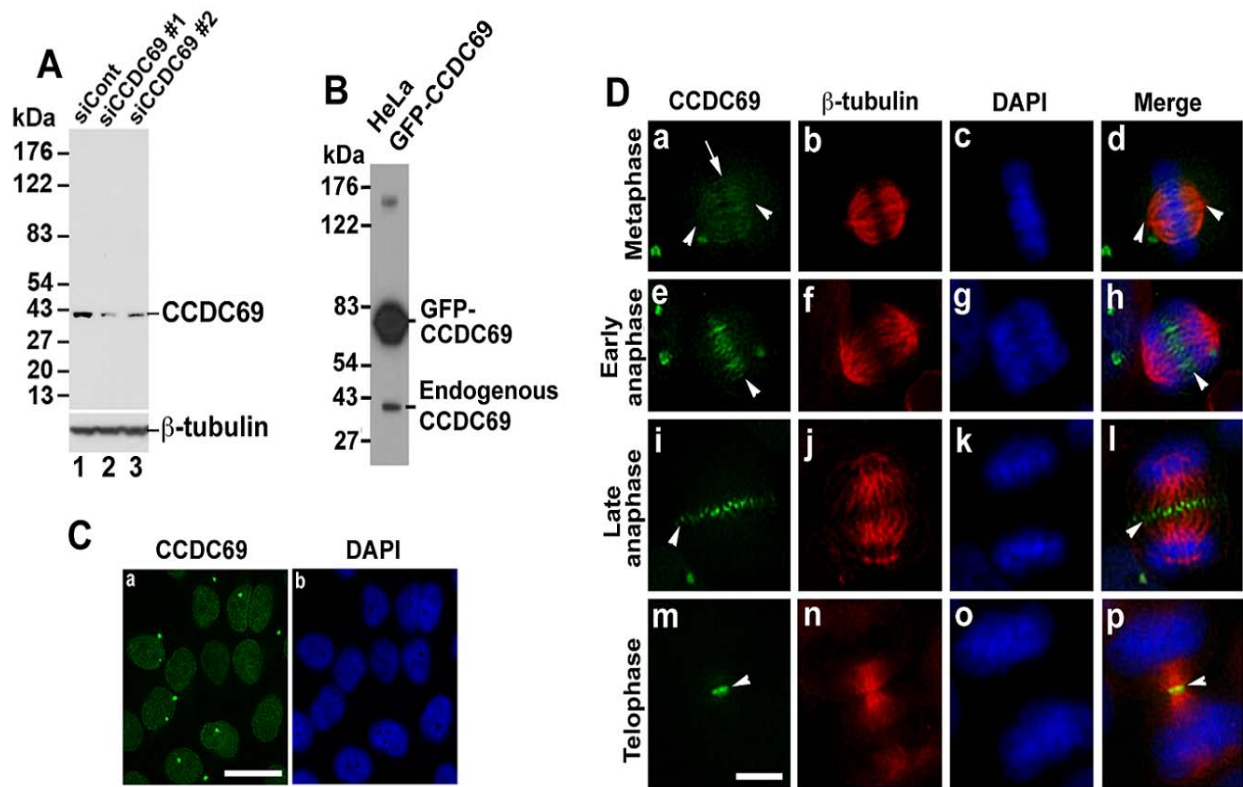
Figure 2-4. Localization of endogenous CCDC69 during cell cycle progression. A) HeLa cells transfected with control (siCont) or CCDC69 (siCCDC69) siRNAs were subjected to immunoblot analysis with the CCDC69 peptide antibody. B) HeLa cells transfected with a plasmid encoding GFP-CCDC69 were subjected to immunoblot analysis with the CCDC69 peptide antibody. C) Untransfected HeLa cells were subjected to immunofluorescence staining with the CCDC69 peptide antibody and DAPI. D) Untransfected HeLa cells were stained with anti-CCDC69, anti-β-tubulin and DAPI. Bar, 25 μm (C) or 5 μm (D).
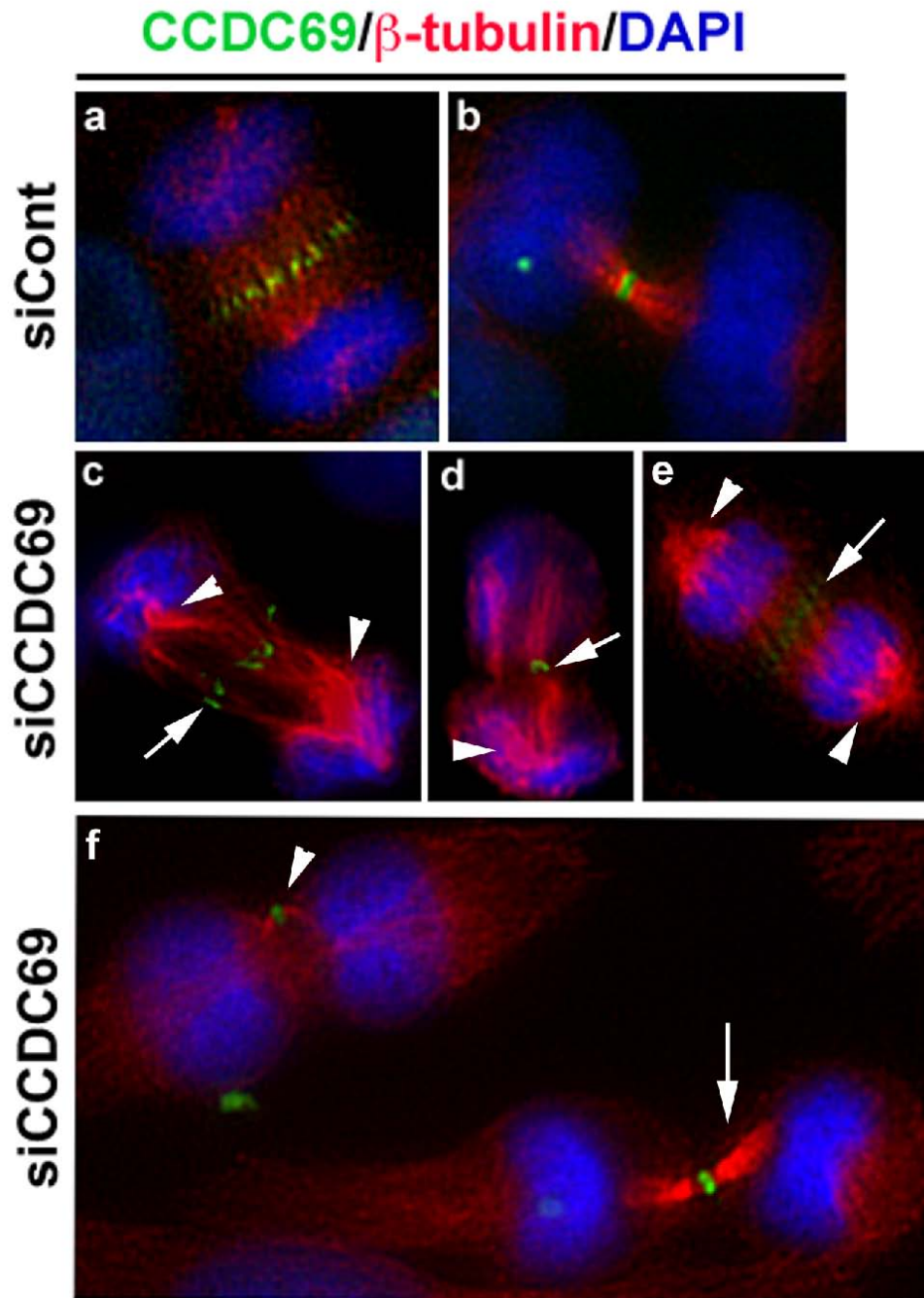
Figure 2-5. Depletion of CCDC69 leads to the formation of aberrant central spindles. HeLa cells transfected with control (siCont; panels a-b) or CCDC69 (siCCDC69; panels c-f) siRNAs were subjected to immunofluorescence staining with anti-CCDC69 (green), anti-β-tubulin (red), and DAPI (blue). The observations were made for n=11/23 cells for c-d, 12/23 cells for e and 4/4 cells for f. Bar, 5μm.
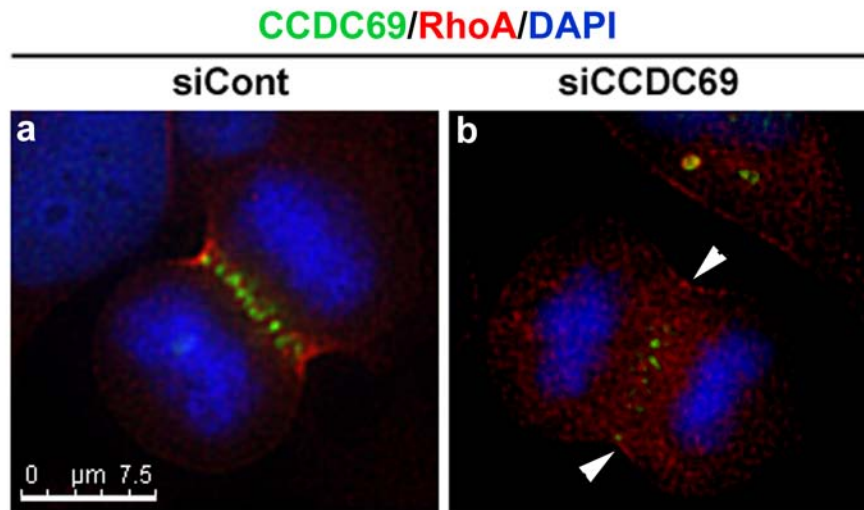
Figure 2-6. Depletion of CCDC69 delocalizes RhoA at the cleavage furrow. HeLa cells transfected with control (siCont; panel a) or CCDC69 (siCCDC69; panel b) siRNAs were fixed with TCA and subjected to immunofluorescence staining with anti-CCDC69 (green), anti–RhoA (red), and DAPI (blue). The observation was made for n=12 cells for b. Bar, 7.5μm. Work done by Dr. Di Wu.
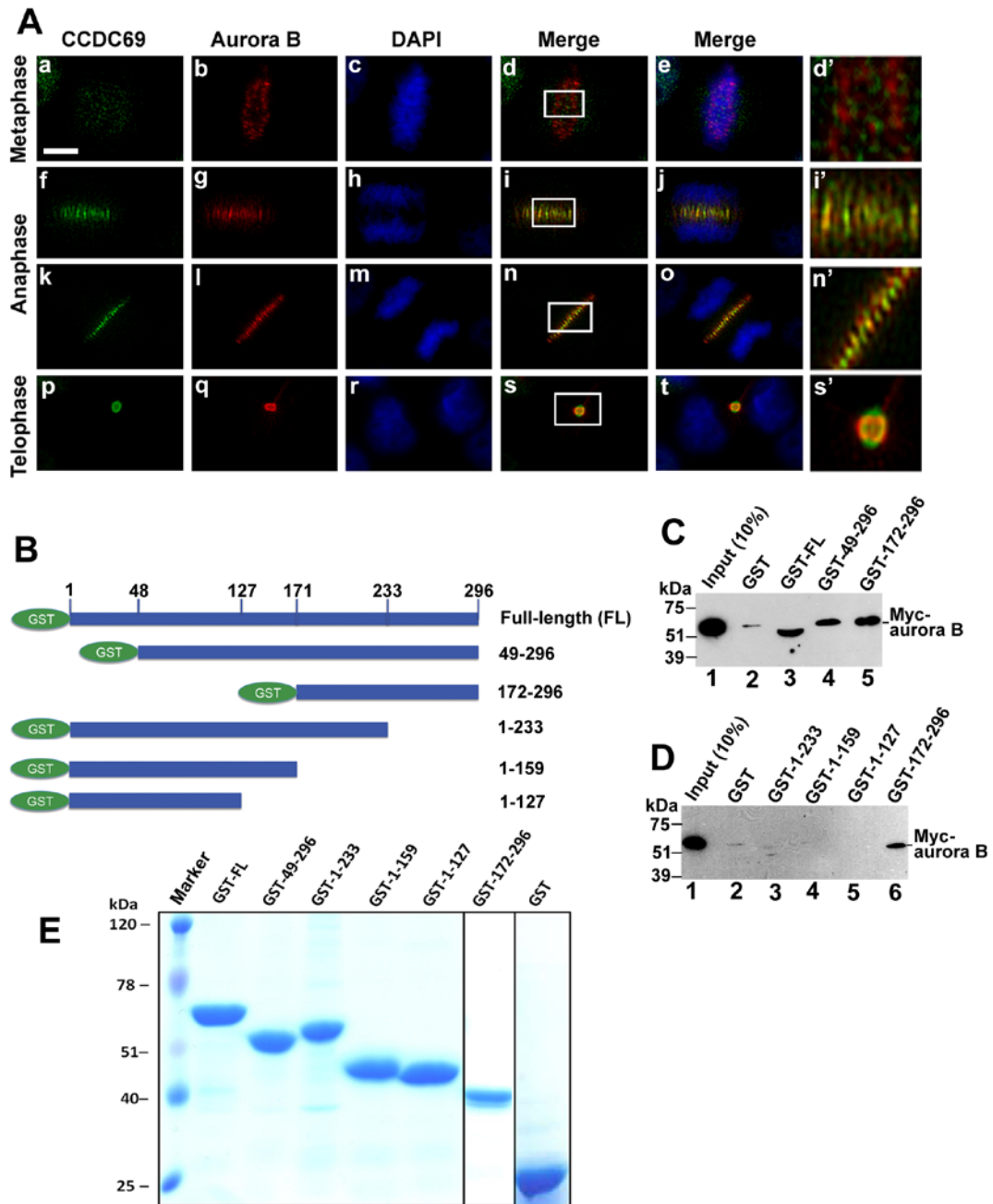
Figure 2-7. CCDC69 interacts with aurora B. A) Untransfected HeLa cells were subjected to immunofluorescence staining with anti-CCDC69 (green), anti-aurora B (red), and DAPI (blue). Panels e, j, o, and t show merge of three channels. Panels d', i', n', and s' are enlarged images that correspond to the insets in panels d, i, n, and s, respectively. Bar 5μm.

B) Schematic diagram of GST-tagged CCDC69 fragments that were used for GST pull-down assays in C and D. C-D) GST-tagged CCDC69 full-length and fragments were used in GST pull-down assays to precipitate the in vitro translated Myc-aurora B. E) A Coomassie staining gel of GST-tagged CCDC69 full-length and fragments that were used in C and D.
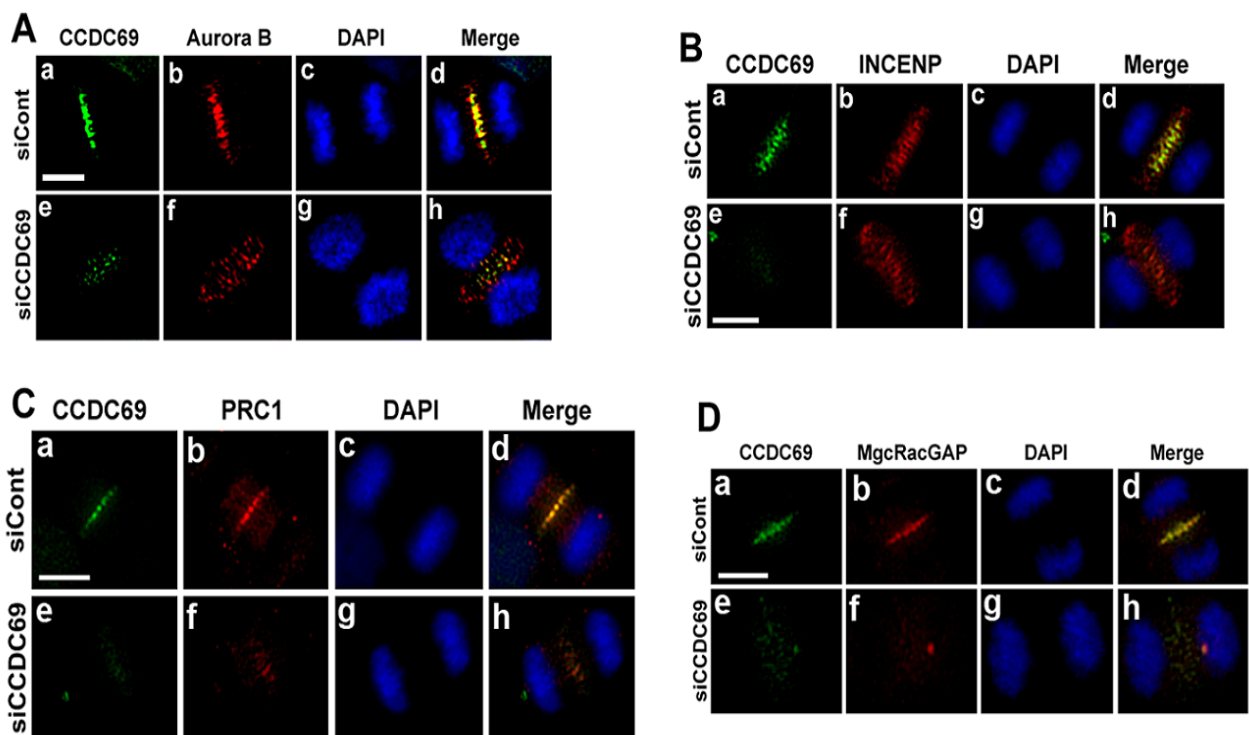
Figure 2-8. CCDC69 is required for the localization of aurora B, INCENP, PRC1, and MgcRacGAP to the central spindle. HeLa cells were transfected with control (siCont) or CCDC69 (siCCDC69) siRNAs. 72 h after transfection, the transfected cells were fixed with paraformaldehyde and subjected to immunofluorescence staining with antibodies as indicated. The chromatids were stained with DAPI. Bar, 5μm. Studies for Figure B,C and D done by Dr. Di Wu.
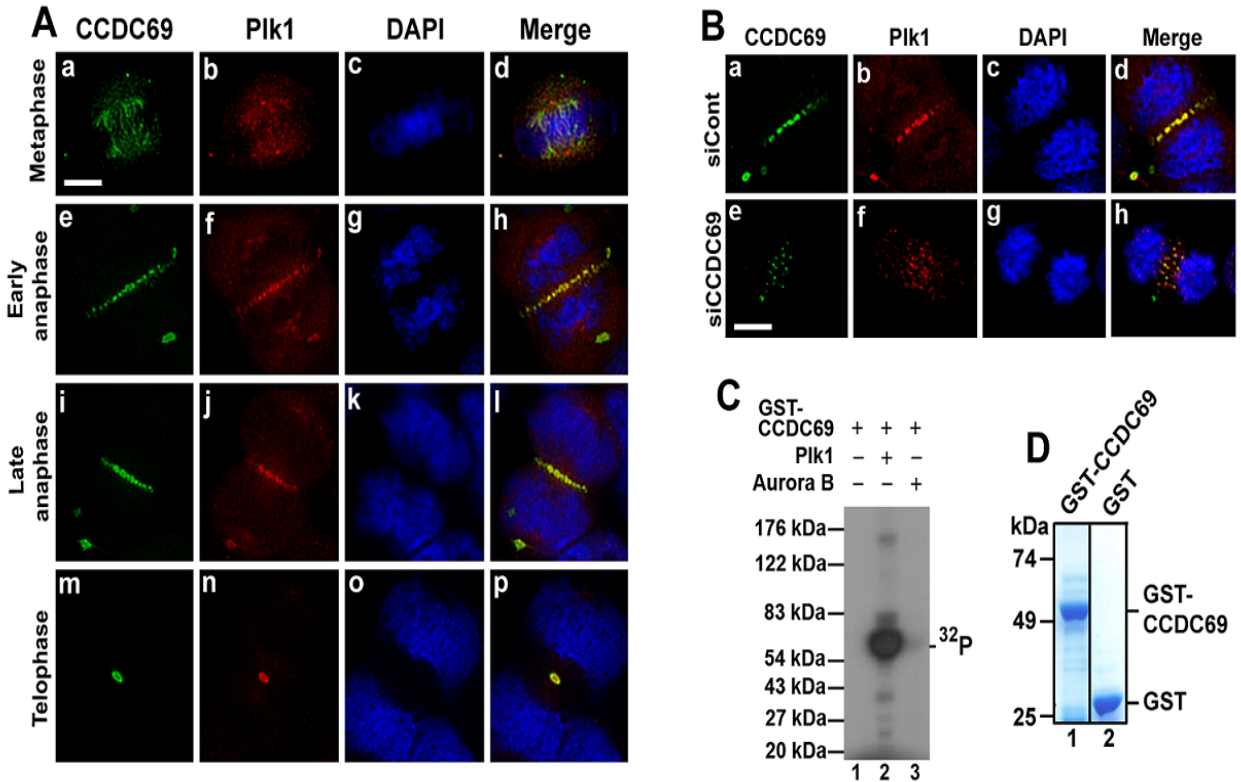
Figure 2-9. CCDC69 is required for the localization of Plk1 to the central spindle. A) Untransfected HeLa cells were fixed with acetone/methanol and subjected to immunofluorescence staining with anti-CCDC69 (green), anti-Plk1 (red), and DAPI (blue). **B)** HeLa cells were transfected with control (siCont) or CCDC69 (siCCDC69) siRNAs. 72 h after transfection, the transfected cells were fixed with acetone/methanol and subjected to immunofluorescence staining with anti-CCDC69, anti-Plk1, and DAPI. Bar, 5μm. **C)** Autoradiogram following SDS-PAGE of samples from in vitro kinase assays carried out to measure the activity of Plk1 and aurora B towards GST-CCDC69. **D)** A Coomassie stained gel of GST-CCDC69 that was used in C. Bar, 5μm.
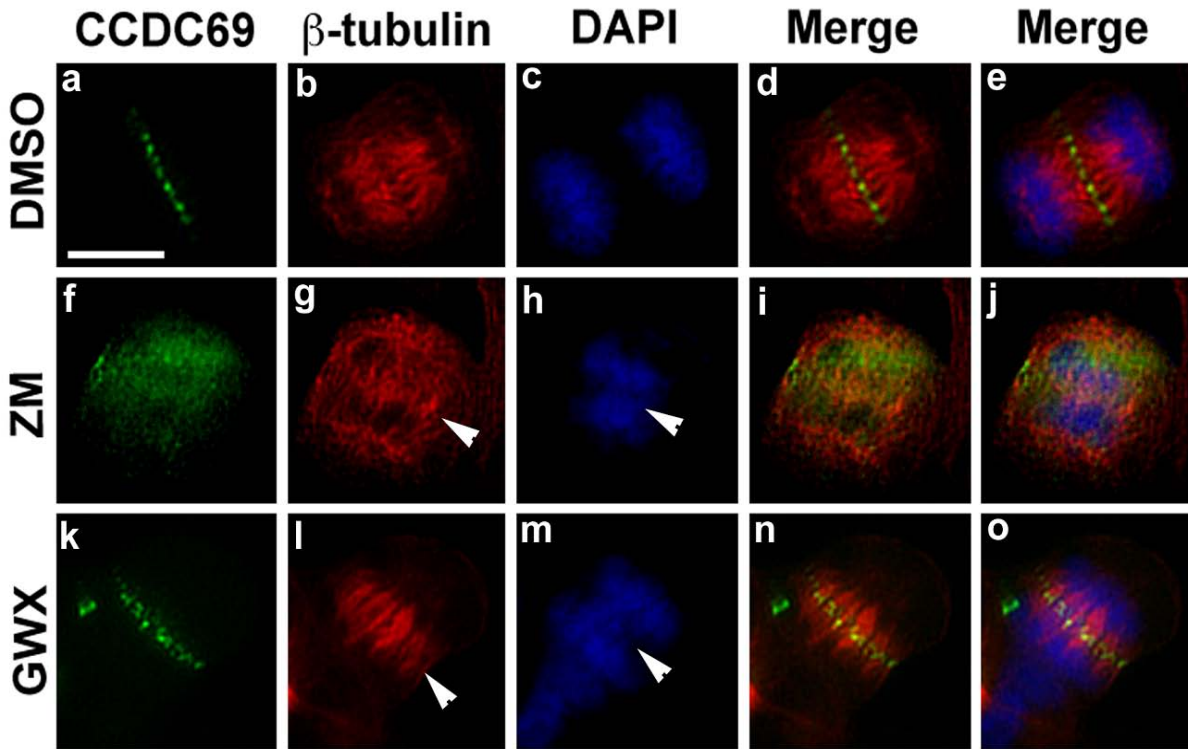
Figure 2-10. Inhibition of aurora B but not Plk1 disrupts the localization of CCDC69 to the central spindle. Untransfected HeLa cells were treated with DMSO (panels a-e), ZM 447439 (ZM; panels f-j), or GW 843682X (GWX; panels k-o) for 25 min. The treated cells were fixed with paraformaldehyde and subjected to immunofluorescence staining with anti-CCDC69 (green), anti-β-tubulin (red), DAPI (blue). Merge of green and red channels is shown in d, i, n and merge of green, red, and blue channels is shown in e, j, o. Bar 7.5μm.

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Appendix A - Sequence comparison of human, mouse and *Xenopus* CCDC69

```
hCCDC69    1 MGCRHSRLSSCKPPKKKRQEPEPEQPPRPEPHELGPLNGDTAITVQLCASEEAERHQKDI 60
mCCDC69    1 MGCRQSRHS...................................................  9
xCCDC69    1 MGCKTSKMCCPQLRKKKRQKAHQGGLTSQELNDLNAKTQGPNEVLQKIKEYEQEIRDLLQ 60

hCCDC69   61 TRILQQHEEEKKKWA...........QQVEKERELELRDRLDEQQRVLEGKNEEALQVLRA 110
mCCDC69   10 ...........RGKRA...........EKVE-ETQTELLEALDKEGRILEGRHEEAGQVPQT 48
xCCDC69   61 KHQEEKTALADAHKADVEARTLELQAQAQKDRDAETAKLLSEQAATMKAEMEEKFAELQK 120

hCCDC69  111 SYEQEKEALTHSFREASSTQQETIDRLTSQLEAFQAKMKRVEESILSRNYKKHIQDYGSP 170
mCCDC69   49 SNAQEKVSLSDCIQEAKASLQNTCASHVSPQEATQAKMNKVDGSILSRLYRNHIQDYGSP 108
xCCDC69  121 SFEQEKVSLTQTHQQFTDALQETVDELNSQLASFREKMKRVEESVLRQDYRRHIQDHGSP 180

hCCDC69  171 SQFWEQELESLHFVIEMKNERIHELDRRLILMETVKEKNLILEEKITTLQQENEDLHVRS 230
mCCDC69  109 GPFWEQELESLHHVIEMKNERIHELEKQLFLLEMLKEKNLILALKNTTLRQEVEDLQFQA 168
xCCDC69  181 GQFWEQELQSLHFVIEMKSELIREQDKRLRNHESTMERNLVLEERSRTLQQENEALKVQT 240

hCCDC69  231 RNQVVLSRQLSEDLLLTREALEKEVQLRRQLQQEKEELLYRVLGANASPAFPLAPVTPTE 290
mCCDC69  169 GNRLTMSRQLRKDLLQD...LEKESQNGHCCSRRRSH.................... 202
xCCDC69  241 QKQGAVTVRLSEELLSTQVSLEKQIHRCEQLQREKEQNLYRAVNGDAPQQFSLQSNAQEL 300

hCCDC69  291 VSFLAT                                                   296
mCCDC69      ......
xCCDC69  301 PVMVL.                                                   305
```
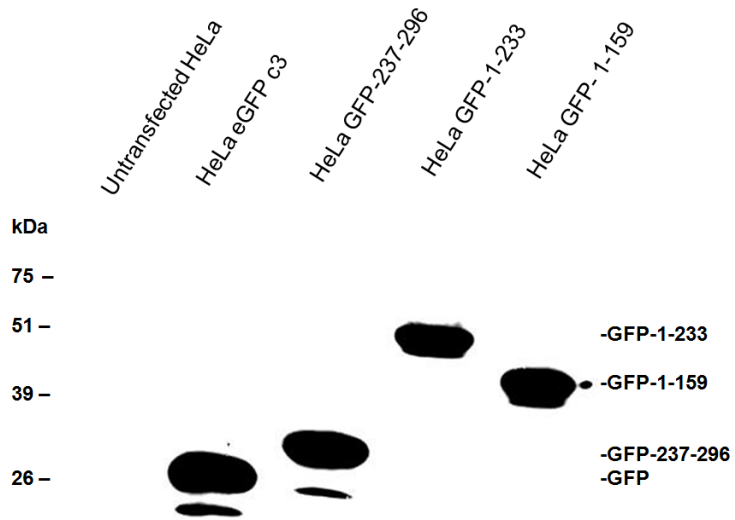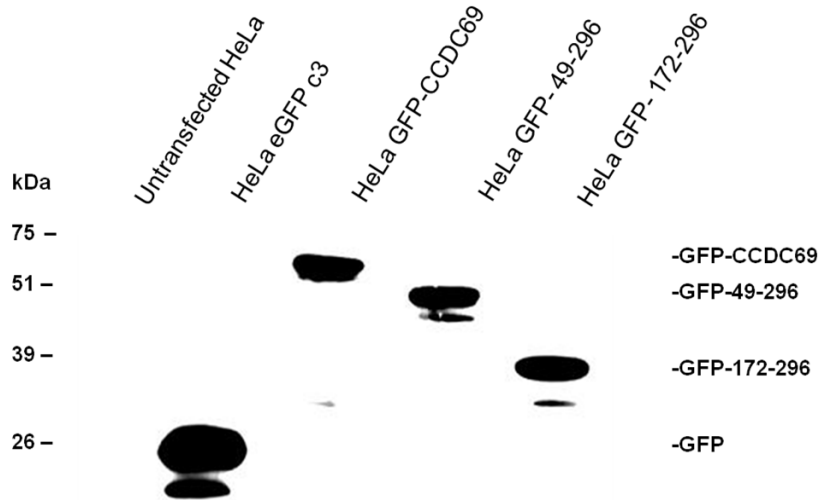
Appendix B - Immunoblot analysis of HeLa cells transfected with plasmids encoding GFP-CCDC69 or GFP-CCDC69 truncation mutants

Appendix C - List of Abbreviations

APC : Anaphase Promoting Complex

ATP : Adenosine Tri Phosphate

Bub : budding uninhibited by benzimidazoles

CCDC69 : Coiled-Coil Domain-Containing protein 69

Cdc25 : cell division cycle 25

CDK : Cyclin Dependent Kinase

cDNA : complementary DNA

CENP : CENtromere Protein

Chk1 : Checkpoint kinase 1

Chk2 : Checkpoint kinase 2

CPC : Chromosome Passenger Complex

DMSO : Dimethyl Sulfoxide

DNA : Deoxyribo Nucleic Acid

DTT : Dithiothreitol

Ect2 : Epithelial cell transforming sequence 2 oncogene

EDTA : Ethylene Diamine Tetraacetic Acid

EGFP : Enhanced Green Fluorescence Protein

EGTA : Ethylene Glycol Tetraacetic Acid

G0 phase : Gap zero phase

G1 phase : Gap 1 phase

G2 phase : Gap 2 phase

γ-TURC : gamma- Tubulin Ring Complex

GDP : Guanosine Di Phosphate

GEF : guanine nucleotide exchange factor

GTP : Guanosine Tri Phosphate

Ig G : Immunoglobulin G

INCENP : Inner centromere protein

KIF11 : Kinesin family member 11

KIF2A : Kinesin heavy chain member 2A

KIF2B : Kinesin family member 2B

M phase : Mitosis phase

Mad : Mitotic arrest deficient 1

MAP : Microtubule-Associated Protein

MAST : Microtubule Associated Serine/Threonine kinase

MCAK : mitotic centromere-associated kinesin

$MgCl_2$: Magnesium Chloride

MgcRacGAP : Rac GTPase activating protein

Mklp : mitotic kinesin-like protein

MOPS : 3-(N-morpholino)propanesulfonic acid

MPP1 : membrane protein, palmitoylated 1

MTOC : MicroTubule Organizing Center

NIMA : Never In Mitosis gene A

Op18 : Oncoprotein 18

PKA : Protein Kinase A

PBD : Polo Box Domain

Plk : Polo like kinase

PRC1: protein regulator of cytokinesis 1

Rb : RetinoBlastoma

Rho : Ras homolog gene family

RNA : Ribonucleic Acid

ROCK : Rho associated, coiled-coil containing protein kinase 1

SDS PAGE : Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis

siRNA : silencing RNA

Slk : Ste20-like kinase

S phase : Synthesis phase phase