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Characterization of Human Cdc14B's function in centrosome cycle control

Jun Wu
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To the Graduate Council:

I am submitting herewith a dissertation written by Jun Wu entitled "Characterization of Human Cdc14B's function in centrosome cycle control." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Yisong Wang, Major Professor

We have read this dissertation and recommend its acceptance:

Brynn Voy, Hayes McDonald, Bruce Mckee, Sundar Venkatachalam

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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Characterization of Human Cdc14B's function in centrosome cycle control

A Dissertation Presented for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Jun Wu

December 2008

DEDICATION

I dedicate this dissertation to my parents Hongfen Xu and Lusheng Wu, my wife Di Xu and daughter Olivia Wu, for their love and support.

ACKNOWLEDGMENTS

Firstly, I would like to thank my advisor, Dr. Yisong Wang, for his encouragement and guidance throughout my research, especially during difficult times in the lab due to lack of funding. I would also like to thank my committee members, Dr. Brynn Voy, Dr. W. Hayes McDonald, Dr. Bruce Mckee and Dr. Sundar Venkatachalam, who have advised me not only in my committee meetings but also in our numerous discussions along the course of my research. I thank all members of our lab for constructive advice. I am especially grateful to Dr. Yie Liu and Dr. Hyekyung P. Cho whom I have learned great amount of scientific knowledge and experimental skills. I wish to thank Dr. John Dunlap for his invaluable assistance with electronmicroscopic work. Finally, I would like to thank my friends also labmates Dr. Richard Giannone and David B. Rhee for their encouragement, support and helpful discussions.

ABSTRACT

Centrosomes are the only non-membranous organelles in most vertebrate cells and their major function is to nucleate microtubules, hence often recognized as the microtubule-organizing center (MTOC). Much like chromosome centrosome duplicates only once during the S phase of each cell cycle. The fidelity and timing of this duplication event will ensure equal division of duplicated chromosomes into the daughter cells. As a consequence, numerical and/or structural centrosome abnormalities will cause chromosome missegregation and lead to the generation of multiple mitosis and ultimately chromosomal instability, which typify many cancers.

The molecular mechanism of centrosome duplication remains unclear. Previous studies found that a fraction of human proline-directed phosphatase Cdc14B associates with centrosomes. However, Cdc14B's involvement in centrosome cycle control has never been explored. In this study, we identify Cdc14B as a negative regulator in centrosome cycle control: depletion of Cdc14B by RNA interference leads to centriole amplification in both HeLa and normal human fibroblast BJ and MRC-5 cells; ectopic expression of Cdc14B leads to stepwise loss of centrioles and attenuates centriole amplification in HU/APH arrested S phase cells and cells treated with proteasome inhibitor Z-L_3VS. This inhibitory function requires centriole-associated Cdc14B catalytic activity. In addition, our data suggests counterbalancing effects between Cdc14B phosphatase and kinases such as Plk4, Cdk2/Cyclin-E/A in centrosome duplication control potentially through modulating phosphorylation status of their common downstream effectors, HsSas-6 and B23 respectively. Taken together, these results

suggest a potential function for Cdc14B phosphatase in maintaining the fidelity of centrosome duplication cycle.

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LIST OF SYMBOLS AND ABBREVIATIONS

ADP	Adenosine diphosphate
Ana	Anaphase
APC	Anaphase promoting complex
APH	Aphidicolin
BSA	Bovine serum albumin
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CHO	Chinese Hamster Ovary
CKI	CDK inhibitor
CMV	Cytomegalovirus
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DOX	Doxycycline
DXR	Doxorubicin
EGFP	Enhanced green flurescent protein
FBS	Fetal bovine serum
FEAR	Cdc fourteen early anaphase release
GFP	Green flurescent protein
GST	Glutathione-S-transferase
HRP	Horseradish Peroxidase

HU	Hydroxyurea
IP	Immunoprecipitation
MAPK	Mitogen-activated protein kinases
MEF	Mouse embryonic fibroblast
Meta	Metaphase
mRNA	mesenger RNA
MT	Microtubule
MTOC	Major microtubule organizing center
Neo	Neomycin
NES	Nuclear Export Signal
MEN	Mitotic exit network
NLoS	Nucleolar localization signal
NT	Non-targeting
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCM	Pericentriolar material
PCR	Polymerase chain reaction
Plk	Polo-like kinase
PP	Protein phosphatase 1
Pro	Prophase
PTP	Protein tyrosine phosphatase
rDNA	ribosomal DNA

RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Sgo1	Shugoshin1
shRNA	short hairpin RNA
siRNA	small interfering RNA
SPD	Spindle pool body
Telo	Telophase
Tet	Tetracycline
TetO2	Tetracycline operator 2
WT	Wild type
YFP	Yellow flurescent protein
γ -TuRC	γ-tubulin ring complex
γ -TuSC	γ-tubulin small complex
GAP	GTPase activating protein
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
Flp	CDC fourteen-like phophatase
SIN	Septation initiation network
SCF	Skp, Cullin, F-box

Chapter 1

Introduction to Centrosome Biology

1.1 Introduction:

Since its discovery in 1876, centrosome has attracted the attention of many scientists from various backgrounds to study its structure, function and reproduction. However, after more than 100 years of extensive studies, many aspects of this fascinating organelle still remain a mystery (Rustem and Claude, 2007).

The term of "centrosome" was first coined by Theodor Boveri during the late 19th century based on the observation that they are small phase-dense bodies located at the center of the cells, surrounded by a relatively larger region of less dense materials (Nigg, 2002). Theodor Boveri was a pioneer of centrosome biology who during his studies addressed many of the fundamental problems in this field, notably the mode of centrosome duplication and its relevance to human development and cancer. However, throughout the early decades of the 20th century, the centrosome had proven refractory to molecular analysis mainly due to its low abundance and tiny size. With the revolutionizing technological advances in biology in the late 20th century such as forward and reverse genetics, mass spectrometry, and the combination of live-cell imaging and laser microsurgery, electron microscopy, molecular cloning and etc, we have gained detailed insights into how centrosome is structurally organized, its detailed composition and how

it functions. These results also open up doors for new studies into the role of the centrosome in the origin of cancer and other human diseases, its impact on stem cell and developmental biology, human reproduction, and last but not least, its relevance to the propagation of intracellular parasites (Nigg, 2004).

Centrosome is unique in that it is the only organelle in most animal cells that doesn't have a membrane. It is usually in close proximity to the nucleus. Centrosome functions primarily as the major microtubule organizing centers (MTOC) of animal cells. It governs most microtubule related functions: In the interphase cell, it facilitates the nucleation of microtubules in the cytoplasm which in turn regulate cell polarity, shape, mobility, adhesion and intracellular transportation; at the onset of mitosis, centrosome helps to instruct the formation of a biopolar spindle which is indispensable for the separation of sister chromatids during cell division (Nigg, 2002).

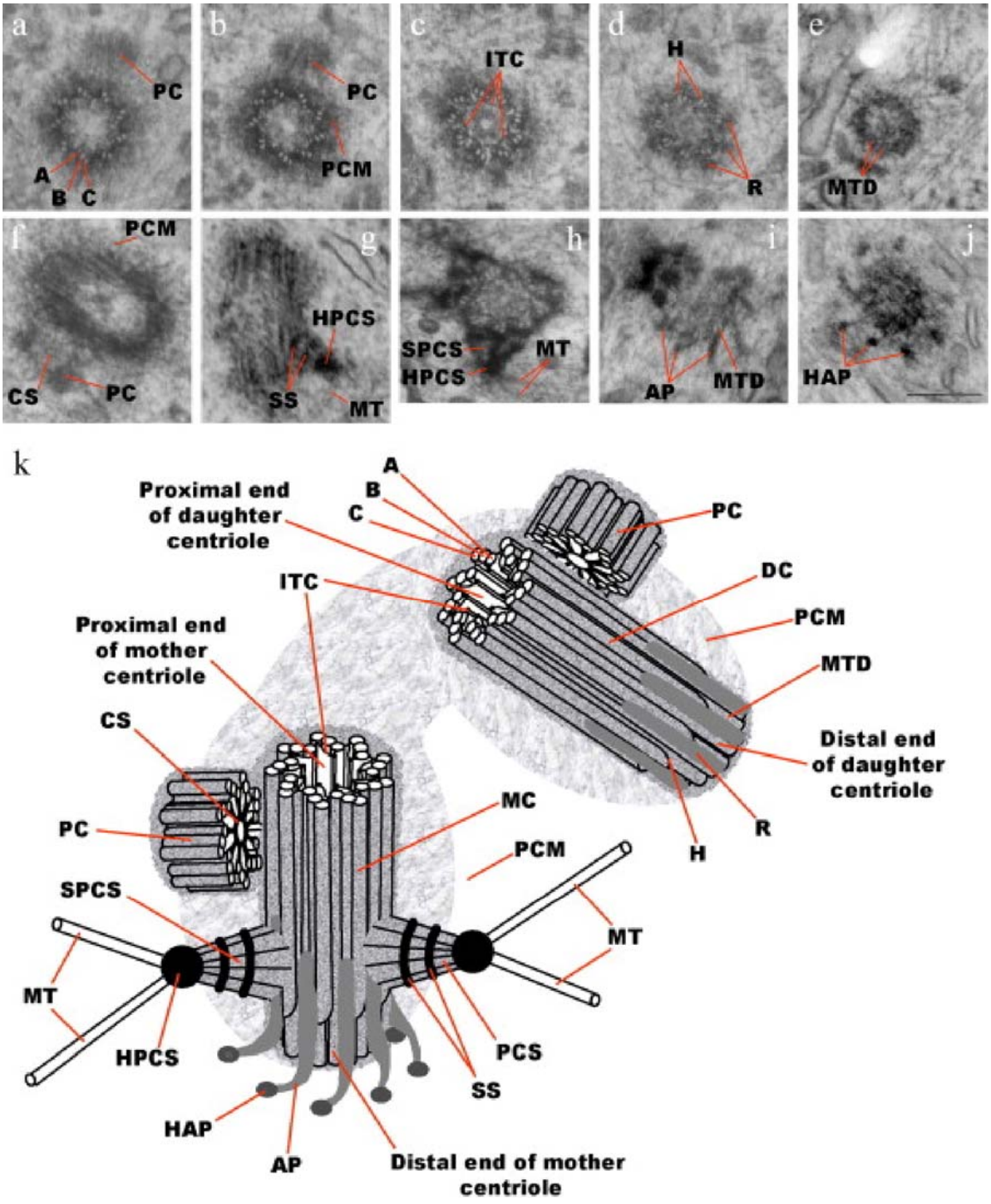
During S-phase of each cell cycle, cells make exactly two copies of its DNA, and during mitosis the equal segregation of all duplicated chromosomes to the two daughter cells depends upon the formation of a bipolar mitotic spindle. Since spindle polarity in higher animal cells is usually dependent on the number of centrosomes serving as major MTOCs, the cell must have exactly a pair of centrosomes by the onset of mitosis, therefore much like chromosome centrosome duplicates only once during the S phase of each cell cycle. The fidelity and timing of this duplication event will ensure equal division of duplicated chromosomes into the daughter cells. As a consequence, numerical and/or structural centrosome abnormalities will cause chromosome missegregation and

lead to the generation of multiple mitosis and ultimately chromosomal instability, which typify many cancers (Erich, 2006). In fact, numerical and structural centrosome aberrations have been implicated in virtually almost all types of cancer cells (Badano et al., 2005; Saunders, 2005).

1.2 Centrosome Structure

Centrosome's ultrastructure was investigated as early as the middle 1960s by electron microscopy. As a result, a detailed description of many aspects of centrosome composition was already available (de Harven, 1968; Fulton, 1971; Rustem and Claude, 2007; Stubblefield, 1967). Structurally, centrosome is comprised of a pair of barrel-shaped centrioles situated at the core of the centrosome and surrounded by an electron-dense proteineous matrix, the pericentriolar material (PCM) (Fig 1.1). Centriole is among the most highly conserved structures in animal cells. It possesses a remarkable nine-fold radial symmetry, consisting of an array of parallel microtubule triplets arranged like blades of a turbine (Azimzadeh and Bornens, 2007; Marshall, 2007). It is a cylindrical structure about 500nm in length and 200nm in diameter (Bornens, 2002; Paintrand et al., 1992). However, there are variations of this structure among different species. In vertebrates, typical nine triplet microtubules are found; whereas in *D.melanogaster* and *C.elegans* they mostly are consist of doublet and singlet microtubules. Each centriole is polarized along the proximal-distal axis with two functional different ends: The proximal end ("minus" end of the centriolar MTs) where the new centriole usually starts to grow and the distal end ("plus" end of the centriolar MTs) which can be the site of cilia origin

Figure 1.1: Centrosome structure. Centrosome structure in an animal cell at the end of G1-phase, beginning of S-phase. From "a" to "j", electron microscopy images (top line – serial sections of daughter centriole, second line – selected sections of mother centriole, scale bar 200 nm) illustrating different centrosome structures shown on the centrosome scheme – "k". MC – mother (mature) centriole, DC – daughter centriole; PC – procentriole; PCM – pericentriolar material (pericentriolar matrix); A – microtubule of triplet; B – microtubule of triplet; C – microtubule of triplet; H – hook of C microtubule; MTD – A–B microtubule duplex (in distal part of centriolar cylinder); ITC – internal triplets connections system; CS – cartwheel structure (axis with spokes); PCS – pericentriolar satellite (=sub-distal appendage); HPCS – head of pericentriolar satellite; SPCS – stem of pericentriolar satellite (connected to three triplets in this case); SS – striated structure of pericentriolar satellite stem; MT – microtubule; AP – appendage (=distal appendage); HAP – head of appendage; R – rib. (Image courtesy of Uzbekov R and Prigent C., 2007).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Proximal end of daughter centriole
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Proximal end of mother centriole
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MT
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PCS
SS
HAP
AP
Distal end of mother centriole

(Azimzadeh and Bornens, 2007). The two centrioles within each centrosome, however, differ in their structures. Only the mature centriole (The maternal, or mother centriole) has appendages close to its distal end ("AP" in Fig 1.1) as well as sub-distal appendages; the immature centriole (The daughter centriole) has only electron dense ribs along the microtubule triplets ("R" in Fig 1.1). The two sets of appendages on mother centriole are thought to be required for anchoring microtubules. Mother and daughter centrioles are linked through their proximal ends in part by large coiled-coil linker proteins of the pericentrin family (Azimzadeh and Bornens, 2007; Rustem and Claude, 2007).

Under the electron microscope, the PCM surrounding the centrioles appears as an amorphous, electron-dense fibrous lattice (Dictenberg et al., 1998; Nigg, 2002). Even though a complete inventory of PCM components has not been established, in a typical human centrosome, the PCM contains over one hundred proteins (Andersen et al., 2003). Most of the components responsible for the major centrosomal functions, for example, the γ-tubulin ring complexes that play indispensable role in microtubule nucleation, reside in the PCM. Other PCM components are less well conserved although many contain predicted coiled-coil domains, suggesting their structural functions to act as scaffolds for the recruitment of cell cycle regulatory proteins, such as protein kinases, phosphatases, components of the ubiquitin-dependent proteolytic machinery and etc (Doxsey et al., 2005; Fry, 2004; Jackman et al., 2003; Kramer et al., 2004).

1.3 The Centrosome Cycle

Traditionally, the centrosome–centriole duplication–segregation cycle has been broken down into four consecutive steps: centriole disengagement, centriole duplication, centrosome elongation and maturation, and sister centrosome separation (Fig 1.2). These steps have been defined morphologically by detailed light and electron microscopy studies following the progression of the cell cycle.

1.3.1 Centriole Disengagement

In a metaphase cell, each of the two spindle poles is characterized by the presence of one centrosome comprising a mother-daughter centriole pair. They are tightly associated with each other through an inter-centriolar linking structure and typically arranged in an orthogonal configuration. Upon exit from M phase (or during early G1 phase) the tight link between the two centrioles is lost, in a process now referred to as 'disengagement' (formerly 'disorientation') in which there is slight separation and loss of orthogonal relationship between the pair of centrioles (Nigg, 2007b). This process has been reported as a potential licensing mechanism for the two centrioles to undergo a new round of duplication. Also, this process was suggested to be requiring the activity of separase, a caspase related protease that also drives the separation of sister chromatids prior to anaphase by cleavage of cohesion (Tsou and Stearns, 2006a). During G1, a different, highly dynamic linker structure is formed between the two disengaged centrioles that involves the tethering of filaments associated with the proximal ends of the two centrioles (Bahe et al., 2005; Yang et al., 2006).

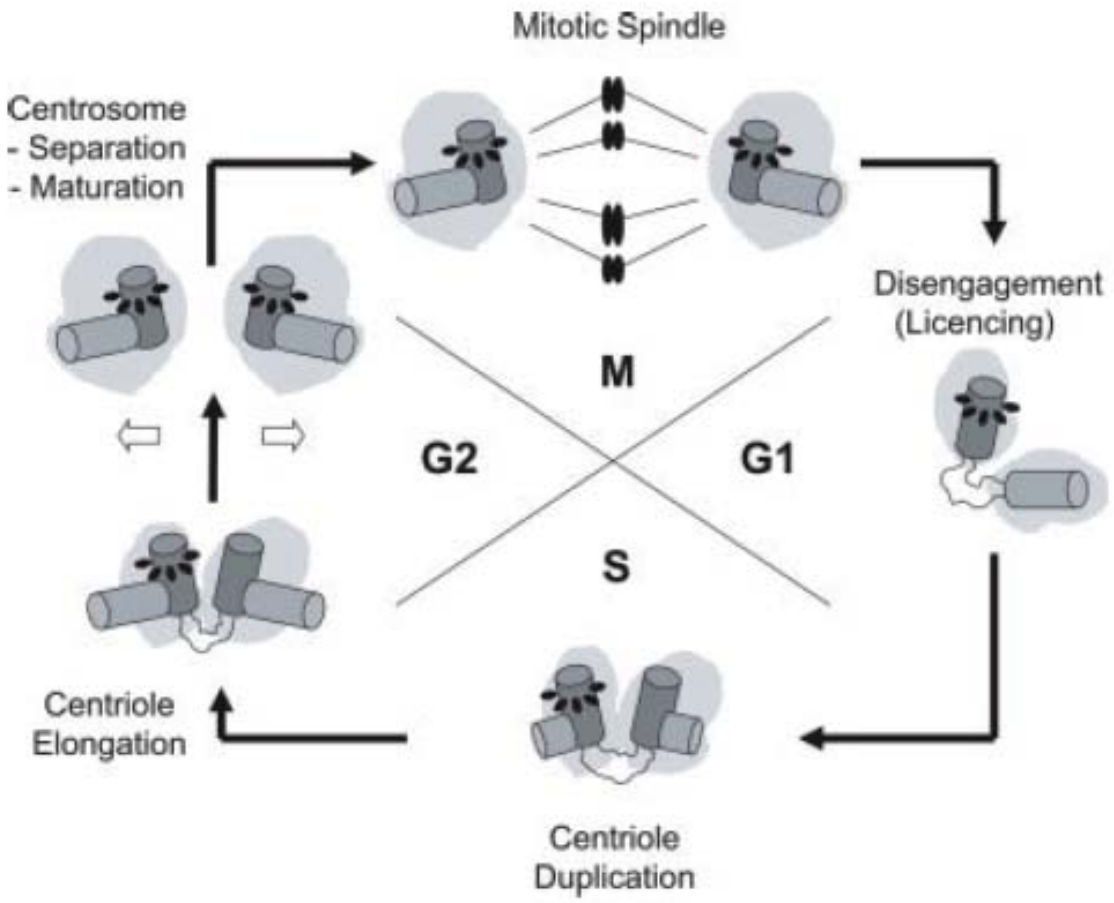


Figure 1.2: Schematic view of the centrosome cycle. (Image courtesy of Nigg EA, 2006)

1.3.2 Centriole Duplication

Careful temporal analysis of CHO cells expressing green fluorescent protein GFP-labelled Centrin-1 led to the conclusion that daughter centriole assembly initiates around the time of the transition between the G1 and S phases of the cell cycle (Thomas M. Durcan, 2008). During this period new daughter centrioles, also referred to as procentrioles, begin to grow at an orthogonal angle next to each maternal centriole close to their proximal ends, establishing again the tight 'base-to-side' connections between mother and daughter centrioles. Thus, duplication is semi-conservative in nature from the perspective of the whole centrosome, but conservative from the perspective of the centriole alone (Hartwell and Kastan, 1994).

Although daughter centriole biogenesis in vetebrate cells remains poorly understood, substantial progress has recently been made in other organisms. In particular, the ease of functional screening of genes using RNA interference (RNAi) has proven to be effective in the identification of molecules that are involved in centriole assembly in *C. elegans*. And as a result, the molecular mechanisms underlying centriole duplication have been best studied in this organism. *C.elegans* centrioles are smaller and simpler compared with the human ones, harboring a central tubule surrounded by 9 singlet rather than triplet microtubules (MTs) (Pelletier et al., 2006). Despite differences in the structure, the centriole/centrosome duplication cycle seems to be conserved. Like human centrosome, generation of new daughter centrioles also happens in S phase in *C.elegans*. Five essential proteins for centriole reproduction have been identified in *C. elegans*. These proteins are recognized as a conserved centriole-assembly protein module including a

protein kinase, Zyg-1 (O'Connell et al., 2001a), and four putative structural proteins: Spd-2, Sas-4, Sas-5, and Sas-6 ((Delattre et al., 2004; Kemp et al., 2004; Leidel et al., 2005; Leidel and Gˆnczy, 2003; Pelletier et al., 2004). With the exception of SPD-2, which also functions in centrosome maturation, reportedly, the rest of the molecules are exclusively required for centriole duplication. Through elegant electron tomographic reconstruction of early *C.elegans* embryos, the initial stages of procentriole assembly have been revealed. In brief, there are two distinct steps as illustrated in Fig 1.3: First, a central tube forms and elongates next to the base of the mother centriole which requires the function of Spd-2, Zyg-1, Sas-5 and Sas-6; second, singlet microtubules assemble around the peripherals of the central tube which is dependent upon Sas-4. Moreover, systematic kinetic analysis and epistasis experiments indicates that the five proteins mentioned are assembled sequentially onto nascent procentrioles (Delattre et al., 2006; Pelletier et al., 2006): the initial recruitment of Spd-2 to the mother centriole mediated by cyclin-dependent kinase 2 (Cdk2) is required for the subsequent centriolar recruitment of the kinase Zyg-1 onto procentrioles; Zyg-1 in turn enables the remaining three proteins to localize to the centrioles; Coiled-coil molecules Sas-6 and Sas-5 are recruited next to form the inner centriole tube that is essential for the binding of Sas-4; Sas-4 is important for subsequent production of the surrounding MTs (Delattre et al., 2006; Pelletier et al., 2006). Also, Sas-4 has been suggested to play an important role in recruiting PCM components such as Spd-5 and γ-tubulin (Dammermann et al., 2004).

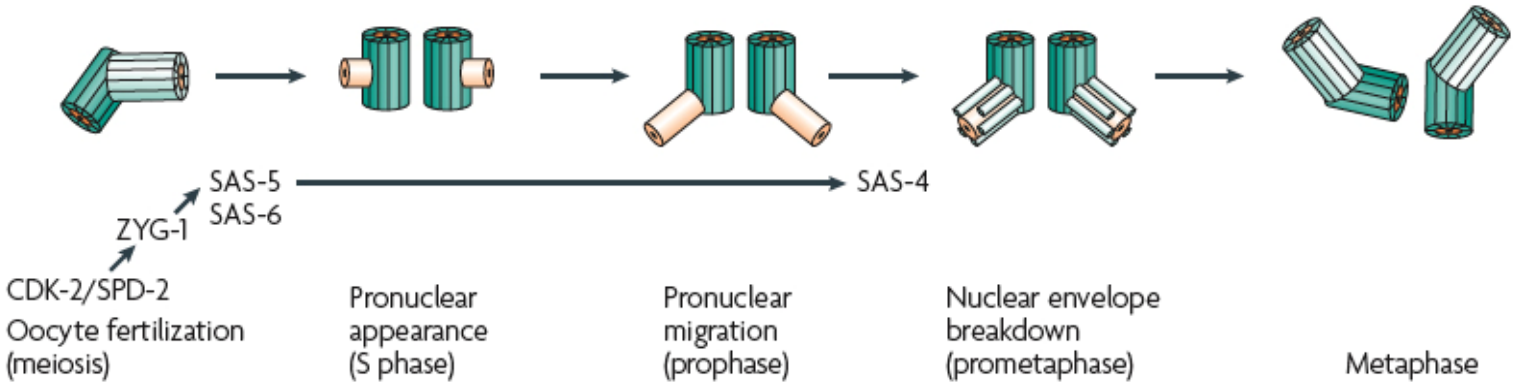


Figure 1.3: Procentriole assembly in *C. elegans*. (Image courtesy of Bettencourt-Dias M and Glover DM, 2007)

Recent studies in other organisms, such as *D.melanogaster* and human, have shed lights on the counterparts of these molecules in centriole duplication. Except for Sas-5 of which orthologs have yet to be found, the rest four proteins all have orthologs in both human and *D.melanogaster*. Thus the centriole assembly pathway is most likely to be conserved during evolution. Cep192, the mammalian Spd-2 ortholog, is a major regulator of PCM recruitment, centrosome maturation, and centriole duplication (Zhu et al., 2008); Sak, also known as Plk4 (C Fode, 1994; Swallow et al., 2005), has been identified as a key regulator of centriole duplication in both *D.melanogaster* (Bettencourt-Dias et al., 2005) and human cells (Habedanck et al., 2005a). Although lacking sequence homology, it is possible that Sak/Plk4 represents a functional homolog of *C.elegans* Zyg-1. Human Sas-6 (HsSas-6) localizes to the centrosome. Its absence leads to a lack of centriole duplication whereas overexpression triggers centrosome amplification, suggesting a crucial role of HsSas-6 in centriole assembly. Homolog of nematode Sas-4 is also essential in centriole duplication in *D.melanogaster* (Peel et al., 2007; Rodrigues-Martins et al., 2007) Recently, through siRNA screening and immunoelectron microscopy studies, a centriole assembly pathway in human cells has also been delineated (Kleylein-Sohn et al., 2007a). The depletion of nearly 30 candidate proteins (Andersen et al., 2003) by siRNA has identified HsSas-6, Cpap (the putative homolog of *C.elegans* Sas-4), Cep135, and Cp110 as being indispensable for centriole biogenesis. Following activation of Plk4 on the surface of the parental centriole, HsSas-6, Cpap, Cep135, and γ-tubulin are rapidly recruited to the procentrioles. γ-tubulin is potentially responsible for centriolar microtubules nucleation while Cpap and Cep135 probably play scaffolding roles. Finally, Cp110 will be recruited and form a cap like structure underneath which the α-/β-tubulin

will be inserted for the growth of the centrioles. This study strengthens the concept that centriole biogenesis is governed by an evolutionarily conserved mechanism (Delattre et al., 2006).

1.3.3 Centriole elongation

Elongation of newly formed procentriole spans from late S phase until it reaches the maximal length during the following cell cycle. The mechanisms underlying centriole elongation are poorly understood. It was suggested that rare tubulin isoforms were involved in the elongation of centrioles (Dutcher, 2003). For example, in *Chlamydomonas*, the tubulin isoform ε-tubulin mutant Bld-2 forms short centrioles made of singlet MTs instead of triplets implicating its role in centriole elongation. ε-tubulin is conserved in mammals and has been proposed to be needed for both centriole duplication and elongation (Azimzadeh and Bornens, 2007; Dutcher, 2003). Another interesting observation is that in the presence of low Cdk1 activity *D.melanogaster* wing imaginal discs had cells in which the daughter centriole was surprisingly longer than the mother centriole. This suggests that the levels of CDKs are likely to regulate centriole elongation (Bettencourt-Dias and Glover, 2007).

1.3.4 Centrosome Maturation and Separation

Maturation and separation of the two centrosomes start around the G2 to M transition marked by acquisition of several maturation markers, recruitment of pericentriolar material as well as an increase in MTOC activity.

Disassembly of the fibrous linker between the two centrosomes at the onset of mitosis is thought to be necessary for centrosome separation. C-Nap1, found at the proximal end of each parental centrioles but not procentrioles, is proposed to serve as the docking site for the formation of this linker. C-Nap1 interacts with another coiled- coil protein called rootletin, a fiber-forming molecule that emanates from the proximal ends of centrioles. However, electron microscopy studies showed that C-Nap1 and rootletin do not seem to account for the continuous linker between the parental centrioles suggesting other proteins are also participating in the formation of the fibrous linker required for centrosome cohesion. The disjunction of the two centrosomes is regulated in part by phosphorylation of C-Nap1 by a centriole-associated NIMA-related kinase (Nek2) during G2 phase of the cell cycle. This phosphorylation release C-Nap1 from the centrioles and leads to the disconnection between the two centrosomes thereby allowing them to separate. The activities of Nek2 kinases are counteracted by protein phosphatase 1 (PP1) which is inactivated at the onset of mitosis (Bahe et al., 2005; Fry et al., 1998; N R Helps, 2000; Yang et al., 2006). Also, Mailand and co-workers have reported that another phosphatase Cdc14A is also involved in centrosome separation (Mailand et al., 2002d). This centrosome-associated phosphatase must act at a different level than protein PP1 because its overexpression leads to precocious centrosome separation and down regulation of which leads to a failure in centrosome separation. The maturation of the procentrioles extends about one and a half cell cycles including two successive mitoses and completed with the acquisition of distal and sub-distal appendages (Azimzadeh and Bornens, 2007).

1.4 Centrosome Functions

1.4.1 Centrosome Mediated MT Events.

Microtubules are made up of two tubulins: α- and β-tubulin whose orientation gives microtubules an intrinsic polarity. Microtubules are involved in many cellular processes, including intracellular protein transportation, cell polarity, mobility, and maintenance of cell shape, cell division and the formation of both meiotic and mitotic spindles. Centrosome is recognized as the major MTOC of most animal cells. The PCM is a key structure that promotes the formation and organization of cytoplasmic microtubules during interphase and mitosis and is responsible for nucleating, anchoring and releasing microtubules. Although many protein components of the PCM have been described, only a subset of these are involved in nucleating microtubules: Pericentrin plays a role in the formation of a lattice-like structure along with several other proteins (Bettencourt-Dias and Glover, 2007; Bornens, 2002) that docks proteins such as γ-tubulin (Zheng et al., 1995) to mediate the nucleation of microtubules. γ-tubulin is a highly conserved protein in eukaryotes and exists in the γ-tubulin small complex (γ-TuSC), a core tetrameric complex composed of two γ-tubulin molecules. A ring structure comprising four or so these γ-TuSC sub-complexes known as the γ-tubulin ring complex (γ-TuRC) was found to be responsible for nucleating microtubules in all cells studied so far (Hannak et al., 2002). γ-TuRC is subjected to cell cycle dependent regulation and as a result conformation and activities are different between interphase and mitotic microtubules. In interphase, γ-TuRC nucleates fewer but longer microtubules while mitotic microtubules are shorter, larger in number, and highly dynamic. This difference

in the MT-nucleating ability of the centrosome between interphase and mitosis seem to be controlled by a balance of factors that either restrict or promote the recruitment of MT organizing molecules. Notably, protein phosphorylation and dephosphorylation play an important part in maintaining this balance: among other protein kinases, Plk1 (Polo-like kinase-1) and Aurora A promote MT nucleation, and this is counteracted by phosphatases such as protein phosphatase-1 (PP1), protein phosphatase-4 (PP4) and etc (Blagden and Glover, 2003; Trinkle-Mulcahy and Lamond, 2006).

Following their nucleation by the γ-TuRC, MTs are either released into the cytoplasm or recaptured and anchored at the centrosome. MT nucleation and MT anchoring are two separate processes that require different classes of molecules. Several different MT anchoring mechanisms have been proposed. The subdistal appendages of the mother centriole are thought to be a major site for anchoring MTs; PCM also has been proposed to perform this function. The MT anchoring activity of subdistal appendages requires ninein, as well as other molecules including centriolin, dynactin and EB1 (Badano et al., 2005; Bornens, 2002; Mogensen et al., 2000). Ninein is a component of the subdistal appendages of the mother centriole. It connects the centriole via its C terminus, whereas its N terminus interacts with the γ-TuRC (Delgehyr et al., 2005). MT release from centrosomes requires the activities of MT severing proteins, such as katanin, which has been shown to sever MTs *in vitro* and *in vivo* (Ahmad et al., 1999; Doxsey, 2001; Hartman et al., 1998).

1.4.2 Organization of Cilia and Flagella

In postmitotic cells, the centrosome migrates to the cell surface, and one of the centrioles differentiates into a basal body that nucleates microtubules to form a cilium (Dawe et al., 2007). Cilia and flagella are highly conserved structures that consist of microtubule axonemes that most commonly in the configuration of either (9+0) or (9+2): nine outer doublet microtubules and two (or none) central pair microtubules. The term cilia and flagella are sometimes used interchangeably despite minor differences such as number, different modes of movement and etc. Cilia are found on virtually all cells within the human body. They protrude from the cell surface and can either enable movement of the cell itself, or facilitate the sensing of chemical and mechanical signals surrounding the cells. A growing body of data suggests that cilia and flagella are indispensable for various cellular and developmental processes that include cell motility, propagation of morphogenetic signals in embryogenesis and sensory perception (Badano et al., 2005).

1.4.3 Centrosome in Cell Cycle Regulation

In addition, the centrosome also plays a crucial role in cell cycle regulation. It has been suggested that centrosomes can serve as multiplatform scaffolds for the binding of many regulatory complexes including many checkpoint proteins. And these proteins have been reported to participate in cytokinesis as well as G1 to S-phase (G1–S), G2 to M-phase (G2–M), metaphase to anaphase (M–A) transitions (Jackman et al., 2003; Mikule et al., 2007; Uetake et al., 2007).

1.4.3.1 Centrosome in Cytokinesis Control

Cytokinesis is the final stage of the cell cycle and is the process that two daughter cells are generated from one mother cell. As shown in Fig 1.4, the general processes involved in cytokinesis in eukaryotes are (Guertin et al., 2002):

- Division site is first chosen, most commonly at the cell equator, and the subsequent assembly of the cleavage furrow is assembled at the division site.
- Formation of contractile ring (also referred to as the actomyosin ring) that contains actin, myosin, and other proteins.
- Ingression or contraction of the actomyosin ring, generating a membrane barrier between the cytoplasmic contents of each daughter cell.
- Formation of a structure called midbody when the ingressing furrow constricts the components of the spindle midzone
- Abscission, the final event of cytokinesis marked by the sealing of the furrow, generating two completely separate cells.

One of recent studies showed that when centrosomes were removed from mitotic cells by laser ablation, mitotic spindles were still assembled but about 30% to 50% of the mitotic cells failed to complete cytokinesis (Khodjakov et al., 2000). Cells appeared to either remain attached by intercellular bridges or abort cytokinesis, forming binucleate cells. In another study in which centrioles were labeled with Centrin-GFP, the final events of

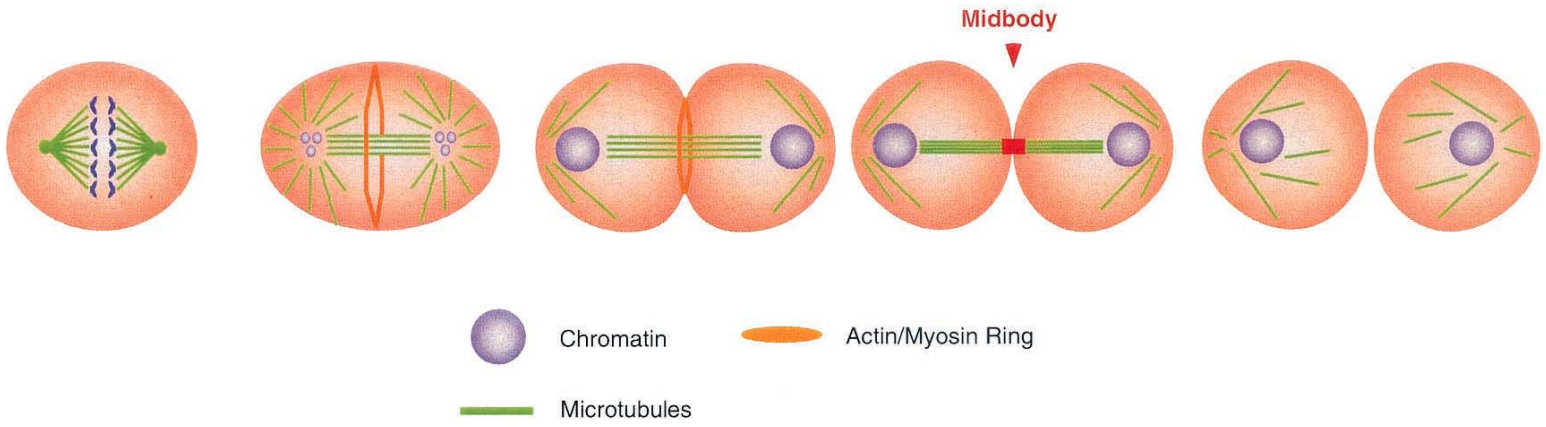


Fig 1.4: General mechanisms of cytokinesis in animal cells. (Image courtesy of Guertin, D.A. *et al*, 2002)

cytokinesis correlated with movement of the maternal centriole to the intercellular bridge. However, the abscission, final step of cytokinesis, didn't finish until the mother centriole moved from the intercellular bridge back to the center of the cell. These observations highly suggest that centrosomes were involved in the activation of the final stages of cytokinesis.

1.4.3.2 Centrosome in G1/S Progression

Recent studies have proposed an intriguing possibility that G1/S transition is dependent on centrosomes. The same techniques used to reveal centrosome function in controlling cytokinesis have also identified a centrosome requirement for S-phase entry (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). When the centrosomes are removed by either microsurgery or laser ablation, cells that do make it through cytokinesis tend to arrest in G1 phase and never progress into the subsequent S phase. More interestingly, if only one of the centrosomes is removed during prophase: the acentrosomal daughter cell becomes arrested in G1 phase; however the centrosome-containing daughter cell progresses to the next mitosis without any delay (Khodjakov and Rieder, 2001). One attractive possibility is that these cells simply did not go through cytokinesis completely which led to the activation of a checkpoint that monitors aberrant centrosome numbers or maybe the presence of excessive DNA (Andreassen et al., 2001). Alternatively, centrosomes may be required in a positive fashion to promote processes required for G1/S transition. This may occur through recruitment or concentration of molecules that are essential for the initiation of DNA synthesis (Hinchcliffe et al., 1999; Lacey, 1999). Even though there is still ongoing debate of whether centrosomes directly

participate in G1/S transition or centrosome defects trigger checkpoints that monitor the completion of mitosis, one obvious outcome of having a centrosomal requirement for G1/S progression is to ensure proliferating cells receive appropriate number of functional centrosomes after each cell cycle.

1.4.3.3 Centrosome in G2/M Progression

Early research suggested that centrosome plays a role in progression into mitosis. The evidence came from experiments when injected with centrosomes, G2-arreasted starfish oocytes started progressing into mitosis and mitotic entry in Xenopus eggs were also accelerated (Perez-Mongiovi et al., 2000; Picard et al., 1987). More recent studies have started to reveal the role for the centrosome in the G2/M transition in mammalian cells. It is widely known that the G2/M transition requires activation of Cdk1/cyclin B. Although cyclin B1 is present throughout the cytoplasm before prophase, active Cdk1/cyclin B1 was first detected at the centrosome during prophase before its appearance in the nucleus (Jackman et al., 2003). This observation implicated that centrosome might function as docking station where regulatory proteins such as Cdk1/cyclin B are accumulated for triggering mitosis (Doxsey et al., 2005; Hames, 2004).

1.4.3.4 Centrosome in Metaphase-to-Anaphase Transition

After mitotic entry, the next critical transition point in the cell cycle is the metaphase to anaphase transition. This is in part under the control of the spindle assembly checkpoint which prevents the initiation of anaphase until all successful attachment of

spindle microtubules to the kinetochores of chromosomes have been achieved (Hames, 2004; Musacchio and Hardwick, 2002). The main target of the spindle assembly checkpoint is the multi-subunit ubiquitin ligase known as the anaphase promoting complex or cyclosome (APC/C) (Hames, 2004; Page and Hieter, 1999). Securin and Cyclin B are among the many substrates of APC/C that will be polyubiquitylated and targeted for proteasome-mediated degradation. Destruction of securin promotes the onset of anaphase by releasing separase which in turn cleaves the centromeric cohesin molecules that hold the sister chromatids together (Nasmyth, 2002); Degradation of Cyclin B, on the other hand, promotes mitotic exit. In order for APC/C to recognize its substrates, an additional adaptor subunit is required: During metaphase to anaphase transition, the adaptor needed is the Cdc20 (Fizzy/Fzy) protein; and at the time of mitotic exit, the adaptor protein is the Cdh1 (Fizzy-related/Fzr) (Morgan, 1999). The current view on how the spindle assembly checkpoint prevents anaphase onset is that checkpoint proteins, notably Mad2, Bub3 and BubR1, form a mitotic checkpoint complex (MCC) with Cdc20 preventing it from interacting with and activating the APC/C (Hames, 2004; Hwang et al., 1998; Kim et al., 1998; Sudakin et al., 2001; Tang et al., 2001).

Kinetochores, as have already been extensively documented and well accepted, play an essential role in regulating the metaphase to anaphase transition (Agnes L. C. Tan, 2005); How centrosomes function as a molecular scaffold for sensing and regulating this transition hasn't been studied in details. It is interesting to know where exactly the APC/C-Cdc20 gets activated: is it mainly activated at the kinetochores or possibly can it first be activated at centrosome/spindle pole. Early evidence showed that some APC/C

components such as Cdc16 and Cdc27 as well as Cdc20 localized to the centrosome in fixed cells, however, these observations were circumstantial and other studies have shown these proteins to be highly dynamic and existed in a number of other locations. The first clear indication that centrosome/spindle pole was responsible for the metaphase to anaphase transition came from live cell imaging of GFP-tagged cyclin B destruction in Drosophila embryos (Raff, 1999). The loss of GFP-Cyclin B starts at the spindle pole before spreading up the spindle to the chromosome. Other reports in HeLa cells (Clute and Pines, 1999) and yeast cells (M Yanagida, 1999) confirmed this result. Even more convincing is the finding that in a particular *Drosophila* mutant ('centrosomes fall off ', cfo), cyclin B destruction occurred on unattached centrosomes but not on the spindles, resulting embryos arrest in anaphase (Wakefield et al., 2000). This experiment provides powerful evidence that destruction of cyclin B begins on spindle poles. In addition to Cyclin B it is possible that destruction of securin, another APC/C substrate, also is initiated at the centrosome/spindle pole although the evidence is not strong (Hagting et al., 2002).

Besides centrosomes' potential role in regulating destruction of APC/C-Cdc20 substrates required for the initiation of anaphse, other studies have suggested that the centrosome protein γ-tubulin and human pericentrin B homolog, Pcp1p, are also involved in the metaphase to anaphase transition in addition to their roles in microtubule nucleation and anchoring (Prigozhina et al., 2004; Rajagopalan et al., 2004).

1.5 Centrosome Abnormalities and Human Diseases

At the turn of the 20th century Boveri's visionary research suggested that cells with abnormal centrosomes were precursor turmor cells. During the past decade, Boveri's early description of centrosome abnormalities in cancer cells has found enormous recognition and been extended to a large number of different human tumors (Brinkley, 2001; Duensing, 2005; Lingle WL, 2005; Nigg, 2002; Pihan et al., 2003). Given centrosome's critical involvement in cell cycle control it's not surprising that it has a role in tumorigenesis. The fact that centrosome abnormailites have been observed in nearly all type of cancers includeing breast, liver, colon, cervical, bone marrow, prostate and etc (Nigg, 2004), and overexpression of certain centrosome components resulted in aneuploidy (Katayama et al., 2003; Lingle WL, 2002) have led to the conclusion that centrosome plays an important role in cancer development and progression.

Generally speaking, centrosome abnormailites can be classified into two broad categories: numerical and structural aberrations. Although their origins and consequences may differ, Numerical centrosome aberrations are frequently accompanied by structural irregularities and both of them can promote aneuploidy and potentially result in cancer formation.

1.5.1 Numerical Centrosome Aberrations

Numerical centrosome abnormalities are defined as an erroneous number (either less than or greater than two) of fully functional centrosomes in mitotic cells that can interfere with the formation of the normal bipolar mitotic spindle for segregating sister

genomes. The exact counting of centrosome numbers, however, is not an easy task. Serial-section electron microscopy can ultimately solve the problem but impractical, therefore most studies have to rely on immunostaining using antibody labeling. Although proven to be method of choice, results from these studies have to be interpreted with caution. Factors to consider include not only antibody quality, cell type, the resolution of the optical device in use, but also the orientation of the centrosome relative to the direction of viewing, and the fact that PCM components associate unevenly with mother and daughter centrioles, respectively. Moreover, possible PCM fragmentation is another factor and as a result, not every positive staining with antibodies targeting PCM components necessarily represents a complete centrosome. Despite all these complications, there is no doubt that numerical centrosome aberrations, particularly extra copies of centrosomes (also referred to as supernumerary centrosomes or centrosome amplification) often lead to genome instability which is the hallmark of cancer (Erich, 2006; Lingle WL, 2005).

Majority of cancer cells with supernumerary centrosomes correlate with loss of p53 or retinoblastoma (Rb) tumour suppressors. This is likely because in these cells, a more permissive condition for cells with amplified centrosomes to proliferate has been established. In addition, deficiency of the breast cancer gene BRCA1 and the overexpression of Aurora-A kinase in transiently transfected cultured cells also result in centrosome amplification in certain cancer cells (Fukasawa et al., 1996; Pihan et al., 1998; Xu et al., 1999). Centrosome amplification caused by deregulation of these centrosome number regulators might arise directly from overreplication of centrosomes

or indirectly from cytokinesis defects. These will be covered in more details in later chapters.

1.5.2 Structual Centrosome Aberrations

Structual aberrations of centrosome, on the other hand, are recognized as defects in centrosome composition that can inhibit the formation of normal bipolar spindles. In addition, disrupted centrosome integrity can indirectly affect multitude of signaling pathways and contribute to the cancer formation. Unlike numerical aberrations manifested only as two phenotypes: less or more than two centrosomes per cell, phenotypes of structural aberrations include enlarged centrosome size (Due to excessive accumulation of PCM around centrioles), formation of acentriolar bodies, alterations in the phosphorylation state of PCM proteins, ectopic appearance of centrosome related bodies (CRB) and etc (Nigg, 2002). Most likely, structural centrosome aberrations are caused by deregulated expression of genes coding for centrosomal components or altered posttranslational modifications and activity of these centrosomal proteins. For most tumors, the functional consequences of structural centrosomal abnormalities are either reduced or enhanced microtubule nucleation capacity depends on the identity and modification state of the deregulated PCM components.

Chapter 2

Cdc14B, a primer

2.1 Introduction

The Cdc14 gene was first discovered in a genetic screen for genes required for the cell division cycle (CDC genes) in the budding yeast *S.cerevisiae* (Hartwell LH, 1974). From this screening, Cdc14 was characterized as an essential gene in the regulation of cell cycle events between metaphase and late telophase. Since its discovery, our understanding of the function of Cdc14 has been dramatically expanded. Cdc14 gene has been cloned in many different organisms and shown to be encoding a novel family of dual specificity phosphatases (D'Amours and Amon, 2004). Now we know that the dual specificity phosphatase Cdc14 is conserved from yeast to mammals. It functions in a variety of cell cycle events although its requirements vary from one species to another (Trinkle-Mulcahy and Lamond, 2006).

Although Cdc14 protein contains other sequences, its phosphatase activity was found to be indespensible for almost all of its known functions. Recent studies have unvealed many different functions of Cdc14 in different organism including mitotic exit, spindle assembly, rDNA resolution, centrosome seperation, embryonic division, microtubule bundling and etc. In spite of their similarities in biochemical properties, the functions of Cdc14-like proteins seem to vary dramatically between species. Next, I will discuss the

function of Cdc14 in different organisms in more detail.

2.2 Cdc14 in *Saccharomyces Cerevisiae*

Cdc14 is essential for the survival of budding yeast *Saccharomyces cerevisiae* since it is absolutely required for the cell to exit from mitosis. The mitotic functions of Cdc14 are associated with its ability to reverse CDK dependent phosphorylation events by dephosphorylating CDK targets. Changes in subcellular localization constitute an important mode of regulation for Cdc14 activity. For most of the cell cycle throughout G1, S phase, G2 and metaphase, Cdc14 is sequestered in the nucleolus by a competitive inhibitor Net1 (Also referred to as Cfi1). Net1p is a core component of the nucleolar RENT complex (Shou et al., 1999b; Straight et al., 1999; Visintin et al., 1999a) and has been proposed to function by occluding the active site of Cdc14, limiting its access to substrates. As a result, Cdc14 phospatase activity is suppressed (Traverso, E. E. et al, 2001). From early anaphase till the end of mitosis, Cdc14 starts to dissociate with Net1 and moves out from nucleolus to nucleoplasm and cytoplasm. The dissociation of Cdc14 is controlled at least by two different signaling pathways: Cdc fourteen early anaphase release (FEAR) and mitotic exit network (MEN) (Fig 2.1). At the onset of anaphase, FEAR pathway first initiates the release of a fraction of the total pool of Cdc14 from Net1 to the nucleoplasm as well as cytoplasm where Cdc14 is found both at the spindle pole body (SPBs, the yeast centrosomes) and mitotic spindle (Pereira et al., 2002; Pereira and Schiebel, 2003; Stegmeier et al., 2002; Yoshida et al., 2002). This process is thought

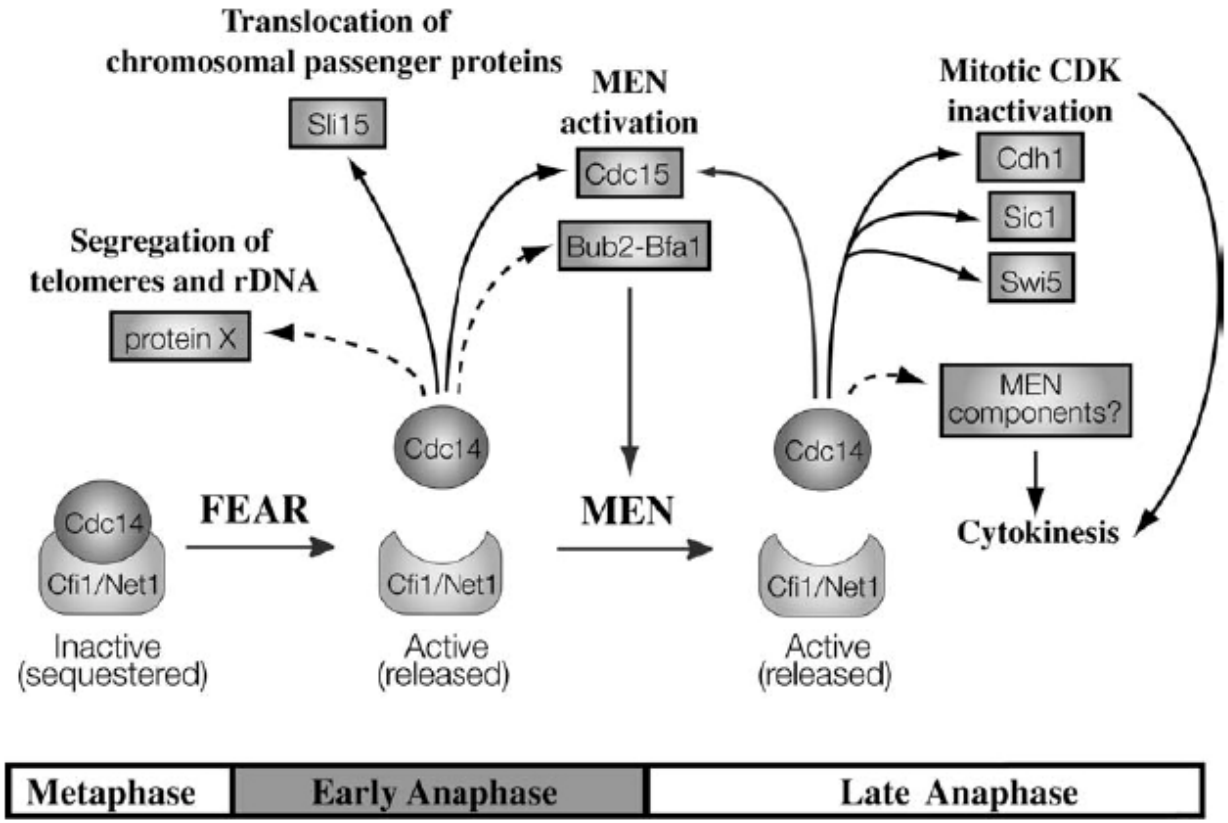


Figure 2.1: Substrates and functions of Cdc14 during mitosis. The known targets of Cdc14 in the regulation of these processes are shown in the gray boxes. Solid arrows indicate reasonably well-established Cdc14 targets; dashed arrows indicate more speculative ones. (Image courtesy of Frank Stegmeier and Angelika Amon, 2004)

to require phosphorylation of both Cdc14 and Net1 (Azzam et al., 2004; Visintin et al., 2003). After the initial release, Cdc14 would return to the nucleolus unless the MEN newtork is activated to sustain its release. As part of the feed forward mechanism, Cdc14 released by the FEAR network in early anaphase stimulates MEN activity, and Cdc14 released by the MEN further activates the MEN itself in a positive feedback loop. This two coupled processes result in the rapid release of Cdc14 from the nucleolus (Visintin et al., 2008). Once released, Cdc14 becomes active and antagonizes Cdk1 (also known as Cdc28p) activity by dephosphorylates Cdk1's substrates and promotes mitotic exit and cytokinesis.

In recent years, it has become clear that Cdc14 induces not only mitotic CDK inactivation and exit from mitosis but regulates a variety of other cellular events such as rDNA and telomere segregation, mitotic spindle dynamics, and cytokinesis. Remarkably, the execution of these diverse events relies on temporal Cdc14 activation mediated by the two different regulatory networks. Although both help the release of Cdc14 from its inhibitor, there are qualitative and quantitative differences between FEAR and MEN networks (Stegmeier and Amon, 2004). Cdc14 released by the FEAR network perform different functions during mitosis than Cdc14 released by the MEN.

2.2.1 Mitotic Exit Network

The MEN network was the first signaling network shown to activate Cdc14 by regulating its subcellular localization (Shou et al., 1999a; Visintin et al., 1999b). The MEN resembles a Ras-like GTPase signaling cascade that consists of: A GTPase, Tem1p;

A putative Guannine-Nucleotide Exchange factor (GEF), Lte1p; A two-component GTPase activating protein (GAP), Bub2-Bfa1; Two protein kinases, Cdc15p and Dbf2p; A Dbf2-associated factor: Mob1; A scaffold protein, Nud1; and the protein phosphatase Cdc14 (Fig 2.2).

Tem1 functions close to the top of the MEN pathway. Its GTPase activity is negatively regulated by the GAP complex Bub2-Bfa1 and positively regulated by Lte1. The activated form of Tem1, which is likely to be the GTP-bound, is thought to transmit a signal to the protein kinase Cdc15 (Sohrmann et al., 1998). Cdc15, in turn, activates the protein kinase Dbf2, which acts together with the Dbf2-associated factor Mob1 to activate MEN (Mah AS, 2001). Mutation in any of these components can prevent exit from mitosis with cells arresting in late anaphase/telophase.

Temporal and spatial regulation of the components of the MEN controls its activation at the end of mitosis. Notably, Lte1 localized in the bud cortex (Bardin et al., 2000; Pereira et al., 2000) while other components of MEN were anchored by the scaffold protein Nud1 at spindle pole body destined to migrate into the daughter cell when the spindle elongates during nuclear division (Ulrike Gruneberg, 2000). The close spatial association between Tem1 and Bub2–Bfa1 maintains Tem1 in its inactive GDP-bound form; the spatial separation between Tem1 and Lte1 prevents premature activation of MEN. Thus the onset of mitotic exit controlled by MEN won't happen until the anaphase spindle is properly oriented and nuclear segregation is achieved.

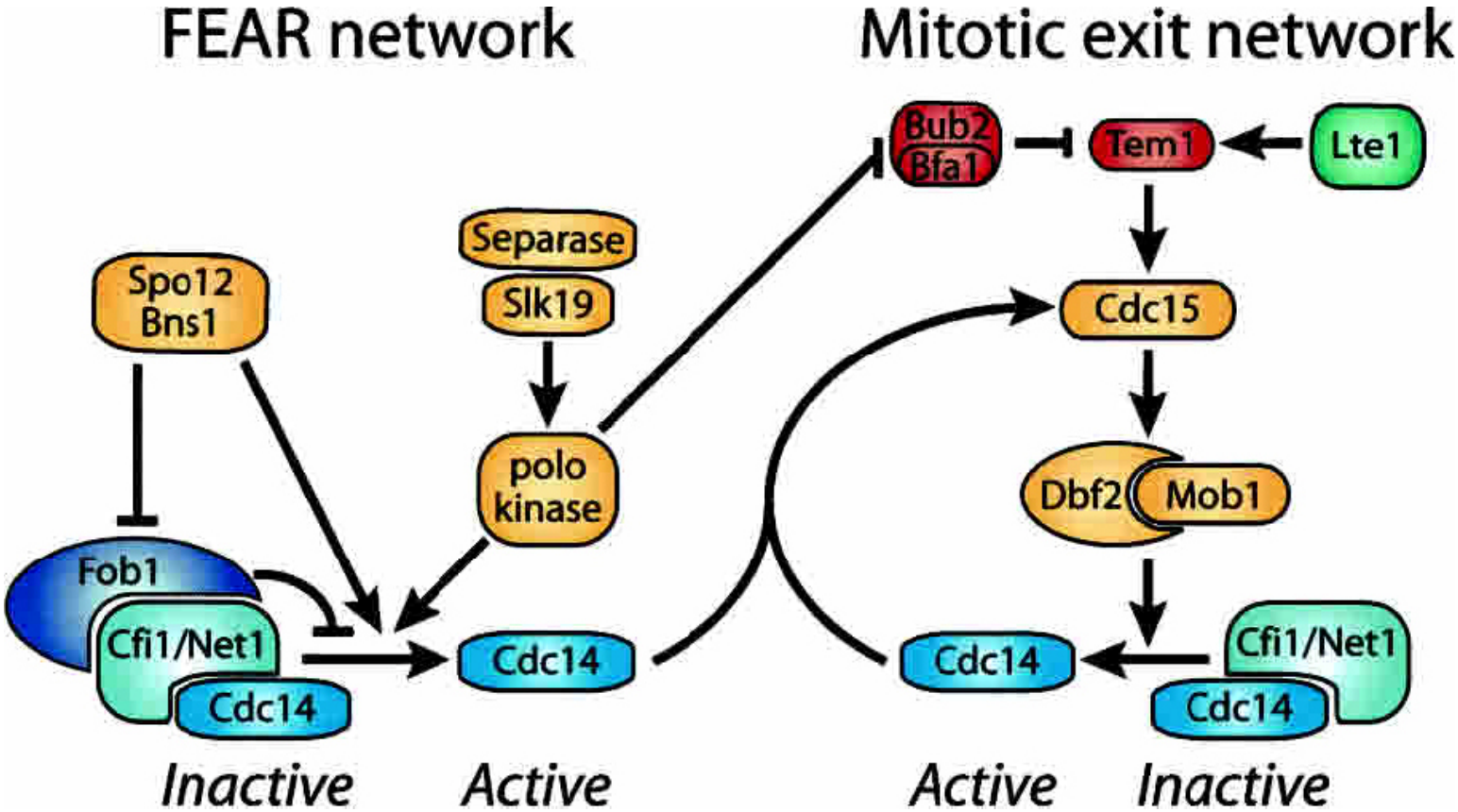


Figure 2.2: The FEAR and MEN networks. The ultimate function of these two networks is to activate Cdc14 by releasing it from its inhibitor Net1/Cfi1. Lines ending with an arrowhead indicate the stimulation of the downstream effector, whereas lines ending with a perpendicular bar indicate the inhibition of the target protein. (Image courtesy of Damien D'Amours and Angelika Amon, 2004)

Cdc14 activated by the MEN network is mainly responsible for promoting exit from mitosis. Several observations, however, indicate that Cdc14 and the MEN also regulate cytokinesis independently of their mitotic exit function.

2.2.2 FEAR network

Recent studies revealed the existence of an additional pathway regulating Cdc14 during early anaphase. This came from observations that Cdc14 was still transiently released from the nucleolus in cells when MEN activity is nonexistent (Pereira et al., 2002; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Yoshida et al., 2002). Factors involved in this transient release of Cdc14 were subsequently identified and are collectively referred to as the FEAR network (Stegmeier et al., 2002).

The known FEAR network to date is consisted of five positive factors including a Seperase (Esp1 in yeast), the kinetochore/spindle protein Slk19, Spo12 and its close homolog Bns1and the polo kinase Cdc5 (Stegmeier et al., 2002; Visintin et al., 2003); and two negative regulators Securin (Pds1 in yeast), an inhibitor of Separase, and the nucleolar protein Fob1 (Cohen-Fix and Koshland, 1999; Stegmeier and Amon, 2004; Sullivan and Uhlmann, 2003; Tinker-Kulberg and Morgan, 1999) (Fig 2.2).

Esp1 encodes a protease that is important for sister chromatid separation. This protease acts by cleaving a component of cohesion which holds sister chromatids together thereby enabling chromosome segregation (Nasmyth, 2002). As a component of the FEAR network, Esp1 is also responsible for Cdc14 release from the nucleolus. Surprisingly, its

protease activity is not required (Sullivan and Uhlmann, 2003). In agreement with this, the cleavage of Slk19, another FEAR network component, at the metaphase to anaphase transition by Esp1 is also not required for Cdc14 release from the nucleolus and its mitotic exit function (Stegmeier et al., 2002). And now we know that in the context of FEAR network Slk19 functions together with Esp1 rather than be controlled by Esp1 through cleavage. Slk19 is in a complex with Esp1 and is required for targeting Esp1 to kinetochores and the spindle midzone (Sullivan et al., 2001), and this is important for Cdc14 release from the nucleolus in early anaphase. Importantly, both the protease-dependent and protease-independent functions of Esp1 are inhibited by the securin Pds1 (Sullivan and Uhlmann, 2003). Therefore, Pds1 is the common negative regulator for prohibiting both the onset of sister chromatid separation and FEAR network-mediated Cdc14 release.

Most strikingly, Spo12, a protein with no known biochemical function, was also identified as a component of the FEAR network (Jensen et al., 2002b). Spo12 is a nucleolar phosphoprotein that is found to physically interact with Fob1, an inhibitor of the FEAR network which localizes to the same rDNA region and binds directly with Net1 to prevent release of Cdc14 (Iwabuchi M, 2000; Stegmeier et al., 2004). At onset of anaphase, phosphorylation of Spo12 can potentially trigger a conformational change in Fob1, which in turn will disrupt the interaction between Cdc14 and its inhibitor Net1, thus rendering Cdc14 release from nucleolar (Stegmeier et al., 2004).

The polo kinase Cdc5 acts at more than one stage to regulate mitotic exit. It is both a component of the FEAR network and a key regulator of the MEN, as a consequence, Cdc5 mutant have Cdc14 sequestered in the nucleolus at all times (Geymonat et al., 2003; Hu F, 2002; Hu et al., 2001a; Pereira et al., 2002; Stegmeier et al., 2002). Cdc5 effects on the MEN network at both upstream and downstream of Tem1. In later stage of mitosis when MEN network is active, Cdc5 contributes to Cdc14 release from the nucleolus by phosphorylating the Tem1 inhibitor complex Bub2-Bfa1 (Hu et al., 2001a). Also, Cdc5 promotes Dbf2 kinase activation. The function of Cdc5 in the FEAR network, however, is inconclusive. Recent studies suggest that Cdc5's kinase activity is required for FEAR network function since it can promote the phosphorylation of both Net1 thereby reducing its affinity for Cdc14 (Shou et al., 2002; Visintin et al., 2003; Yoshida and Toh-e, 2002).

Information regarding the functional relationship among FEAR network components is rather limited. According to a recent model, the FEAR network can be divided into two sub-branches: One branch with Esp1, Slk19, and Cdc5; the other includes Spo12, Bns1 and Fob1. Both branches are required to promote Cdc14 release from its inhibitor during early anaphase although their modes of action may differ.

Although Cdc14 transiently released by the FEAR network is not essential for cell survival, it helps to regulate several mitotic events: It stimulates MEN activity through either dephosphorylation of Cdc15 or inactivation of the Bub2-Bfa1 complex (Jaspersen and Morgan, 2000; Stegmeier et al., 2002); It regulates the subcellular localization of chromosomal passenger proteins, which possibly contributes to the stabilization of the

mitotic spindle during early anaphase (Pereira and Schiebel, 2003); It is also important for division of nucleoli and chromosome segregation, specifically in the resolution of highly repetitive rDNA and telomere regions (D'Amours and Amon, 2004). In addition, FEAR network components and Cdc14 are also required for the maintenance of genomic integrity and viability (D'Amours and Amon, 2004; Hartwell and Smith, 1985).

2.2.3 Inactivation of the MEN and FEAR Network

The inactivation and re-sequester of Cdc14 after mitotic exit has been completed is as important for successful cell division as its activation during anaphase. FEAR network activity appears to be limited to a very brief time during early anaphase, as Cdc14 re-enters into the nucleolus during late anaphase in cells lacking a functional MEN network (Pereira et al., 2002; Stegmeier et al., 2002; Yoshida et al., 2002). The inactivation of the MEN network is accomplished in multiple ways: Cdc14 dephosphorylates both Bfa1 and Lte1, which potentially restore the GAP activity of Bub2-Bfa1 toward Tem1 resulting in the release of Lte1 from the bud cortex (Geymonat et al., 2003; Hu et al., 2001b; Jensen et al., 2002a; Pereira et al., 2002; Seshan et al., 2002); The decreased concentration of Lte1 in the bud prevents Tem1 activation (Bardin et al., 2000; Jensen et al., 2002a; Seshan et al., 2002); Moreover, the released Cdc14 activates APC/C^{Cdh1}, which targets the MEN activator Cdc5 for degradation (Charles et al., 1998; Cheng et al., 1998; Shirayama M, 1998); Finally, Amn1, expressed specifically in daughter cells following Cdc14 activation, is thought to inhibit MEN function by competing Cdc15 for Tem1 binding (Wang et al., 2003).

2.2 Cdc14 in *Schizosaccharomyces Pombe*

In fission yeast *Schizosaccharomyces pombe* the Cdc14 homologue Clp1 (cdc fourteen-like phophatase, also called flp1) is also regulated by cell cycle dependent changes in its subcellular localization. It functions primarily to regulate the G2/M transition and cytokinesis through inactivation of mitotic CDKs (Cueille et al., 2001; Trautmann et al., 2001). It achieves the former by inactivating another phosphatase, Cdc25 and the latter through septation initiation network (SIN), a signaling pathway homologous to the MEN network and is essential for cytokinesis in fission yeast (Fig 2.3).

Like budding yeast cdc14, Clp1/Flp1 is largely sequestered in the nucleolus except some of it also detected at spindle pole bodies during interphase. Unlike its counterpart in budding yeast, Clp1/Flp1 is released from the nucleolus in early mitosis (prophase) and spreads throughout the nucleoplasm, kinetochores, the mitotic spindle, and the medial actomycin ring, a highly dynamic structure that controls the ingression of the division plane (Clifford et al., 2008; Trautmann et al., 2004).

The cellular mechanism for release of Clp1/Flp1 during prophase remains to be clarified. Although the initial release of Clp1/Flp1 from the nucleolus at mitotic entry is not dependent on SIN, this signaling network is required to maintain Clp1/Flp1 in its released state when the cytokinesis checkpoint has been activated (Cueille et al., 2001; Trautmann et al., 2001). Therefore, maintenance of budding yeast Cdc14 and Clp1/Flp1 in its

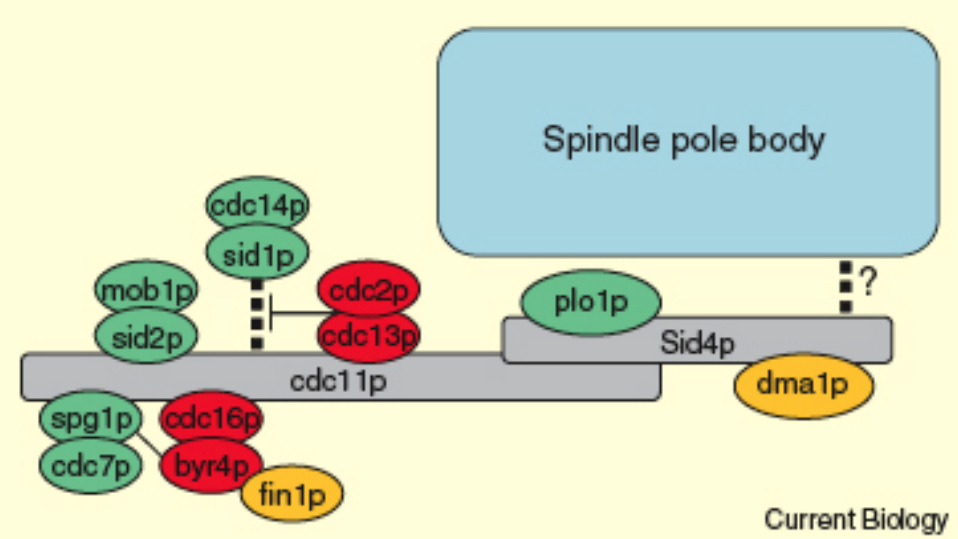


Figure 2.3: The SIN pathway in *S. pombe.* (Image courtesy of Andrea Krapp *et al*., 2004)

released state shares conserved pathways. Interestingly, at the end of the mitosis, the return of Clp1/Flp1 to the nucleolus is dependent on SIN network. In addition, a recent study showed that under replication stress, Cds1, a checkpoint kinase, triggered nuclear accumulation of the fission yeast Cdc14 phosphatase homologue (Diaz-Cuervo and Bueno, 2008).

Although might not be the only mechanism of regulation, the changes in subcellular localization of Clp1/Flp1 are likely to contribute to its activation. Once released, like Cdc14 in budding yeast, Clp1/Flp1 antagonizes mitotic CDK (Cdc2p in fission yeast) activity. However, the mechanisms behind mitotic CDK inactivation and the cellular processes regulated by Clp1/Flp1 and Cdc14 in budding yeast appear to be quite different (Stegmeier and Amon, 2004). Unlike Cdc14, Clp1/Flp1 is not essential for mitotic exit but predominantly counteracts mitotic CDK activity during G2/M transition. In budding yeast, Cdc14 is responsible for dephosphorylating and activating both Cdh1 and Sic1. Cdh1 is the APC/C (an E3 ubiquitin ligase) activator and Sic1 is the Cdk1 inhibitor. As a result, the mitotic CDKs are targeted for destruction by activated APC/C^{Cdh1} while their activities inhibited by activated Sic1; In fission yeast, the Cdc14 homolog Clp1/Flp1, however, antaganizes mitotic CDK activity by promoting inhibitory phosphorylation of a conserved tyrosine residue Y15 on the fission yeast Cdk, Cdc2 (Trautmann et al., 2001). Clp1/Flp1 promotes this inhibitory tyrosine phosphorylation by activating the protein kinase Wee1, which in turn phosphorylates Cdc2, and by dephosphorylating, destabilizing, and inactivating the Cdc25 phosphatase, which dephosphorylates the same site on Cdc2 (Wolfe BA, 2004). At mitotic entry, Cdc2 participates in its own regulation

by activating the mitotic inducing phosphatase Cdc25, and inhibiting the opposing kinase, Wee1. By disrupting this Cdc2 positive feedback loop, Clp1 contributes to Cdc2 inactivation.

Recent data suggests that the Clp1's activity during mitosis subjects to be regulated by the mitotic kinase Cdc2 (Wolfe et al., 2006). When phosphorylated, Clp1's phosphatase activity is attenuated. The full activation of Clp1 was proposed to occur in two consecutive steps: first, upon mitotic entry, Clp1 is released from the nucleolus and localizes to the kinetochores, the mitotic spindle, and the medial actomycin ring. At this stage, some Clp1 phosphatase activity must exist since it is required at kinetochores for monitoring chromosome biorientation (Trautmann et al., 2004); As Cdc2 activity declines during anaphase progression, autodephosphorylation of Clp1 reverses the Cdc2 mediated inhibitory phosphorylation and lead to full activation of Clp1. Activated Clp1 in turn will dephosphorylate other Cdc2 substrate(s).

2.4 Cdc14 Homologues in Metazoans

Homologues of Cdc14 exist in the majority of all eukaryotes and have been characterized at least to some extent in *C.elegans, Xenopus*, *Drosophila* and human. However, their functions and regulations are not well understood.

In the nematode *Caenorhabditis elegans*, CeCdc14 localizes to the central spindle and the midbody, similar to Clp1/Flp1 in *S. pombe*, but the phosphatase was found absent from

the nucleolus and centrosomes (Stegmeier and Amon, 2004). According to one study, CeCdc14 is also required for cytokinesis but not mitotic exit (Gruneberg et al., 2002). In this study, Gruneberg *et al* showed depletion of Cdc14 using RNA interference caused a striking mitotic phenotype: the failure in cytokinesis and embryonic lethality (Kipreos, 2004). The observed phenotype is in part due to the complete loss of the central spindle. CeCdc14 can potentially dephosphorylate Zen4, a mitotic kinesin-like protein that is essential for central spindle formation in *C.elegans.* This dephosphorylation is required for Zen4 to localize to the central spindle and perform its function (Kipreos, 2004). In contrast, however, a recent study by Saito *et al* reached the opposite conclusion (Saito et al., 2004). In this study, mitotic phenotype and embryonic lethality are not observed in both CeCdc14 null worms and depletion of CeCdc14 using RNAi approach (Saito et al., 2004). The different outcomes of these two studies are possibly due to off-target effect of RNAi and/or the strains used in two studies differ in their susceptibilities to loss of CeCdc14 (Kipreos, 2004). Surprisingly, the latter study revealed an additional unique role of CeCdc14 in G1 for preventing cell cycle re-entry during prolonged periods of developmentally regulated quiescence (Saito et al., 2004). Genetic and biochemical analyses suggest that Cdc14 functions upstream of the CDK inhibitor CKI-1 and prevent extra cell divisions during periods of cell quiescence by stabilizing CKI-1(Saito et al., 2004).

Cdc14 homologs in *Xenopus* and *Drosophila* haven't been studied in detail. The *Xenopus* homologs of cell cycle regulator phosphatase include XCdc14α and XCdc14β and both are closely related to human Cdc14A. XCdc14α/β are both localized to nucleoli and the

centrosome in interphase, and concentrated at the mitotic centrosomes. XCdc14α/β maybe required for normal cell division since injection of antibodies raised against XCdc14α/β into Xenopus embryos blocks cell division (Kaiser et al., 2004). In addition, a recent study suggested that XCdc14α potentially is also involved in regulating G2/M transition through dephosphorylation of XCdc25 and abscission (Krasinska et al., 2007). In the case of *Drosophila*, a recent overexpression screen revealed Cdc14's possible role in cytokinesis.

Human cells express two homologs of Cdc14, termed Cdc14A and Cdc14B that are both functional homologs of yeast Cdc14 (Li et al., 1997b; Vazquez-Novelle et al., 2005a). After more than a decade of research, however, the functions of these phosphatases in cell cycle progression remain poorly understood. Although Cdc14A and Cdc14B share about 50% sequence identity, they show two notable differences: Cdc14B has a unique 54 amino acid N terminal extension; Cdc14A and Cdc14B differ also in their C terminal domains.

Until recently, most studies focused on Cdc14A. The Cdc14A gene is located on band 1p21 on chromosome 1 and consists of 16 exons spread over 170 kbp of DNA (Paulsen et al., 2006). Cdc14A can rescue Cdc14 mutants in both *S. cerevisiae* and *S. pombe* suggesting that human Cdc14A and yeast Cdc14s share some conserved functions (Li et al., 1997c; Vazquez-Novelle et al., 2005b).

Human Cdc14A localizes dynamically to the interphase centrosomes while abscent from

mitotic centrosomes suggesting a role for Cdc14A in centrosome cycle control. The evidence for Cdc14A's centrosomal function comes from the observation that overexpression of Cdc14A resulted in premature centrosome splitting, while depletion of Cdc14A using siRNA techniques led to a delay in centrosome separation (Kaiser et al., 2002b; Mailand et al., 2002c). The physiological substrate(s) of Cdc14A in centrosome cycle control hasn't been identified; however, one of the potential candidates is Nek2 kinase. Overexpression of Nek2 kinase can also cause premature centrosome splitting similar to that observed in cells with deregulated Cdc14A. Potentially, Cdc14A can activate Nek2, which in turn promote centrosome splitting at mitotic entry (Kaiser et al., 2002b).

Besides its centrosome function, Cdc14A is also suggested being involved in cytokinesis regulation since during mitosis, it concentrates at the spindle midzone and the midbody (Gruneberg et al., 2004; Kaiser et al., 2002b). In consistant with this, downregulation of endogenous Cdc14A using siRNA prevented cells from undergoing cytokinesis (Mailand et al., 2002c).

In addition, Cdc14A has been implicated in participating in other functions of cellular regulations: Cdc14A was shown to dephosphorylate human APC cofactor Cdh1 and activate APC/C^{Cdh1}. When activated, APC/C^{Cdh1} in turn will promote ubiquitination of mitotic cyclins, allowing the mitotic exit to happen (Bembenek and Yu, 2001); Also, Cdc14 is capable of dephosphorylating the INCENP protein *in vitro*, suggesting its role in the regulation of the translocation of chromosomal passenger proteins in mammals

(Gruneberg et al., 2004); Moreover, Cdc14A was shown to interact with the tumor suppressor p53 and dephosphorylate the Ser315 site of p53 which phosphorylated by Cdk2/Cyclin-A and aurora kinase A, implying that Cdc14A may act as an counterbalancing phosphatase for those kinases (Li et al., 2000; Paulsen et al., 2006); Last but not the least, Cdc14A can also reverse Cdk1 phosphorylation of Cdc25A on Serines 115 and 320, suggesting its role in the cell cyle regulation of Cdc25A stability (Esteban V, 2006).

In contrast to Cdc14A, much less is known about the function of human Cdc14B. Previous studies have shown that the localization pattern of Cdc14B differs greatly from Cdc14A: During interphase, majority of Cdc14B localizes to nucleoli while a small portion of it found at centrosomes; During mitosis, Cdc14B was found to localize throughout the nucleous and cytoplasm and associate with the equator of central spindles in anaphase and concentrated at the center of midbody during telophase and cytokinesis (Cho et al., 2005b). The dramatic differences between their localization profiles suggest that Cdc14A and Cdc14B perform different tasks in the cell.

Several Cdc14B related functions have been suggested: Our lab has previously demonstrated that Cdc14B is a microtubule binding and bundling protein. Ectopic expression of cytoplasmic Cdc14B leads to microtubule bundling and stabilization in interphase cells, and impairs microtubule dynamics from microtubule organization centers (Cho et al., 2005b). Intriguingly, this function of Cdc14B doesn't rely on its phosphatase acvitity.

Cdc14B has also been implicated in the maintenance of nuclear architecture. More than 30 years the hypothetical nuclear matrix or karyoskeleton has been baffling the science community. The proposed function of this filamentous structure within the nucleus is to regulate nuclear structure and function. Nalepa *et al* showed in their studies that Cdc14B phosphatase was tightly associated with the filamentous structure stretching from the nucleolar periphery to nuclear envelope. This association was dependent on a bipartite signal on Cdc14B that directed it to the intranuclear filaments. Therefore, Cdc14B may serve as a marker of intrinsic nuclear scaffold and Cdc14B-labeled filaments may represent a physiological relevant nuclear structure that is critical for the maitenance of proper nuclear structure (Grzegorz Nalepa, 2004).

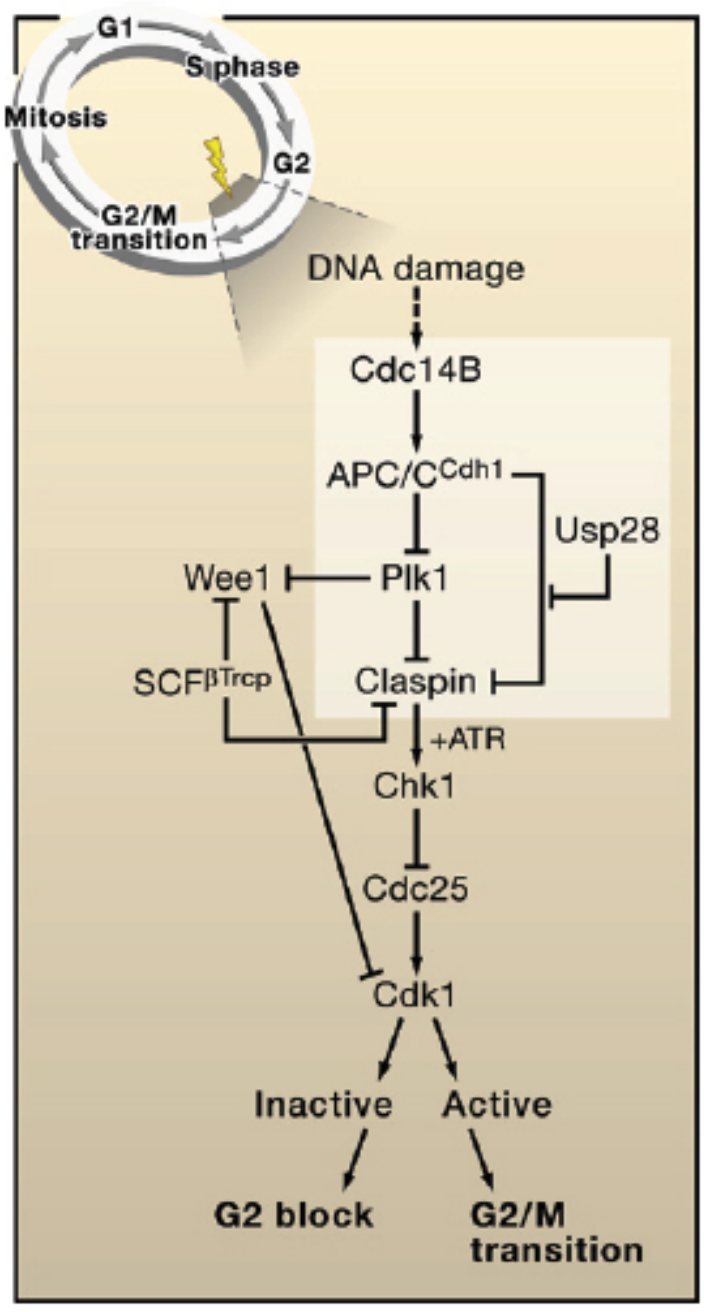
Another possible albeit controversial function of human Cdc14B is in the regulation of mitotic exit. According to one study, Cdc14B but not its homolog Cdc14A provokes mitotic exit by dephosphorylating Sirt2 which targets Sirt2 for degradation by the 26S proteasome (Dryden et al., 2003a); However, another study showed both Cdc14B and Cdc14A can dephosphorylate Sirt2 on serine 368, a site targeted by Cdk1 kinase. And cellular proliferation is dependent on phosphorylation status of this particular site. However, in this study, degradation of Sirt2 by the 26S proteasome in response to Cdc14B overexpression wasn't observed. The differences between these two studies may due to different cell lines used (North and Verdin, 2007); More interestingly, a recent study reached a completely opposite conclusion that Cdc14B is dispensable for mitotic exit in human cells. In their study, Berdougo *et al* homozygously disrupted the Cdc14B locus in human somatic cells. Surprisingly, Cdc14B null cells were viable and lacked

defects in mitotic exit (Berdougo E, 2008). A possible explanation for this discrepancy is that Cdc14B's function in mitotic exit regulation may be partially complemented by Cdc14A (Berdougo E, 2008).

In addition, Cdc14B was shown to regulate S phase entry or G1 length in an Skp2-dependent manner (Rodier G, 2008). Skp2 is the $p27^{Kip1}$ ubiquitin ligase receptor which targets p27 for degradation. The activity of SCF^{Skp2} is regulated by the APC/C^{Cdh1}. Cdc14B, but not Cdc14A, can dephosphorylate Skp2 on serine 64 and promote the degradation of Skp2 by APC/C^{Cdh1} at the M to G1 transition. Also, depletion of Cdc14B stabilizes Skp2 in HeLa cells and accelerates the progression from mitosis to S phase, implicating Cdc14B's role in regulating G1 length (Rodier G, 2008).

Last but not least, a most recent study revealed a surprising role of Cdc14B in DNA damage induced G2 checkpoint control (Fig 2.4). In this elegant study, Bassermann *et al* demonstrated that a network of proteins regulating mitotic exit in budding yeast had been conserved in human but rewired to serve a different purpose which is G2 checkpoint control in response to DNA damage (De Wulf and Visintin, 2008). Majority of Cdc14B protein is sequestered in the nucleolus during interphase. Upon DNA damage, Cdc14B was shown to translocate to the nucleoplasm, where it physically interacted and activated APC/C^{Cdh1} by reversing the CDK-mediated phosphorylation of Cdh1 (Bassermann et al., 2008). In turn, activated APC/C^{Cdh1} promoted degradation of Plk1 which resulted in the stablilization of Claspin, an activator of the DNA damage induced G2 checkpoint.

Figure 2.4: The cell cycle and G2 DNA damage response in human Cells. DNA damage during the cell cycle triggers the DNA-damage checkpoint and results in a block of the cell cycle (right) at G2 via the inhibition of mitotic cyclin-dependent kinase (Cdk1) activity. Bassermann et al. find a new regulatory pathway of the G2 DNA-damage checkpoint (shaded box) in which the phosphatase Cdc14B activates the ubiquitin ligase anaphase-promoting complex or cyclosome (APC/C^{Cdh1}) to degrade the Polo-like kinase 1 (Plk1) and arrest the cell cycle. Plk1 is required for cell-cycle reentry as its kinase activity triggers inactivation (by the ubiquitin ligase $SCF^{\beta TrCP}$) of Cdk1 inhibitors such as Wee1 and Claspin, an adaptor protein required by the kinase ATR to maintain Chk1 inhibition of Cdc25, an activator of Cdk1. During the G2 cell-cycle arrest, APC/CCdh1 specifically degrades only Plk1 and not other substrates such as Claspin due to the action of the deubiquitinating enzyme Usp28. Upon DNA repair, Cdk1 is reactivated to allow cell-cycle progression from G2 to mitosis. (Image courtesy of Peter De Wulf and Rosella Visintin, 2008)


G1
S phase
Mitosis
G2
G2/M transition
DNA damage
Cdc14B
APC/C Cdh1
Usp28
Wee1
Plk1
SCF βTrcp
Claspin
+ATR
Chk1
Cdc25
Cdk1
Inactive
Active
G2 block
G2/M transition

Although the functions of both human Cdc14B and Cdc14A phosphatases remain elusive, expression profile analysis using microarray approaches revealed that alteration of Cdc14A and Cdc14B expression were frequently observed in cancer cells, such as prostate cancer, breast cancer, MCL, and acute myeloid leukemia (Ashida et al., 2004b; Martinez et al., 2003b; Neben et al., 2005b; Yu et al., 2004a); Moreover, in response to anti-cancer drugs, Cdc14B was found to be down regulated in several cancer cell lines (di Pietro et al., 2003; Zhao et al., 2004); and low Cdc14B expression also correlated with tumor grade and bad prognosis, suggesting that Cdc14B may act as a tumor suppressor in certain tissues (Rodier G, 2008).

Chapter 3

Cdc14B associates with centriole in a cell cycle dependent manner

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3.1 Introduction

Normally, centrosomes are tiny, single copy non-membranous organelles located near the cell center – hence its name. In animal cells a typical centrosome consists of two main structures: A pair of mircrotubule-based cylindrical structures known as centrioles, which are surrounded by a fibrogranular matrix of protein aggregates termed peri-centriolar material (PCM).

Besides their major roles in supporting the centrosome structure and centrosome biogenesis, the two centrioles can also function as basal bodies for the assembly of cilia and flagella protruding from the surface of most vertebrate cells (Basto et al., 2006). In mammals, each centriole consists nine triplets of microtubules, which are arranged into a cylindrical structure that spans ~200 nm in diameter and ~500 nm in length, with additional filaments, fibers, and dense material attached to both the outside and inside of the microtubular cylinder (Doxsey, 2001); (Beisson and Wright, 2003). In addition to its major structural constituents: α-tubulin and β-tubulin, only a small number of proteins have been found to localize to the centriole in mamamalian cells which include but not limited to: centrin, ε-tubulin, centriolin, ninein, and poly (ADP-ribose) polymerase-3

(PARP-3) (Augustin et al., 2003; Chang and Stearns, 2000; Mogensen et al., 2000; Ou et al., 2002; Paoletti et al., 1996; Piel et al., 2000; Uzawa et al., 1995). In contrast, the PCM harbors hundreds of proteins that perform many diverse functions: involvement in multiple signaling pathways; regulation of different cellular processes; and of course its well-established role as the primary microtubule-organizing center. Among these proteins, γ-tubulin ring complexes (γ-TURCs) act as templates for nucleating microtubules (Moritz et al., 1995); peri-centrin scaffolds appear to be in contact with γ-TURCs and needed for microtubule anchorage (Dictenberg et al., 1998); Other proteins such as PCM-1 may be involved in microtubule anchoring via recruitment and assembly of subsets of centrosomal proteins (Dammermann and Merdes, 2002).

Numerous centrosome-associated proteins have been discovered and their "centrosomal" functions have been characterized. Many centrosomal proteins have been identified through genetic analysis or through the production of autoimmune sera and subsequent purification of centrosomal antibodies (Schatten, 2008). Also, the search for interacting partners of known centrosomal proteins with techniques such as yeast two-hybrid screenings, immunoprecipitation has expanded the list. Most recently, application of high resolution and high sensitivity mass spectrometers in biological research has empowered many groups to analyze protein composition of centrosomes in greater details (Andersen et al., 2003). Albeit promising, proteomic analysis of isolated centrosomes relies a great deal on the initial purification step that still constitutes a challenging goal. Even though centrosomes are by no means the smallest organelles within the cells, the purification is made difficult by: their low copy numbers; they make up only a small proportion of total

cell protein; they are not membrane-bound therefore don't have defined borders; and they often associate tightly with both the cytoskeleton and other subcellular organelles, notably the Golgi apparatus and the nucleus. The current centrosome isolation methods can easily generate false-negatives by removing centrosomal components or false-positives by including contaminants that are not centrosomal origin (Christopher J. Wilkinson, 2004; Schatten, 2008). In addition, the centrosome structure is highly dynamic in nature and undergoes dramatic structural alterations and compositional changes during the cell cycle. This is reflected by the appearance and disappearance of many centrosomal proteins at particular cell cycle stages. For example, at the G2/M transition when the centrosome undergoes maturation, the γ-TURCs is enriched at centrosomes while other proteins such as C-Nap1 will diminish at the onset of mitosis (Fry et al., 1998; Zheng et al., 1995).

Some structural proteins constitute the centrosome core struture. These proteins remain centrosomal bound even after treatment of the centrosome complex with microtubule depolymerizing agents such as cold, nocodazole, colchicine derivatives, and others (Schatten, 2008). Centrosome core proteins are permanently associated with the centrosome structure regardless of changes of different cell cycle stages. In contrast, other centrosomal proteins, mostly regulatory in nature, transiently associate with the core centrosome structure during different cell cycle stages to perform cell cycle-specific functions. These regulatory molecules typically use centrosome as a central docking station or platform, further complicating the analysis of determining the number of centrosome proteins (Schatten, 2008).

Despite these difficulties, many centrosomal proteins have been reported and the number of which varies depending on the methods applied. Mass spectroscopic analysis has identified as many as 500 centrosomal proteins (Andersen et al., 2003) while a survey of published literature suggests a more conservative number of around 100 proteins with about 60 being present in the interphase centrosomes (Christopher J. Wilkinson, 2004).

Cdc14B has been shown to associate with distinctive cellular structures such as the nucleolus, nuclear filament, centrosome, and the spindle midzone and midbody (Cho et al., 2005b; Grzegorz Nalepa, 2004; Kaiser et al., 2002b; Mailand et al., 2002a), and to undergo active nucleocytoplasmic translocation (Bembenek J, 2005). Yet, the functional significance of Cdc14B in centrosome cycle regulation has never been explored. Before further investigation of Cdc14B's centrosomal function, a more detailed localization study is needed to pinpoint where Cdc14B localizes with respect to centrioles/PCM and whether the centrosomal localization pattern differ in each cell cycle stage.

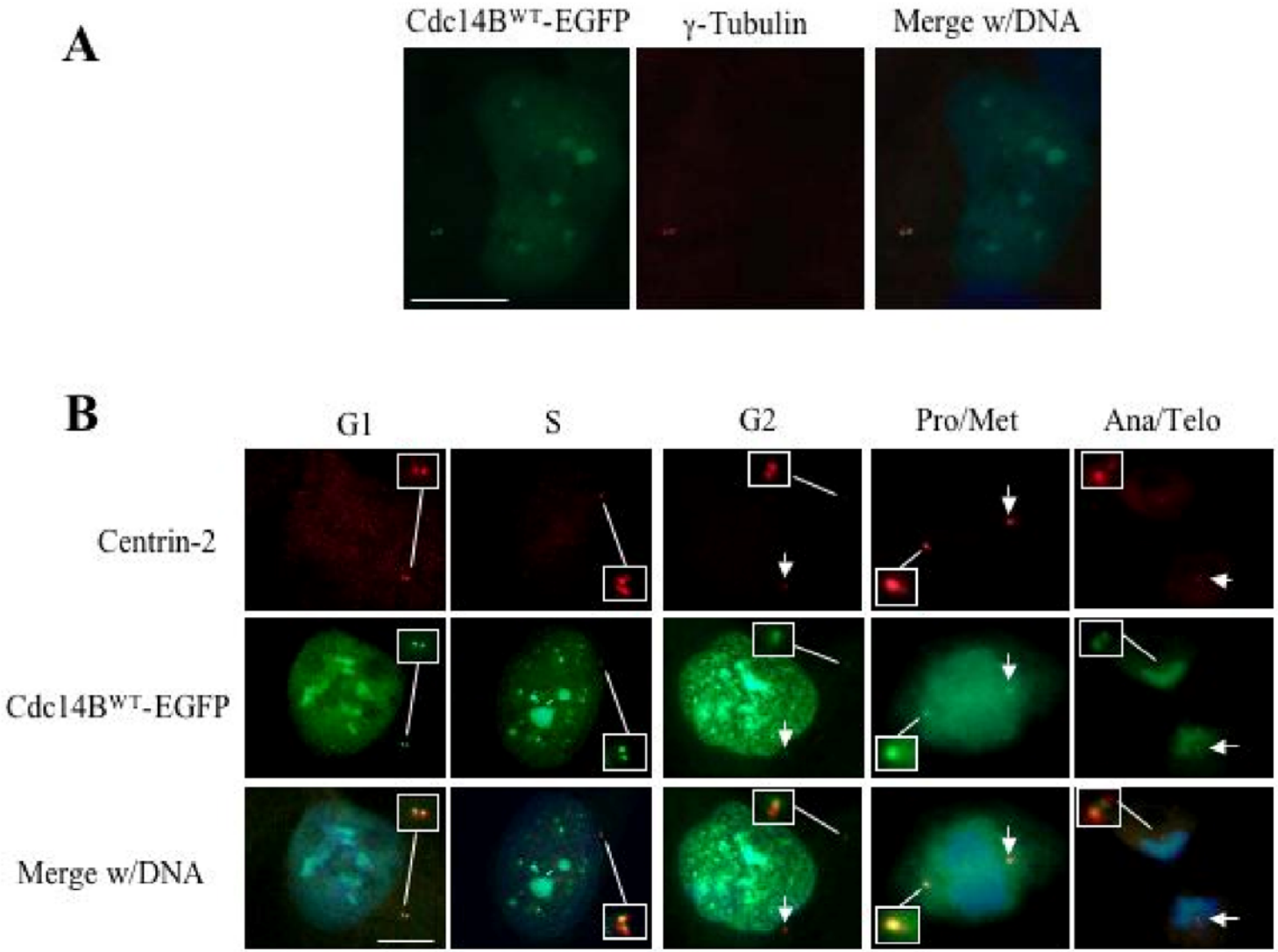
3.2 Results

3.2.1 Cdc14B associates with centriole in a cell cycle dependent manner

First, we isolated a full-lengh Cdc14B cDNA (isofrom 1) from the marathon human heart cDNA library (Clontech), fused it with an enhanced green fluorescent protein (EGFP) tag at the C-terminus and subcloned the Cdc14B^{WT}-EGFP construct into the pBI-tet vector (Clontech). The expression of Cdc14B^{WT}-EGFP is under the control of

a doxycycline-regulatable promoter. We then co-transfected pBI-tet-Cdc14B^{WT}-EGFP plasmid together with the pBabe-puro (a kind gift from Dr. Gerald Evans) into U2OS$^{Tet\text{-}on}$ cells (Clontech) to establish stable clones with the selection of both G418 and puromycin. The expression of Cdc14B^{WT}-EGFP in these stable U2OS$^{tet\text{-}on}$ stable clones can be turned on by the addition of doxycycline (Dox, a tetracycline derivative) to the culture medium. When induced, we found that Cdc14B^{WT}-EGFP is frequently present at centrosomes as judged by co-localization with γ-tubulin, a bona fide centrosome marker (Fig 3.1A). In order to distinguish whether Cdc14B^{WT}-EGFP localizes to centrioles or PCM, we studied the co-localization of Cdc14B^{WT}-EGFP with a mouse monoclonal Centrin-2 antibody used as the centriole marker. Centrin-2 antibody marks the distal ends of both centrioles and procentrioles and doesn't stain on PCM. By immunofluorescence analysis, we found that the Cdc14B^{WT}-EGFP partially co-localized with Centrin-2 throughout the cell cycle (Fig 3.1B), implying that Cdc14B^{WT}-EGFP localizes to the centriole instead of PCM. Also, we noted that the staining pattern of Cdc14B^{WT}-EGFP differed in cells that appeared to be at different stages of the cell cycle, suggesting the possibility that Cdc14B^{WT}-EGFP may differentially localize at either the mother or daughter centrioles. To further explore this possibility, we focused on the cells that are in S phase of the cell cycle when the daughter centriole can be easily distinguished from mother centriole by the size of Centrin-2 dots. And we found that the majority of cells exhibited only one strongly stained Cdc14B^{WT}-EGFP dot at each centrosome. Superimposing Centrin-2 and Cdc14B^{WT}-EGFP signal further demonstrated that the Cdc14B^{WT}-EGFP dot only partially overlap with the stronger/bigger Centrin-2 dot (mother centriole) while there is

Figure 3.1: A fraction of Cdc14B^{WT}-EGFP associates with centrioles. (A) Cdc14B^{WT}-EGFP localizes to centrosomes, Cdc14B^{WT}-EGFP (green) and γ-Tubulin (Red). B) Overlay images illustrate partial colocalization of Cdc14B^{WT}-EGFP (green) with Centrin-2 (red) in U2OS cells during different stages of the cell cycle. Stages of the cell cycle were determined by the Centrin-2-labeled centrioles as well as DAPI-stained DNA (blue). Arrows indicate the Centrin-2 and Cdc14B-GFP labeled centrioles. Magnified images of centrioles were shown in insets. We noticed that Cdc14B^{WT}-EGFP became diffused in mitotic cells and partially overlapped with DNA which might be due to Cdc14B^{WT}-EGFP overexpression. Bar, 5 μm.


A
Cdc14B^WT-EGFP
γ-Tubulin
Merge w/DNA
B
G1
S
G2
Pro/Met
Ana/Telo
Centrin-2
Cdc14B^WT-EGFP
Merge w/DNA

essentially no Cdc14B^{WT}-EGFP signal associated with the weaker/smaller Centrin-2 staining (daughter centriole) (Fig 3.1B). In addition, during G2, prophase and metaphase, Cdc14B^{WT}-EGFP seemingly only partially associates with the "stronger/bigger " Centrin-2 staining within each centrosome indicating that Cdc14B^{WT}-EGFP is preferentially associated with the mother centriole during the S/G2 and early M phases of the cell cycle.

Since Cdc14B^{WT}-EGFP and Centrin-2 staining only partially overlap with each other, we used a second centriole marker anti-C-Nap1antibody that marks the proximal end of centrioles (Mayor et al., 2000) to help potentially pinpoint where Cdc14B^{WT}-EGFP localizes on the centriole. As a result, we found that Cdc14B^{WT}-EGFP also partially co-localized with C-Nap1 staining, confirming that Cdc14B^{WT}-EGFP localizes to centrioles instead of PCM (Fig 3.2). This observation also implicates that Cdc14B^{WT}-EGFP localized to the centriole somewhere between Centrin-2 and C-Nap1 staining and places it somewhere in the middle of centriole. The observation that there was seemingly more overlap or shorter distance between Cdc14B^{WT}-EGFP and C-Nap1 staining compared to Cdc14B^{WT}-EGFP and Centrin-2 staining suggests the possibility that Cdc14B^{WT}-EGFP may localize more toward the proximal end of the centriole.

Also, the combination of immunostaining with both Centrin-2 and C-Nap1 antibodies can help distinguish engaged versus disengaged centrioles based on the numerical ratio between Centrin-2- and C-Nap1- marked centriole dots as illustrated in (Fig. 3.3). Each engaged centriole pair has two Centrin-2 dots while only one C-Nap1 dot (2:1 ratio of

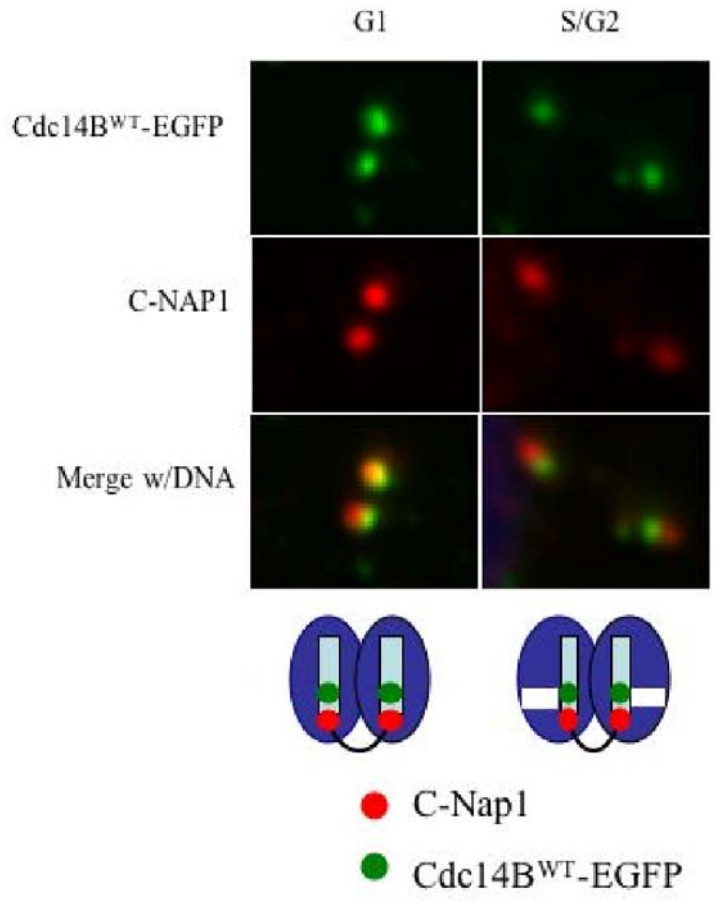


Figure 3.2: Colocalization of Cdc14B^{WT}-EGFP and C-Nap1. Partial colocalization of Cdc14B^{WT}-EGFP (green) with C-Nap1 (red) in U2OS cells. Schematics at the bottoms show potential localization of Cdc14B^{WT}-EGFP relative to C-Nap1 staining which marks the proximal end of the centriole.

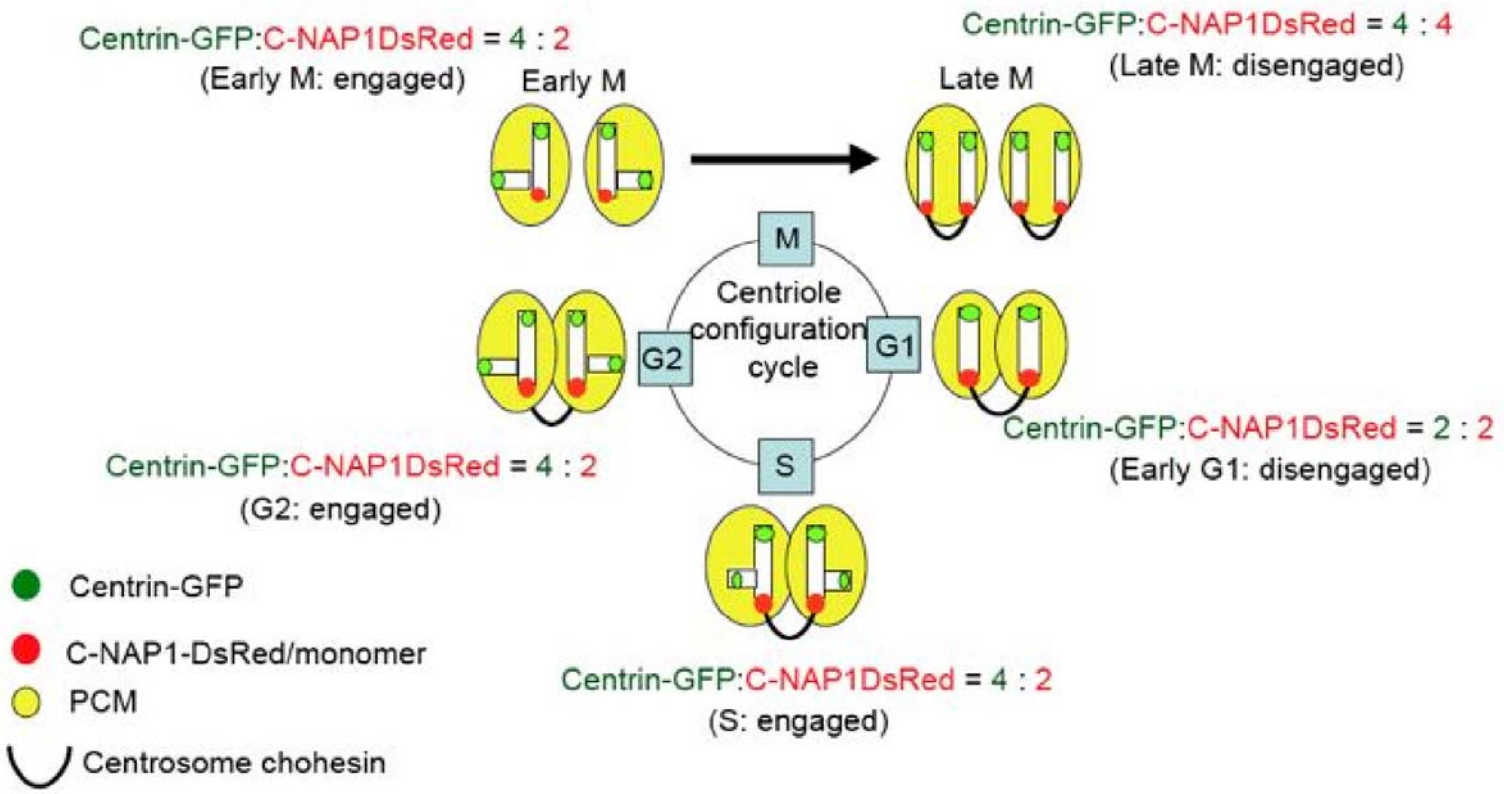


Figure 3.3: Centrin-GFP vs C-Nap1 DsRed during cell cycle. Schematics of centriole cycle depicting how to distinguish engaged centrioles (S, G2, early M) from disengaged centrioles (late M and early G1) based on the localization pattern of Centrin-GFP and C-NAP1-DsRed, and numerical ratio between Centrin-GFP and C-NAP1-DsRed dots.

Centrin-2: C-Nap1); Disenganged centriole pair has one Centrin-2 dot and one C-Nap1 dot per centriole (1:1 ratio of Centrin-2: C-Nap1) (Tsou and Stearns, 2006a). Just like C-Nap1, during the S, G2 and early M phases of the cell cycle when the centriole pair is in the engaged configuration, there is only one Cdc14B^{WT}-EGFP dot at each centrosome, and the numerical ratio between Centrin-2 staining and Cdc14B^{WT}-EGFP is 2:1; While during G1 and late M phases when the two centrioles within each centrosome are in disengaged conformation, there are two Cdc14B^{WT}-EGFP dots associated with each centrosome, and the ratio between Centrin-2 focus and Cdc14B^{WT}-EGFP is 1:1 (Fig 3.1).

To investigate whether a similar localization pattern on centrioles also exists for endogenous human Cdc14B, we performed immunoflurorescence studies with three different commercial available Cdc14B antibodies.

Although a rabbit anti-Cdc14B antibody from Sigma (Cho et al., 2005b) can specifically recognize Cdc14B in western blots, and stains on distinctive cellular structures such as the nucleolus, nuclear filament, the spindle midzone and midbody in agreement with previous reports (Cho et al., 2005b; Grzegorz Nalepa, 2004; Kaiser et al., 2002b; Mailand et al., 2002a), it failed to reveal the centriole-associated Cdc14B in our immunoflurorecence studies. We believe the reason is that this antibody might react with epitope(s) that were not exposed at centrioles under the fixation/extraction conditions tested. In fact, Nalepa *et al* previous reported that the centrosomal Cdc14B only became visible after 3 minutes with 0.5% Triton X-100 extraction prior to paraformaldehyde fixation using another rabbit anti-Cdc14B antibody (Grzegorz Nalepa, 2004).

Second, a mouse Cdc14B polyclonal antibody from Abnova was tested for immunostaining. Surprisingly, in three different fixation conditions tested (3.7% paraformaldehyde, 100% methanol or 0.5% glutaraldehyde), when using Centrin-2 as a centriole marker, Abnova Cdc14B antibody stains exclusively on the centrioles while not found on any other cellular structures as expected. Also, when this antibody was subjected to western analysis, it fails to recognize endogenous Cdc14B and even transfected exogenous Cdc14B (Data not shown). And when the specificity of this antibody was tested in cells where Cdc14B is depleted using siRNA approach, Abnova Cdc14B antibody still stains strongly on the centrioles in immunostaining (Data not shown), suggesting that the mouse polyclonal Cdc14B antibody from Abnova may detect another non-specific centrosomal component instead of Cdc14B. Therefore this antibody was not used in later studies.

Finally, a chicken anti-Cdc14B antibody from Geneway was used. After pre-extraction and methanol fixation of U2OS cells, immunofluorescence analysis revealed that this Cdc14B antibody partially co-localized with both Centrin-2- and C-Nap1- labeled centrioles in addition to nucleolus, nuclear filament, the spindle midzone, midbody and intercellular bridge reported using Zymed Cdc14B antibody (Fig 3.5 & Fig 3.6). Also, the staining pattern of Geneway Cdc14B antibody exactly matches $Cdc14B^{WT}$-EGFP as we have seen in Fig 3.1 and Fig 3.2. More importantly, when Cdc14B is depleted in the HeLa cells, the corresponding centrosomal signal of this antibody is significantly reduced, proving the specificity of this antibody for centriole-associated endogenous Cdc14B (Chapter 4, Fig 4.3). We therefore used this antibody for all our

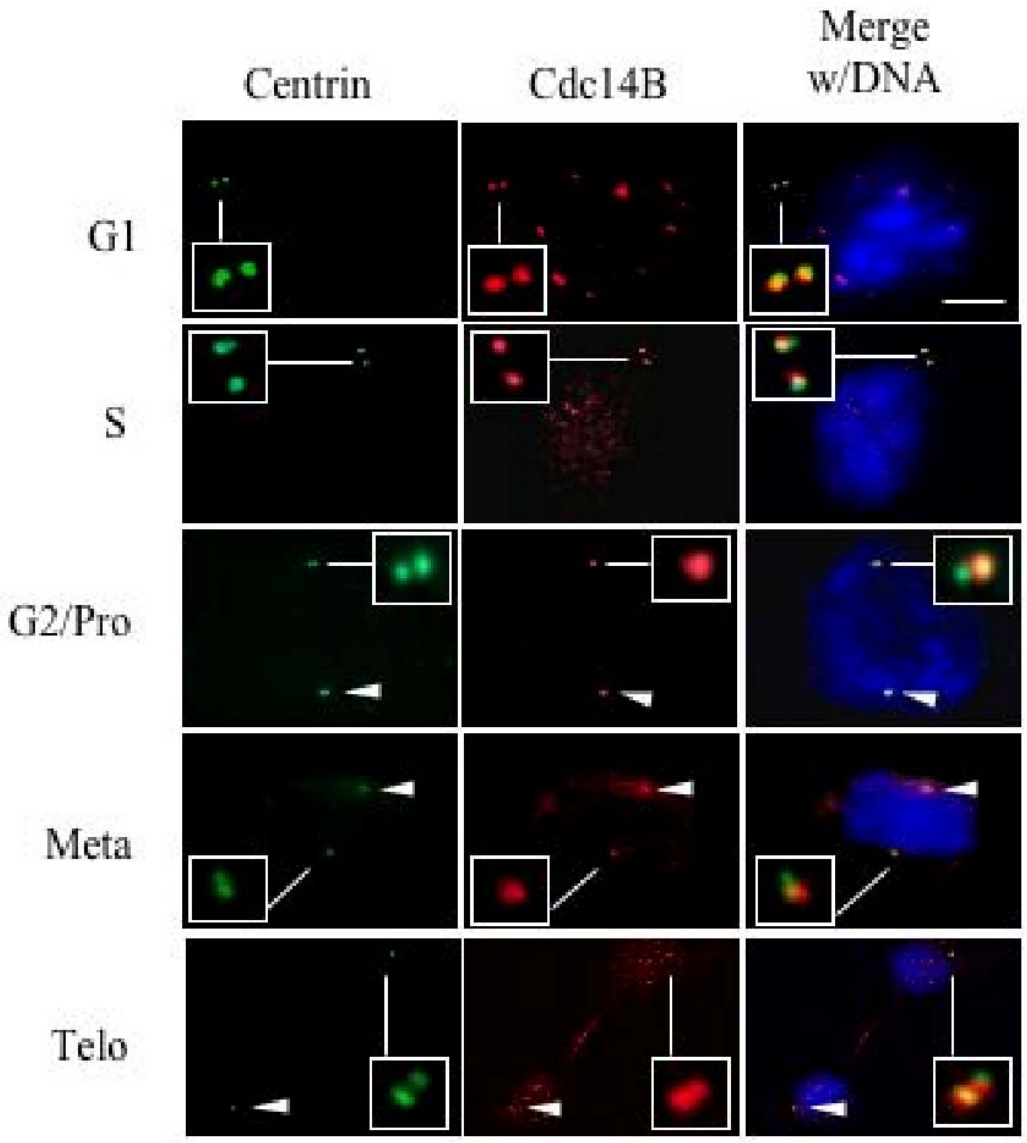


Figure 3.4: A fraction of Cdc14B associates with centrioles. Overlay images depict partial co-localization of endogenous Cdc14B (red) with centrin (green), a centriole marker in U2OS cells during different stages of the cell cycle. Stages of the cell cycle were determined by the centrin-labeled centrioles as well as DAPI-stained DNA (blue). Arrowheads indicate centrin and Cdc14B-GFP labeled centrioles. Magnified images of centrioles were shown in insets. Bar, 5 mm.

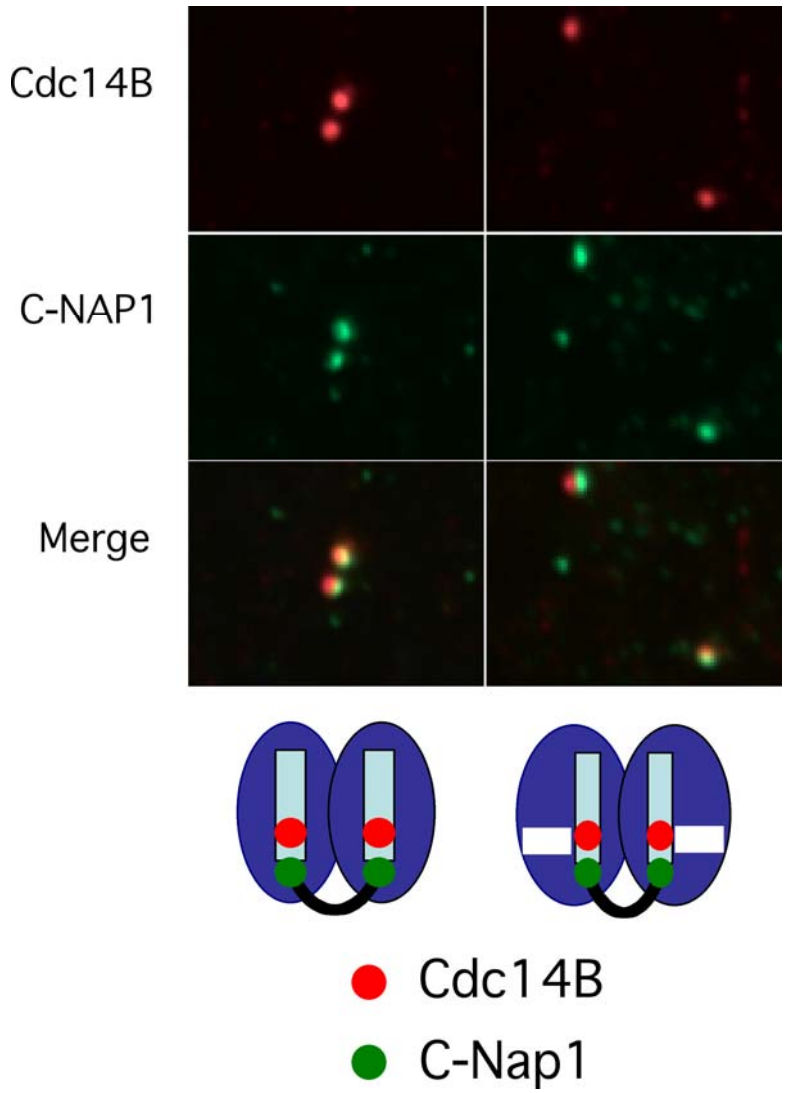


Figure 3.5: Colocalization of endogenous Cdc14B and C-Nap1. Partial colocalization of endogenous Cdc14B (Red) with C-Nap1 (Green) in U2OS cells. Schematics at the bottoms show potential localization of endogenous Cdc14B relative to C-Nap1 staining which situates at the proximal end of the centriole.

immunoflurorescence studies on endogenous Cdc14B.

To futher corroborate our observation that Cdc14B localizes to the centrosomes, we independently evaluate the centrosome association of Cdc14B with purified centrosome fractions from asynchronized HeLa cells by discontinuous sucrose gradient centrifugation according to a published protocol (Moudjou, 1998). Proteins in each fraction were resolved by SDS-PAGE and analyzed by Western blotting. At the expected 60% sucrose density, fractions 14 to 17 contained the most abundant amounts of γ-tubulin. In parallel, Cdc14B was highly enriched in those γ-tubulin containing centrosome fractions when probed with Zymed Cdc14B antibody (Fig 3.7A). Moreover, when a purified centrosome fraction (fraction 15) was subject to immunostaining, we found that Cdc14B (Geneway Cdc14B antibody) but not nucleolin, a noncentrosomal protein, stained positive at the purified centrosomes (Fig 3.7B).

Taken together, these data suggests that a fraction of both Cdc14B^{WT}-EGFP and endogenous Cdc14B specifically localize to the mother centrioles when the centriole pair is in the engaged configuration; in contrast, when the two centrioles are disengaged or disoriented, both Cdc14B^{WT}-EGFP and endogenous Cdc14B associate with each of the disoriented centrioles.

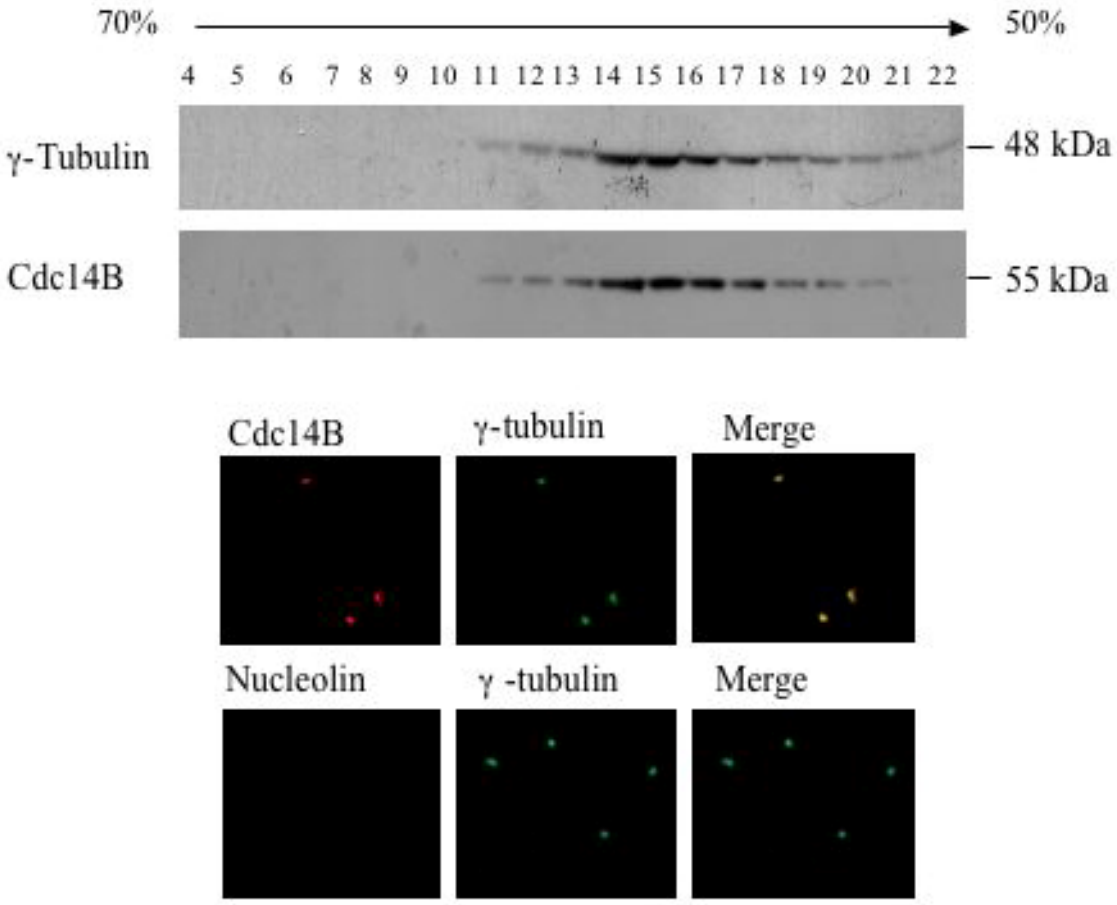


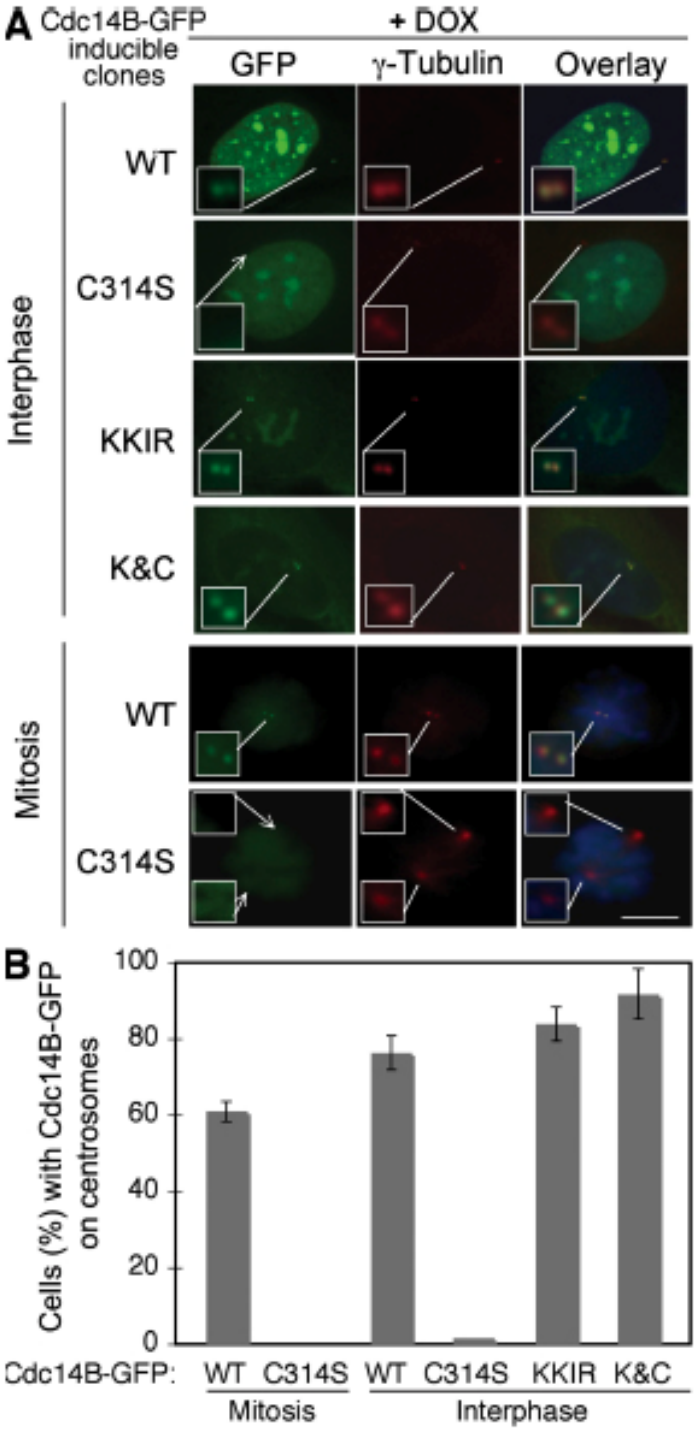
Figure 3.6: Isolated centrosomes contain Cdc14B. Top panel: Cdc14B co-fractionates with γ-tubulin. Lysates of asynchronized HeLa cells were fractionated on a 50 to 70% sucrose gradient. Proteins from fractions 4 to 22 were analyzed by western blot with γ-tubulin and Cdc14B antibodies. Bottom panel: Cdc14B but not nucleolin (a non-centrosomal protein) co-stains with γ-tubulin on purified centrosomes (fraction 15 of top panel).

3.2.2 Cdc14B's phosphatase activity is indispensable for targeting Cdc14B to centrioles.

In yeast, Cdc14's phosphatase activity is required in almost all its known functions. Thus, it's tempting to test whether catalytic activity is also required for targeting human Cdc14B to centrosomes. In a previous study we generated a construct harboring a phosphatase-dead mutant of Cdc14B by mutating the critical cysteine residue within the active site to serine (C314S) and fused it with a C-terminal EGFP tag (abbreviated as Cdc14B^{C314S}-EGFP mutant). The expression of Cdc14B^{C314S}-EGFP is also under the control of a doxycycline-regulatable promoter in pBI-tet vector. To establish stable clones we co-transfected pBI-tet-Cdc14B^{C314S}-EGFP plasmid together with the pBabe-puro (a kind gift from Dr. Gerald Evans) into U2OS$^{tet\text{-}on}$ cells (Clontech) and selected with both G418 and puromycin. In tested stable clones, upon treatment with doxycycline, Cdc14B^{C314S}-EGFP expression was induced, and its centrosomal localization was assessed. As shown in Fig 3.8, majority of Cdc14B^{C314S}-EGFP localized in nuclei/nucleoli and was absent from the centrosomes in virtually all the interphase cells examined. In contrast, wild-type Cdc14B^{WT}-EGFP was found to associate with centrosomes in about 75% of EGFP positive interphase cells.

Although these findings may suggest that catalytic activity is essential for Cdc14B centrosomal localization, its perturbation may also disrupt shuttling of Cdc14B between nucleoplasm and cytoplasm either directly or indirectly since >95% of the

Figure 3.7: Cdc14B centrosomal retention requires Cdc14B phosphatase activity. (A) Cdc14B-GFP fusion proteins were induced by DOX for 72 h in U2OSteton stable cell lines carrying different Cdc14B-GFP constructs as indicated. Centrosomes were visualized by γ-tubulin staining (red) and overlaid with Cdc14B-GFP (green) and DAPI (blue). Cdc14B^{C314S}-GFP was not detectable at interphase or mitotic centrosomes (arrows). Insets represent magnified images of centrosomes. Bar, 5 mm. (B) Histogram shows the percentage of interphase and mitotic cells with the indicated Cdc14B-GFP at centrosomes 72 h after DOX addition. The interphase data represent the means ±SD of three independent experiments and at least 500 cells were counted in each experiment. The mitotic experiment was performed at triplicate and a total of 113 Cdc14BC314S-GFP- and 374 Cdc14BWT-GFP-positive cells were counted respectively.


A
Cdc14B-GFP inducible clones
+ DOX
GFP
γ-Tubulin
Overlay
Interphase
WT
C314S
KKIR
K&C
Mitosis
WT
C314S
B
Cells (%) with Cdc14B-GFP on centrosomes
100
80
60
40
20
0
Cdc14B-GFP: WT C314S WT C314S KKIR K&C
Mitosis
Interphase

Cdc14B^{C314S}-EGFP mutant localized to the nucleolus. It is possible that mutation of a critical residue at the active site may dramatically changed the Cdc14B protein's conformation, therefore potentially interfered with the NES (Nuclear export signal) located at the C terminal part of the Cdc14B protein; or, hypothetically, the mutation disrupted interaction of Cdc14B with other proteins which are required for shuttling Cdc14B out of nucleus. Either way, as a consequence, it could indirectly prevent Cdc14B from reaching centrosomes in interphase cells where the nuclear envelope is still intact. To exclude this possibility, we studied the localization of Cdc14B^{C314S}-EGFP at the centrosomes of mitotic cells where the nuclear envelopes have disassembled. As shown in Fig 3.8, no Cdc14B ^{C314S}-EGFP was found to associate with mitotic centrosomes (0%, n = 113), whereas Cdc14B^{WT}-EGFP associated with a majority (61%, n = 374) of mitotic centrosomes examined. This finding suggests that the failure of Cdc14B^{C314S}-EGFP centrosomal retention is not due to the lack of nucleocytoplasmic shuttling activity and thus supports the possibility that Cdc14B phosphatase activity is indispensable for its centrosome localization.

Amino acids from 7 to 32 (^{7}**RR**SSWAAAPPCSRRCSSTSPGV**KKIR**^{32}) on Cdc14B contain a potential bipartite nuclear localization sequence (K/R2-X10-20-K/R3; in boldface type in the sequence above). We generated a mutant by changing three basic residues at positions 29, 30, and 32 to alanines (Cdc14BKKIR29-32AAIA, abbreviated as Cdc14B^{KKIR} mutant) and subcloned it into pBI-tet vector and also established stable clones. As expected, the nuclear localization of Cdc14B was abrogated with majority of Cdc14B^{KKIR}-EGFP mutant stayed in cytoplasm. All of the cytoplasmic Cdc14B^{KKIR}-

EGFP protein appeared in rings or MT-like bundles and partially colocalized with MTs (Cho et al., 2005b). When we studied the localization pattern of Cdc14B^{KKIR}-EGFP with respect to centrosome, we found in comparison with Cdc14B^{WT}-EGFP, the percentage of cells with centrosomal EGFP signal was significantly increased (Fig 3.8, *t* test; $P < 0.01$). In addition, we previously demonstrated that the cytoplasmic translocation/microtubule bundling ability of Cdc14B^{C314S} mutant was impaired but restored when the additional KKIR mutation was introduced to the Cdc14B^{C314S} mutant (KKIR29-32AAIA+C314S, abbreviated as Cdc14B$^{K\&C}$) (Cho et al., 2005b). Surprisingly, introduction of KKIR mutation also restored Cdc14B^{C314S} localization to both interphase and mitotic centrosomes (Fig 3.8). Because the lack of nuclear/cytoplasmic barrier in mitosis did not help to restore Cdc14B^{C314S} to centrosomes, this finding indicates that the additional KKIR mutation not only helps Cdc14B^{C314S} regain cytoplasmic translocation but may also act as a gain-of-function mutant that facilitates Cdc14B^{C314S} to re-associate with centrosomes in a process that doesn't rely on its catalytic activity.

3.3 Discussion

In mammals, two Cdc14 paralogs, Cdc14A and Cdc14 B have been identified (Li et al., 1997b). Human Cdc14A dynamically localizes to centrosomes and may play a role in centrosome separation and cytokinesis regulation (Bembenek and Yu, 2001; Kaiser et al., 2002b; Mailand et al., 2002a). This is based on the observation that overexpression of Cdc14A causes premature centrosome separation in early S-phase cells, and RNAi-mediated Cdc14A depletion leads to severe centrosome separation defect and thus

cytokinesis failure. Genetic studies demonstrate that the *Xenopus* orthologs of human Cdc14A, called XCdc14a and XCdc14b, are required for embryonic division and localize to nucleoli and centrosomes (Kaiser et al., 2004). In contrast to Cdc14A, Cdc14B has not been studied in detail. Unlike Cdc14A, centrosomal localization of human Cdc14B was a bit controversial based on previous reports. Our lab first reported that a portion of wild type C-terminal EGFP tagged Cdc14B co-localized with γ-tubulin to centrosomes (Cho et al., 2005b). In contrast, Mailand *et al* showed that N-terminal Myc-tagged Cdc14B did not co-localize with centrosomal markers tested (Li et al., 1997b; Mailand et al., 2002a). And the reason for this discrepancy is possibly due to different isoforms of Cdc14B used. In their study, the Cdc14B isoform-2 (accession number AF064104.1, abbreviated as Cdc14B2) was used, however, in contrast, we used the isoform-1 of Cdc14B instead (accession number AF023158.1, abbreviated as Cdc14B). In agreement with this, two other reports also failed to detect Cdc14B2's localization on centrosomes when they fused it with GFP tag either at C-terminus or N-terminus (Kaiser et al., 2002b; Li et al., 1997b). Indeed, in our hands, a wild type Cdc14B2 fused with C-terminal flag tag doesn't show any centrosomal signal either (Data not shown). Although the first 1-448 amino acids of the two isoforms are identical, Cdc14B2 has a longer distinct C-terminus (aa 449-498) compared with Cdc14B (aa 449-459). The additional C-terminal tail may have prevented Cdc14B2 from being recruited to the centrosomes. Using a polyclonal rabbit anti-Cdc14B antibody, Nalepa *et al* documented that after preextraction with Triton X-100 prior fixation, small amounts of endogenous Cdc14B could be visualized on centrosomes (Grzegorz Nalepa, 2004). Since the endogenous rabbit anti-Cdc14B antibody used was designed against 1-54 amino acids of Cdc14B and thus can recognize

both isoforms. And the centrosomal signal might have come from endogenous Cdc14B isoform 1 instead of Cdc14B2.

In order to reveal Cdc14B's centrosomal functions, we first sought to study its centrosome localization in more detail. Although previous studies showed a fraction of both exogenous and endogenous Cdc14B associated with centrosomes, many questions remain unanswered. These questions include but not limited to: where does Cdc14B localize within centrosome, centriole or PCM? Is Cdc14B associated with centrosome in a cell cycle dependent manner? Is Cdc14B's phosphatase activity required for its centrosomal association? In order to address these questions, we studied the localization pattern of both Cdc14B^{WT}-EGFP and endogeous Cdc14B in much more details. First we used two centriole markers: Centrin-2 and C-Nap1 to study whether Cdc14B is a centriole-associated protein. Both of these markers localized to the centriole with Centrin-2 at the distal end and C-Nap1 at the proximal end. By co-immunostaing, our analysis showed that Cdc14B, both exogenous and endogenous forms, partially co-localized with both Centrin-2 and C-Nap1 staining. Careful observation and comparison further enabled us to propose that Cdc14B was sandwiched between Centrin-2 and C-Nap1 and seemingly closer to C-Nap1 in a cell cycle dependent manner (Fig 3.8). In summary, we characterized Cdc14B as a centriole-associated protein and localized close to the proximal end of mother centriole during S, G2 and early M phases and marked the proximal part of both centrioles in late M and G1 phases. Even though the exact localization of Cdc14B on centriole needs to be further investigated by triple-labeling or Immuno-electromicroscopy experiments, our current co-immunostaining studies using

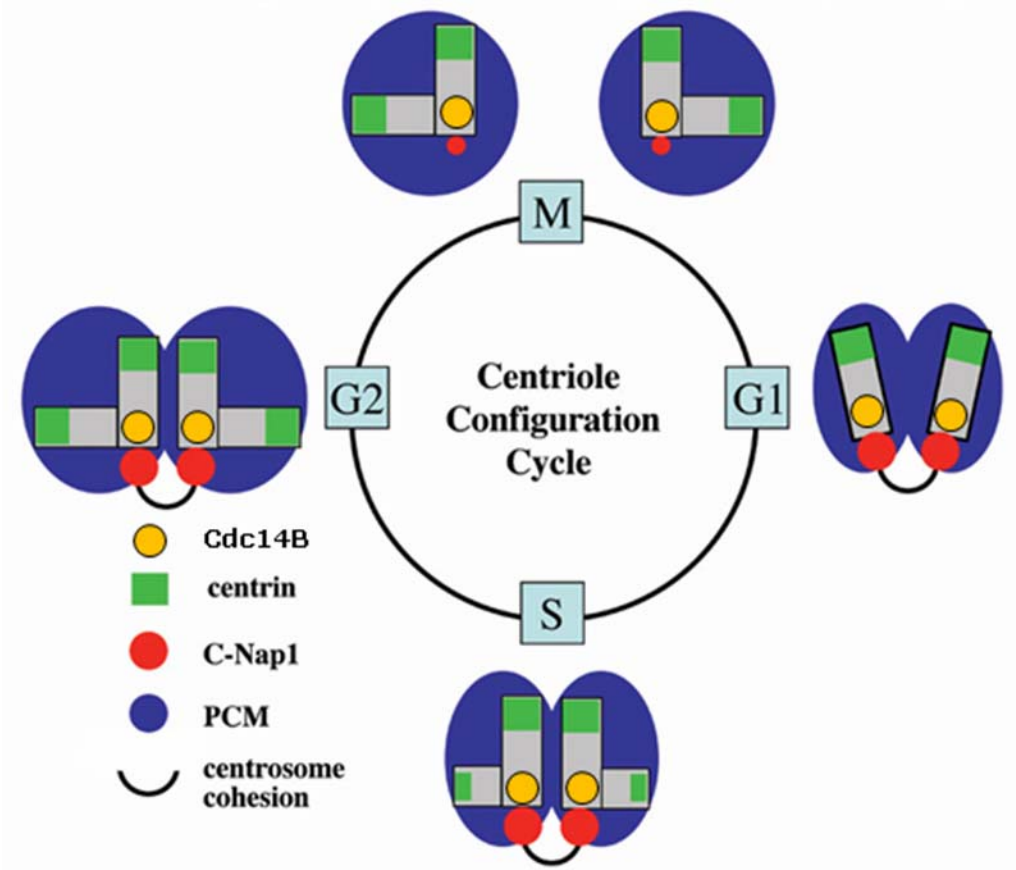


Figure 3.8: Mapping of Cdc14B to centrioles. Hypothetical localization of Cdc14B (Yellow) on centriole. Centrin (Green) marks the distal end and C-Nap1 (Red) marks the proximal end of centriol, PCM (Blue).

both Centrin-2 and C-Nap1 as centriole markers suggested that Cdc14B may localize very close to a structure called "cartwheel" (or "central tube" in *C.elegans* and *Drosophila*) where the procentriole starts to assemble into a new centriole. The cartwheel, a structure with a hub and nine radiating spokes, is proposed to be the centriolar precursor and it is the first structure appearing in the centriole assembly. Recently, several proteins such as SAS-6 have been identified as essential component of the cartwheel and help to stabilize the 9-triplet structure. If Cdc14B is indeed localize to or close to the cartwheel, it may raise the possibility that Cdc14B is involved in centriole biogenesis by potentially regulating its centriole substrate(s) at the cartwheel. Thus, during centriole duplication, Cdc14B will be at the right place to perform its job.

Also, based on our immunofluorescence studies, Cdc14B associates with engaged mother, not engaged daughter, but with both disengaged mother and daughter centrioles when they are in disengaged configuration. The reason for this is unknown. One tantalizing possibility is that Cdc14B may keep the disengaged centrioles in check to prevent overduplication or keep engaged centrioles tied together to prevent re-duplication. Thus, it's tempting to propose that Cdc14B may also be involved in centriole-cohesion. According to a recent model, centriole engagment is proposed to be the intrinsic block to re-duplication while centriole disengagment is a prerequisitive for duplication (Tsou and Stearns, 2006c). Disengagment of centrioles happens at the onset of anaphase and depends on the activity of seperase, a caspase-related protease. Separase is best known for its requirement in promoting sister chromatid separation. The protease cleaves a component of cohesin, the protein complex that holds sister chromatids

together, thereby triggering chromosome segregation (D'Amours and Amon, 2004; Nasmyth, 2002).

One possibility is that separase cuts one or more of its centriole-associated targets that glue centrioles together. Alternatively, it may act indirectly by regulating the activity of another enzyme (a kinase or phosphatase) that in turn triggers centriole disengagement (Nigg, 2006). Recent evidence showed that when a splice variant of Shugoshin1 (sSgo1), a protein that protects sister chromatids from separase activity during prophase, is depleted in human cells, centriole–procentriole pairs disengage prematurely, suggesting sSgo1 potentially could be a substrate for separase in centriole disengagement (Strnad and Gˆnczy, 2008). In budding yeast, it's been well known that Esp1/Separase is required for Cdc14 release from the nucleolus as a component of the FEAR network and thus Cdc14 acts in the downstream of Esp1/Separase in controlling mitotic exit. Therefore, it's tempting to further test whether Cdc14B or Cdc14A can be also acting in the downstream of human separase in controlling centriole disengagment at the onset of anaphase. In line with this, sSgo1 interacts with Plk1 and its centrosomal localization is Plk1 dependent. Centriole disengagement triggered by sSgo1 depletion or expression of a dominant negative mutant is suppressed by ectopic expression of sSgo1 or by RNAi based Plk1 knockdown (Wang et al., 2008). A most recent study, although in a different context, identified the Cdc14B-Cdh1-Plk1 axis in controlling G2 DNA-damage checkpoint implicating Plk1 may act at the downstream of Cdc14B (Bassermann et al., 2008).

Kaiser et al reported previously deletion of the first 54 amino acids resulted in Cdc14B2's

(Isoform-2, here referred to as Cdc14B2) localization to the cytoplasm and centrosomes rather than nucleolus. This data suggested the first 54 amino acids of Cdc14B encode a nucleolar localization signal (NLoS) targeting Cdc14B (both isoforms 1 and 2) to nucleolus. This NLoS may be enough to inhibit Cdc14B2's centrosomal localization by restricting it to the nucleolus, however, may be inadequate to prevent Cdc14B's (isoform 1) centrosomal retention. In general, sequences of Cdc14B can be primarily divided into three regions, the N-terminal region (containing nucleolar targeting domain in Cdc14B), the middle region (phosphatase domain) and the C-terminal region (Ser and Asn rich region). Mutation of the basic amino acid residues of nuclear localization signal (NLS) at the N-terminal region of Cdc14B facilitated, to some extent, microtubule bundling and stabilizing activities of Cdc14B (Cho et al., 2005b). Structural analysis of Cdc14B revealed a novel arrangement of two domains, designated as A- and B-domains in the phosphatase domain, which are conserved within the Cdc14 family members (Gray et al., 2003). The A-domain, which has no sequence similarity to other dual specificity phosphatase, determines substrate specificity but does not possess phosphatase activity. The B-domain, containing the conserved catalytic motifs of protein tyrosine phosphatases (PTPs), has phosphatase activity. The C-terminal Ser/Asn rich region contains a nuclear exit signal (NES) (Kaiser et al., 2002a; Mailand et al., 2002b). After establishing that Cdc14B is a centriole-associated protein, although hasn't been addressed in the current study of ours, next logic step will be mapping domains of Cdc14B required for centrosome association. Deletion mutants will enable us to determine centrosome binding domain(s) and its (their) relationship with the phosphatase domains in Cdc14B. Once centrosome binding domains of Cdc14B are resolved, site-directed mutagenesis to mutate

specific amino acids of the domain and determine the specific amino acids required for centrosome binding will be followed. Determination of Cdc14B centrosome binding domains or motifs will also help us to understand or design experiments to Fig out how Cdc14B centrosome localization is regulated during the cell cycle and thus its function in centrosome cycle regulation.

In the current study, however, we sought to determine whether Cdc14B's phosphatase activity is required for its centrosomal association. The idea behind this is that in budding yeast, almost all the Cdc14 related functions rely on its phosphatase activity. Our study did show that a phosphatase-dead mutant of Cdc14B (Cdc14B$^{C/S}$-EGFP) failed to localize to both interphase and mitotic centrosomes indicating catalytic activity is indeed required for Cdc14B's loading onto centrosome. However, whether this mutation is within the Cdc14B centrosomal localization signal is not known. The mutation could either directly or indirectly inhibits Cdc14B association with centrosomes. By changing a critical residue at the active site, the Cdc14B protein conformation might have undergone a dramatic change that resulted in dissociation of Cdc14B either with centrosome or with other proteins that can recruit Cdc14B to centrosomes.

3.4 Summay

In summary, we identified a fraction of both endogenous Cdc14B and Cdc14B^{WT}-EGFP asscociates with centrioles throughout the cell cycle. During cell cycles when two centrioles are in engaged configuration (S, G2 and early M phase), Cdc14B seemingly

only associates with mother centriole; in cell cycles when two centrioles are disengaged (G1 and late M phase), Cdc14B assocatiates with both centrioles. Further, the association of Cdc14B with centrioles requires an intact phosphatase activity.

3.4 Experimental Procedures

3.4.1 Cloning and site-directed mutagenesis

Cdc14B open reading frame (ORF) cDNA was amplified by PCR from the Marathon human heart cDNA library (Clontech) using the following primers: forward, 5'-ACTCCCGGGTCCATGAAGCGGAAAAGCGAGC-3'; and reverse, 5'-AGTCCCGGGTTAACGCAAGACTGTTTTAGTCC-3'. The PCR product was digested with SmaI endonuclease and cloned into the SnaBI site of pMX-pie vector (a kind gift from Gerry Nolan) carrying an N-terminal 6-myc epitope tag. Sequencing analysis of the resulting plasmid confirmed that the Cdc14B cDNA was identical to hCdc14B isoform-1 in GenBank (AF023158.1), except for a T-to-C transition at position 52 from the first ATG. The inducible expression system of Cdc14B was generated by subcloning the C-terminal EGFP-tagged (enhanced green fluorescent protein) Cdc14B into pBI-tet vector (Clontech). All of the Cdc14B mutants (C314S, KKIR29-32AAIA, and KKIR29-32AAIA plus C314S) were generated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and verified by sequencing analysis.

3.4.2 Cell Culture, Transfection and Drug Treatment

U2OS (ATCC), U2OS$^{Tet\text{-}On}$ (BD Biosciences) cells were cultured under 5% CO_2

at 37°C in high-glucose Dulbecco's Modified Eagle's medium (DMEM, Invitrogen), 10% fetal bovine serum (FBS), and antibiotics (100ug/ml penicillin and 50ug/ml streptomycin sulfate). Cells were transfected with either FuGene 6 (Roche) or Lipofectamine Plus (Invitrogen). To establish DOX-inducible Cdc14B-EGFP stable clones, the pBI-tet-Cdc14B^{WT}-EGFP, pBI-tet-Cdc14B^{KKIR}-EGFP , pBI-tet-Cdc14B^{C314S}-EGFP , or pBI-tet-Cdc14B$^{K\&C}$-EGFP plasmids were co-transfected with pBabe-puro into U2OS$^{Tet\text{-}On}$ cells harboring endogenous Cdc14B. Stable clones were obtained after selection with 500 μg/ml G418 and 2 μg/ml puromycin in the absence of DOX.
The DOX-inducible Cdc14B-EGFP stable clones were maintained in 100ug/ml of G418 (Invitrogen) and 2ug/ml of puromycin (Sigma-Aldrich). The expressions of Cdc14B^{WT}-EGFP, Cdc14B^{KKIR}-EGFP, Cdc14B^{C314S}-EGFP and Cdc14B$^{K+C/S}$-EGFP were induced by the addition of 4ug/ml of doxycyclin and cultured up to 96 h. To study the localization of Cdc14B^{WT}-EGFP and Cdc14B^{C314S}-EGFP on mitotic centrosomes, U2OS$^{Tet\text{-}On}$ cells were treated with 0.2 ug/ml nocadazole (Sigma) for 18 h before fixation.

3.4.3 Antibodies and Immunofluorescence

Centrosome markers used include: γ-tubulin (GTU-88 [Monoclonal, Sigma-Alrich] and C-20 [Polyclonal, Santa Cruz Biotechnology, Inc.]); Rabbit anti-Peri-centrin polyclonal antibody (Abcam); Mouse anti-Centrin-2 monoclonal antibody (20H5; a gift from J. Salisbury, Mayo Clinic Foundation, Rochester, MN); Mouse anti-C-Nap1 monoclonal antibody (BD Biosciences). Cdc14B antibodies: Rabbit anti-Cdc14B polyclonal antibody (Zymed Laboratories, Inc.); Mouse anti-Cdc14B polyclonal antibody

(Abnova); Chicken anti-Cdc14B polyclonal antibody (Geneway Biotech, Inc.).

For indirect immunofluorescence, cells were grown on glass coverslip and fixed with either paraformaldehyde or cold 100% methanol. The cells were then permeabilized with PBS/0.5% Triton X-100 for 10 min at room temperature followed by blocking with PBS/1% BSA for 30 min. Centrosomes or centrioles were visualized by immunostaining with antibodies against γ-tubulin, pericentrin, Centrin-2 or C-Nap1 antibodies. For visualization of centriole-associated endogenous Cdc14B, cells were treated with or without 10 μg/ml nocodazole (for better exposure of centrosome Cdc14B) for 2 h, briefly extracted with 0.5% Triton X-100 on ice, fixed with cold 100% methanol, and immunostained with chicken anti-Cdc14B antibody (GenWay Biotech, Inc.). In our hands, methanol fixation preserved GFP signals and, thus, anti-GFP immunostaining was not used to visualize Cdc14B-GFP fusion proteins in the fixed cells. Secondary antibodies including Alexa Fluor 488 and 594 donkey anti-mouse and anti-goat and goat anti-rabbit, and anti-chicken IgY antibodies were obtained from Invitrogen. DNA was counterstained by DAPI. Cells were visualized with a 100 °- Plan Neofluar objective (1.30 oil; /0.17; Carl Zeiss, Inc.) under an epifluorescence microscope (Axioskop 2; Carl Zeiss Inc). Images were acquired with a charge-coupled device camera (AxioCam HRC; Carl Zeiss, Inc.) controlled by Openlab software (version 3.5; PerkinElmer). For confocal microscopy, images were captured with an HCX-PL/APO 63 X 1.32 oil objective (Leica) under a SP2 laser scanning confocal microscope (Leica) equipped with confocal software (LCS version 2.0; Leica). The coverslips were mounted using PermaFlour Mountant media (Thermo Fisher Scientific) and imaging was performed at room temperature.

Image processing was performed using Photoshop CS (8.0).

3.4.4 Centrosome fractionation experiments

Centrosomes were prepared from exponentially growing HeLa cells according to a previously published procedure (Moudjou and Bornens, 1998). In brief, a total of $6x10^8$ cells were treated with 1 μg/ml cytochalasin D and 2.2 μM nocodazole. Cells were lysed in a buffer containing 1 M Hepes, pH 7.2, 0.5% NP-40, 0.5 mM MgCl 2, 0.1% mercaptoethanol, and protease inhibitor cocktail (Roche) and centrifuged at 2,500 *g*. The resulting supernatant was filtered, incubated with 2 U/ml DNase I, and loaded over a 60% sucrose cushion for centrifugation at 10,000 *g* with a SW28 rotor (Beckman Coulter). Concentrated centrosomes were centrifuged again over a discontinuous gradient containing 70, 50, and 40% sucrose solutions at 75,000 *g*. A total of 32 fractions were collected from the bottom of the tube. Each fraction was separated by SDS-PAGE. Centrosome-enriched fractions were determined by immunoblotting with anti-γ-tubulin antibody, and the presence of Cdc14B was judged by immunoblotting with anti-Cdc14B antibody (Zymed Laboratories, Inc.). For immunofluorescence analysis of isolated centrosomes, each 10 μl of fraction 15 was diluted into 4 ml of 10 mM Pipes buffer, pH 7.2, and transferred into a 38.5-ml ultracentrifuge tube (Beckman Coulter) with a specially designed adaptor to fit to a 15-mm round coverslip. The samples were then subjected to centrifugation at 20,000 *g* (10,000 rpm) for 20 min with a SW28 rotor followed by fixation in methanol at 20 ° C for 10 min and immunostaining with antibodies against Centrin (20H5; a gift from J. Salisbury, Mayo Clinic Foundation,

Rochester, MN), Cdc14B (GenWay Biotech, Inc.), and nucleolin (4E2; Research Diagnostics, Inc.), respectively. Finally, coverslips were placed in pure ethanol for 2 min at room temperature, air-dried, and mounted with a drop of Permaflour (Thermo Fischer Scientifi c) on a microscope slide. Images were captured as described in the preceding paragraph.

Chapter 4

Cdc14B phosphatase is a negative regulator for centrosome duplication

4.1 Introduction

As the primary MTOC of animal cells, centrosomes play important role in all microtubule related processes by controlling the number, polarity and distribution of microtubules. These processes include cell polarity, shape, mobility, adhesion as well as intercellular transport. More importantly, centrosomes are crucial for chromosome segregation and cytokinesis during mitosis (Nigg, 2002). When the nuclear envelope breaks down during mitosis, the two sister centrosomes will separate from each other and relocate to each pole of the cell and start to nucleate astral arrays contributing most of the microtubules to the formation of the bipolar spindle. Mitotic centrosomes, through these microtubule arrays, determine spindle polarity, spindle position and spindle orientation within the cell (Nigg, 2004).

In order to be ready to perform its normal function during mitosis, the single interphase centrosome needs to be duplicated once, and only once. Although elementary in concept, to make sure there are no errors occurring during the process of centrosome duplication cell cycle after cell cycle is not as simple as it may appear. The centrosomes must duplicate (exactly once), separate at the right time in relation to nuclear events in the cell

cycle. Considering the multitude of critical cellular functions that depend on the correct number of centrosomes, it's not surprising that the numeral integrity of centrosomes is tightly regulated and involves multiple mechanisms. Although our understanding of the molecular mechanism behind this "once and only once" control is still incomplete, many critical steps have been described: Some suggests an intrinsic mechanism in controlling centrosome reproduction while others illustrated phosphorylation and ubiqutin-dependent proteolysis are indispensible in centrosome number control.

The presence of two centrosomes at the onset of mitosis is crucial for the formation of a bipolar spindle. Extra copies, a condition called centrosome amplification, frequently leads to the formation of multipolar spindle, an aberrant mitotic spindle with more than two spindle poles. This in turn can lead to unequal distribution of chromosomes during mitosis and genomic instability, which is regarded as a major driving force in multi-step carcinogenesis (Nigg, 2004).

4.1.1 The Events of Centrosome Reproduction

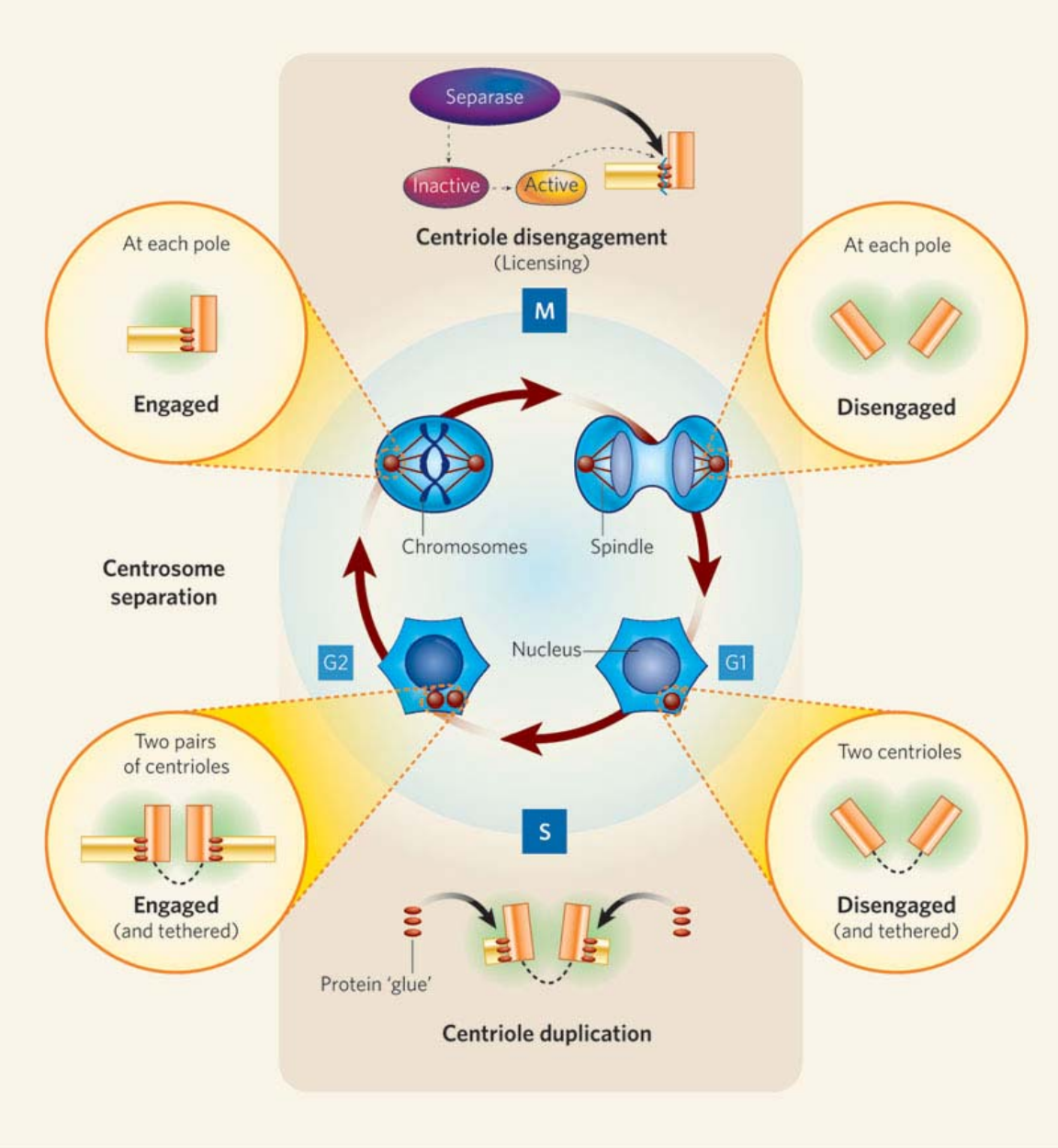
Except in some specialized cell types and unusual conditions in which centrosomes can form *de novo*, the typical centrosome duplication pathway requires a pre-existing centrosome. The classical centrosome duplication cycle can be divided into several consecutive steps: In M phase, one centrosome consists of two centrioles is present at each spindle pole; at the end of mitosis and early G1, the two centrioles lose their orthogonal configuration and become "disengaged", according to recent publications, constitutes a necessary "licensing" step for centrosome duplication in the

next cell cycle. After disengagement, the two centrioles are still loosely connected by cohesion fibers during G1 phase; at the G1/S transition when the activity of Cdk2/Cyclin-E starts to rise centrosome duplication is initiated along with DNA replication. Procentrioles start to grow orthogonally from the proximity of each pre-existing maternal centrioles and continue to elongate throughout S and G2 phase; in early mitosis the connecting fiber between two newly formed centrosomes is severed and each centrosome will migrate toward the cellular pole and participates in mitotic spindle pole formation (Tsou and Stearns, 2006b).

Procentriole assembly normally initiates around the time of the transition between the G1 and S phases of the cell cycle when the activation of the Cdk2/Cyclin-E complex also starts. Thus, it is possible that the centriolar components triggering the onset of procentriole assembly are regulated by the rise in Cdk2/Cyclin-E activity. In agreement with this idea, the first "licensing" model proposed for centrosome duplication by Okuda, M. *et al* in 2000 involved a nucleolar phosphoprotein B23 (also called NPM1, Nucleophosmin or NO38). In this model, B23 was identified as one of the targets of Cdk2/Cyclin-E kinase and could be specifically phosphorylated at the Thr199 postion. This site-specific phosphorylation is important for the initiation of centrosome duplication but not DNA replication(Tsou and Stearns, 2006a). The unphosphorylated form of B23 is present in the loose connection between the two centrioles in cells in the G1 phase of the cell cycle. Upon phosphorylation by the Cdk2/Cyclin-E complex, B23 dissociates from the centrosome, triggering procentriole assembly (Tokuyama et al., 2001)y. Toward the end of mitosis, B23 loses its Thr199 phosphorylation due to some

unknown phosphatase and re-associates with the centrosome. Expression of a non-phosphorlatable mutant of B23 (T199A, Thr199 change to Ala, could not be phosphorylated by Cdk2/Cyclin-E) blocked centrosome reproduction (Tokuyama et al., 2001). The B23T199A mutant remained at the centrosomes, likely accounting for its dominant negative characteristics. In addition, microinjection of anti-B23 monoclonal antibody, which sterically blocked phosphorylation of B23 by Cdk2/Cyclin-E also inhibited the duplication of centrosomes (Tokuyama et al., 2001). It is also worth mentioning that the Cdk2/Cyclin-A complex phosphorylates Thr199 of B23 at a similar efficency with Cdk2/Cyclin-E, and possibly be responsible for preventing re-association of any cytoplasmic B23 to centrosomes during S and G2 phases, continuing to support centrosome duplication (Tokuyama et al., 2001). Thus, phosphorylation of B23 by Cdk2/Cyclin-E/A is part of the molecular machinery that may license centrosome duplication. Consistant with this, Wang et al. further demonstrated that the centrosomal loading of B23 was dependent on the Ran/Crm1 complex, a nuclear-cytoplasmic shuttle system that, when inhibited, resulted in supernumanary centrosomes, similar to the phenotype caused by B23 depletion by RNAi (Wang et al., 2005). Based on the fndings made to date, a model for the role of Cdk2/Cyclin-E/A and B23 in the regulation of the centrosome duplication cycle can be summarized as follow (Fig 4.1): During G1 phase, upon phosphorylation by Cdk2/Cyclin-E, Centrosome-bound B23 dissapears from centrosomes (either through dissociation or degradation), which in turn triggers initiation of centrosome duplication; During S and G2 phases, any residual B23 present in the cytoplasm are prevented from re-association with centrosomes potentially

Figure 4.1: Centrosome duplication cycle. The central panel shows the centrosome duplication–segregation cycle. Each centrosome is shown as a red circle; G1, S, G2 and M are stages of the cell-division cycle. The arrangements of the centrioles at different stages of the cell cycle are shown in the insets. Centrioles are the orange and yellow cylinders; the pericentriolar protein matrix that surrounds them is green; a putative tether connecting parental centrioles is shown as a black dotted line; and the protein structure ('glue') presumed to be responsible for centriole engagement is red. Only disengaged centrioles are competent for duplication, whereas engagement prevents a second duplication. This 'licensing' mechanism thus ensures that centrioles are duplicated only once in every cell cycle. Tsou and Stearns show that the enzyme separase is essential for disengagement of the centrioles. But it is not known whether separase acts directly on the glue proteins itself, or whether it activates some other factor that then divides the centrioles. (Image courtesy of Erich A. Nigg, 2007)


Separase
Inactive
Active
Centriole disengagement
(Licensing)
M
At each pole
Engaged
At each pole
Disengaged
Chromosomes
Spindle
Centrosome
separation
G2
Nucleus
G1
Two pairs
of centrioles
Engaged
(and tethered)
Two centrioles
Disengaged
(and tethered)
S
Protein 'glue'
Centriole duplication

through the activity of Cdk2/Cyclin-A; During late mitosis, B23 re-associates with centrosomes, hence each daughter cell receives one centrosome bound by B23 upon cytokinesis (Masaru, 2002). Albeit interesting, this model fails to explain why centrosomes do not readily re-duplicate during late S, G2 and early mitosis when B23 is not present at centrosome, suggesting Cdk2/Cyclin-E/A-B23 pathway may not be the sole licensing factor for centrosome duplication.

The concepts of a 'license' step for centrosome duplication and a "block" to re-duplication within the same cell cycle in part draw from extensive studies of DNA replication. During DNA replication, it's been generally accepted that the licensing step is the loading of MCM helicases onto DNA to help unwrap the DNA duplex for replication. And once the DNA duplex is unwound and primed, the licensing factors are no longer required (Blow and Dutta, 2005). Also, the "block" to re-duplication in the context of DNA replication is intrinsic to the double helix itself, which is not accessible for another round of replication without a second licensing event in next cell cycle. By analogy to DNA replication, a centrosome-intrinsic block to re-duplication has been proposed (Tsou and Stearns, 2006c). Strong evidence for such a block comes from cell fusion experiments. When a cell in the G1 phase was fused with a cell in the G2 phase, the G1 cell cycle status became dominant and drove the fusion cell into S phase instead of mitosis. During this S phase, a new procentriole assembled next to each centriole coming from the G1 cell, but not next to those originating from the G2 cell (Strnad and Gˆnczy, 2008). It has been suggested that the centrosome-intrinsic block to re-duplication is maintained by the tight association between each daughter-mother centriole pair. This

tight association or centriole "engagement" configuration is established during centriole duplication in S phase and continues till "disengagement' in late M-phase and early G1 that renders mother and daughter centrioles loosely tethered to one another (Nigg, 2006). Evidence of recent elegant work strongly supports the notion that engaged centrioles prevent centriole re-duplication during S and G2 phases whereas disengaged centrioles are prerequisite for the growth of new centrioles from the mature centriole templates (Nigg, 2006; Tsou and Stearns, 2006c; Wong and Stearns, 2003). Also, centriole disengagement at the end of mitosis seemingly requires the activity of separase, a caspase-related protease best known for its requirement in promoting sister chromatid separation by cleavage of cohesion at anaphase (Tsou and Stearns, 2006c). Depletion of separase prevented centriole disengagement from happening in the late anaphase. In supporting this idea, a study by Thein *et al* have recently demonstrated that depletion of the microtubule and kinetochore protein astrin resulted in premature sister chromatid separation as well as centriole disengagement which is consistent with untimely separase activation. Supporting this idea, astrin-depleted cells contain active separase, and separase depletion suppresses the premature sister chromatid separation and centriole disengagement in these cells (Thein et al., 2007). Conversely, when a splice variant of Shugoshin1 (sSgo1), a protein that protects sister chromatids from separase activity during prophase, is depleted in human cells, centriole-procentriole pairs disengage prematurely (Tsang and Dynlacht, 2008). Taken together, these findings suggest that, during mitosis, separase cleaves a substrate, which might be sSgo1, and that this cleavage results in disengagement of the centriole-procentriole, thus licensing each of them for another round of procentriole formation during the subsequent cell cycle.

4.1.2 RNA interference

RNA Interference (RNAi) is among the most important technological breakthroughs in modern biology, revolutionizing the way that researchers study gene function. As a novel biological pathway, RNAi has had significant impact on the ease, speed, and specificity with which the loss of function of specific genes can be directly studied in mammalian systems.

Overexpression has been used extensively to study the function of a particular gene. However, oftentimes the phenotypes due to overexpression don't respresent the real function of the gene of interest. Loss of function studies can be performed using dominant negative constructs because when overexpressing these mutants, the endogenous function of genes can be blocked. However not all the protein has a dominant negative mutant and results may sometimes be hard to interpret. Another method to study loss of function of genes is to generate knockout mice. While proved to be the ultimate way of dissecting a gene's function, this method is extremely labor intensive, expensive, time consuming as well as may result in an embryonic lethal phenotype. Other nucleic acid-based silencing technologies such as oligonucleotide and ribozymes also have been used, yet these may not work for all targets.

Historically, RNAi has been around for a little less than 20 years. It was in the early 1990s when for the first time RNA was found to be able to inhibit protein expression in plants and fungi. This phenomenon was referred to as "posttranscriptional gene silencing" and "quelling" at that time because it was not fully understood how it worked.

It was not until 1998 when Fire and Mellow discovered that when *C.elegans* was fed with double-stranded RNA (dsRNA), the protein expression was inhibited in a sequence-specific manner, thus the term "RNA interference" was coined. Fire and Mellow won the 2006 Nobel Prize in Physiology or Medicine for their discovery of RNA interference.

Since its discovery in *C.elegans* the use of long dsRNAs (>400bp) has been successful in inhibiting protein expression in many organisms including Drosophila (Misquitta L, 1999), zebrafish (Wargelius et al., 1999), Planaria (Sánchez Alvarado A, 1999) and numerous plants (Fukusaki et al., 2004; Jensen et al., 2004; Jørgensen F, 1990). However, dsRNA was limited to lower organisms because delivering long dsRNA to mammalian cells can trigger endogenous nonspecific antiviral responses that target longer dsRNAs for degradation. Further studies demonstrated that small interfering RNA strands (siRNA) are keys to the RNAi process in mammalian cells. siRNA molecule is typically 21 nucleotides in length, processed internally by an enzyme called "Dicer". Subsequently in 2001, it was discovered that delivery of siRNA to mammalian cells avoided nonspecific effects and thus could directly trigger RNAi.

Today we have a much better understanding of entire RNAi pathway. Now we define RNAi (RNA interference) as a process during which a target protein expression can be inhibited by specifically targeting its mRNA for degradation. RNA interference involves multiple steps: The RNAi pathway is initiated by an RNase III family member enzyme dicer, which chops long dsRNA molecules into short fragments of 20–25 base pairs (Agrawal et al., 2003; Bernstein et al., 2001; Elbashir et al., 2001). One of the two strands

of each fragment, known as the guide strand, is then incorporated into the RNAi targeting complex known as RISC (RNA-induced silencing complex) and pairs with complementary mRNA sequences to induce cleavage of the target mRNA molecule by argonaute, the catalytic component of the RISC complex (Bernstein et al., 2001). The target mRNA is cleaved in the center of the region complementary to the siRNA, and broken down into smaller pieces that can no longer be translated into protein (Elbashir et al., 2001).

Besides siRNAs, it has been demonstrated that a small hairpin RNA or short hairpin RNA (shRNA) can be as efficient as siRNAs in inducing RNA interference. (Barton GM, 2002; Brummelkamp et al., 2002; Yu JY, 2002). shRNA is a sequence of RNA that bears a fold-back, stem-loop structure of approximately 19 perfectly matched nucleotides connected by various spacer regions and ending in a 2-nucleotide 3′-overhang. shRNA can be introduced into cells using a vector with a polIII promoter to ensure that the shRNA is consitutively expressed. Once inside the cells, shRNA hairpin structure can be cleaved and integrated into the RISC complex in the same way as the normal siRNA does.

4.2 Results

4.2.1 Ablation of Cdc14B leads to centriole amplification in HeLa cells

To determine whether Cdc14B plays a role in centrosome cycle regulation, we first performed loss-of-function experiments. In this regard, we took three different RNAi approaches to knockdown endogenous Cdc14B.

Short siRNA molecules can be prepared either by direct chemical synthesis of two unmodified 21-oligonucleotide molecules annealed together, or by transcription driven by RNA polymerase promoters. The direct transfection of chemically synthesized siRNA duplexes into cells, originally demonstrated by Tuschl Lab in Rockefeller University's, is currently the most popular approach. However, the success of this technique is heavily dependent on the ability of the model cell system to undergo transfection and to sustain the RNAi effect. Also, the transient presence of siRNA in the cell renders this technique less feasible for long-term studies. Vector-based siRNA technology, in contrast, involves cloning a small DNA insert of about 70 bp into a commercially available or custom-made vector. This vector can be transfected into the cell, where the DNA insert expresses a short hairpin RNA to specifically target your mRNA of interest for degradation.

As a first approach, we used a vector-based RNAi approach to knockdown endogenous Cdc14B. In search of the optimal siRNA target sequence to deplete the endogenous Cdc14B, we have tested as many as 17 different oligos and finally we found that an oligo corresponding to nucleotide position 1234-1254 relative to the Cdc14B start codon was most effective in ablating endogenous Cdc14B expression in HeLa cells (designated as $Cdc14B^{1234}$ siRNA). We cloned this siRNA oligo into pSuperior-neo-GFP vector purchased from Oligoengine whose expression is driven by the H1 promoter. With pSuperior vectors, expression of $Cdc14B^{1234}$ siRNA is repressed in the absence of tetracycline and induced when tetracycline is added to the culture medium. This is due to the prescence of a TetO2 (tetracycline operator 2) site serving as the binding site for two molecules of the Tet repressor. In the absence of tetracycline, the Tet repressor forms a

homo-dimer that binds with extremely high affinity to the TetO2 sequence (Hillen and Berens, 1994). This in turn represses transcription of the RNA hairpin precursor of Cdc14B^{1234} siRNA. Upon addition, tetracycline binds with high affinity to the Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The Tet repressor tetracycline complex then dissociates from the Tet operator and allows transcription of the RNA hairpin precursor of the Cdc14B^{1234} siRNA duplex. After successful cloning, the resulting plasmid was transfected into TREx-HeLa cells that already has stably incorporated a construct expressing Tet-repressor. Transfected TREx-HeLa cells were selected under both G418 and blasticidin to obtain Cdc14B knockdown stable clones. After several weeks' selection, several clones were obtained. The levels of siRNA expression and gene knockdown will typically vary widely among cells. Therefore, we first try to validate with these stable clones whether expression of endogenous Cdc14B can be successfully knocked down or not when induced with tetracycline. Western blot analysis showed a reduction of endogenous Cdc14B expression at the protein level in those two clones (clones #2 and #3) in the presence of tetracycline while in stable clone #1, there is no suppression of Cdc14B protein expression as shown in Fig 4.2A. Also, the same siRNA plasmid could also knockdown exogenous Cdc14B-EGFP expression in U2OS^{Teton} cells confirming target specificity of the Cdc14B^{1234} siRNA (Fig 4.2A). Moreover, immunostaining with the anti-Cdc14B antibody revealed that centrosomal Cdc14B expression was greatly reduced in Cdc14B^{1234}siRNA stable clone #3 cells (Fig 4.3A).

Figure 4.2: Depletion of Cdc14B leads to centriole amplification in HeLa cells. (A) Western blot analysis using anti-Cdc14B antibody showed Cdc14B knockdown in TREx-HeLa Cdc14BsiRNA clones #2 and #3, but not in TREx HeLa and clone #1**.** The same Cdc14BsiRNA also led to Cdc14B-GFP knockdown in U2OSteton cells visualized by immunoblot with anti-GFP antibody. β-actin was used as a loading control. (B) Images of supernumerary centrioles. Centrioles were visualized by anti-centrin antibody and DNA by DAPI (not shown). Insets, magnified images of centrioles. Bar, 4 μm. (C) Percentage of cells with > 4 centrioles was calculated from the experiments shown in A and B (left panel). Note that the data shown here does not include polyploid cells with >4 centrioles described in Fig. S3A. (D) Top panel: Immunoblot shows the level of endogenous Cdc14B and Cdc14A in HeLa cells transfected with siGLO control oligos (lane 1), Dharmacon SMARTpool Cdc14BsiRNAs (lane 2), pSuper empty vector (lane 3) and pSuper-Cdc14BshRNA-E9 (lane 4). Bottom panel: HeLa cells were transfected with the corresponding oligos and vectors as in top panel. Percentages of cells with > 4 centrioles were calculated from experiments shown in B (middle and right panels). All the data are presented as the means $\pm$ SD of three independent experiments. At least 300 cells were counted in each experiment. Note that the indicated fold changes in A and D (top panel) were calculated based on the densitometric values of each lane normalized against the β-actin loading controls.

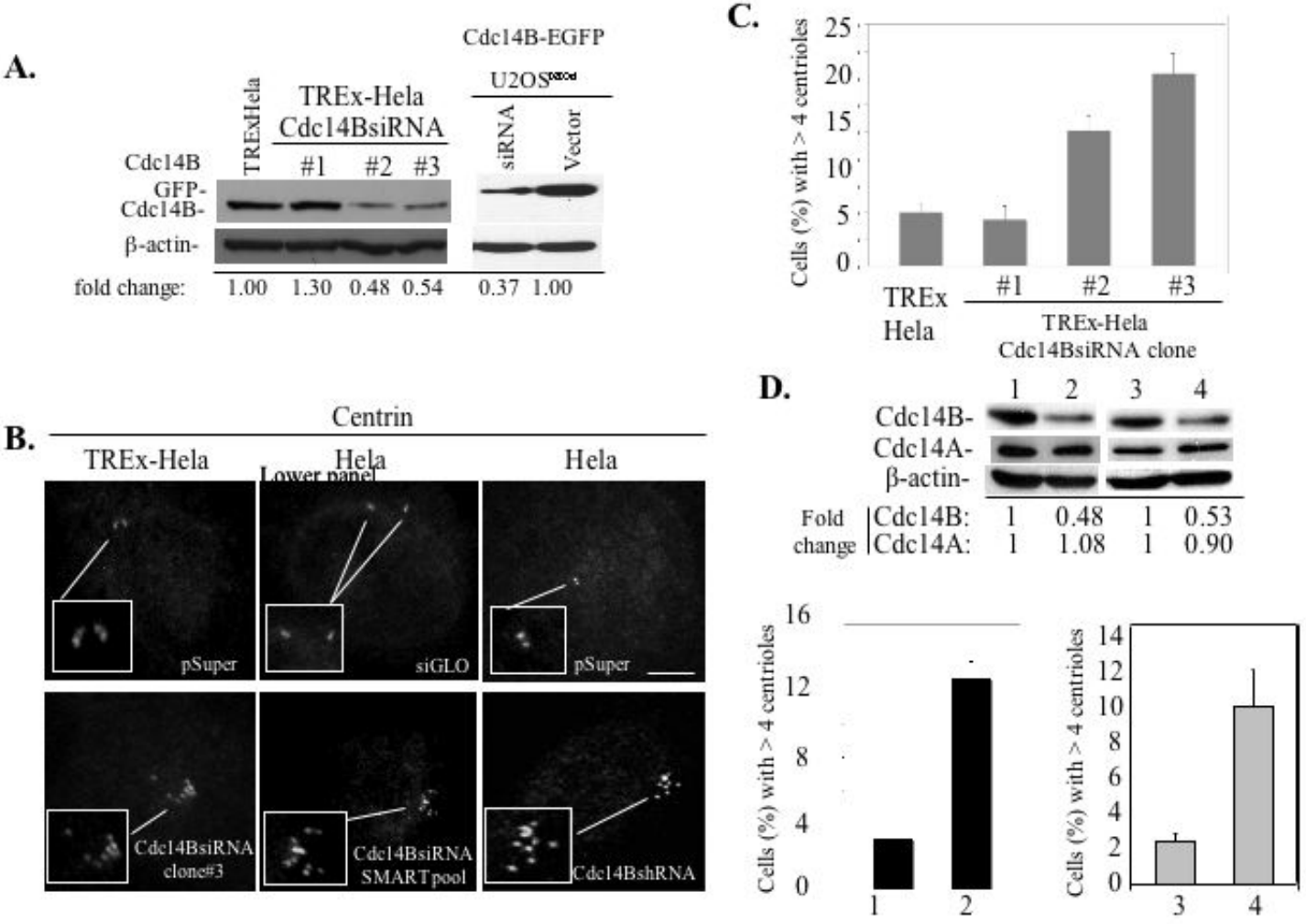
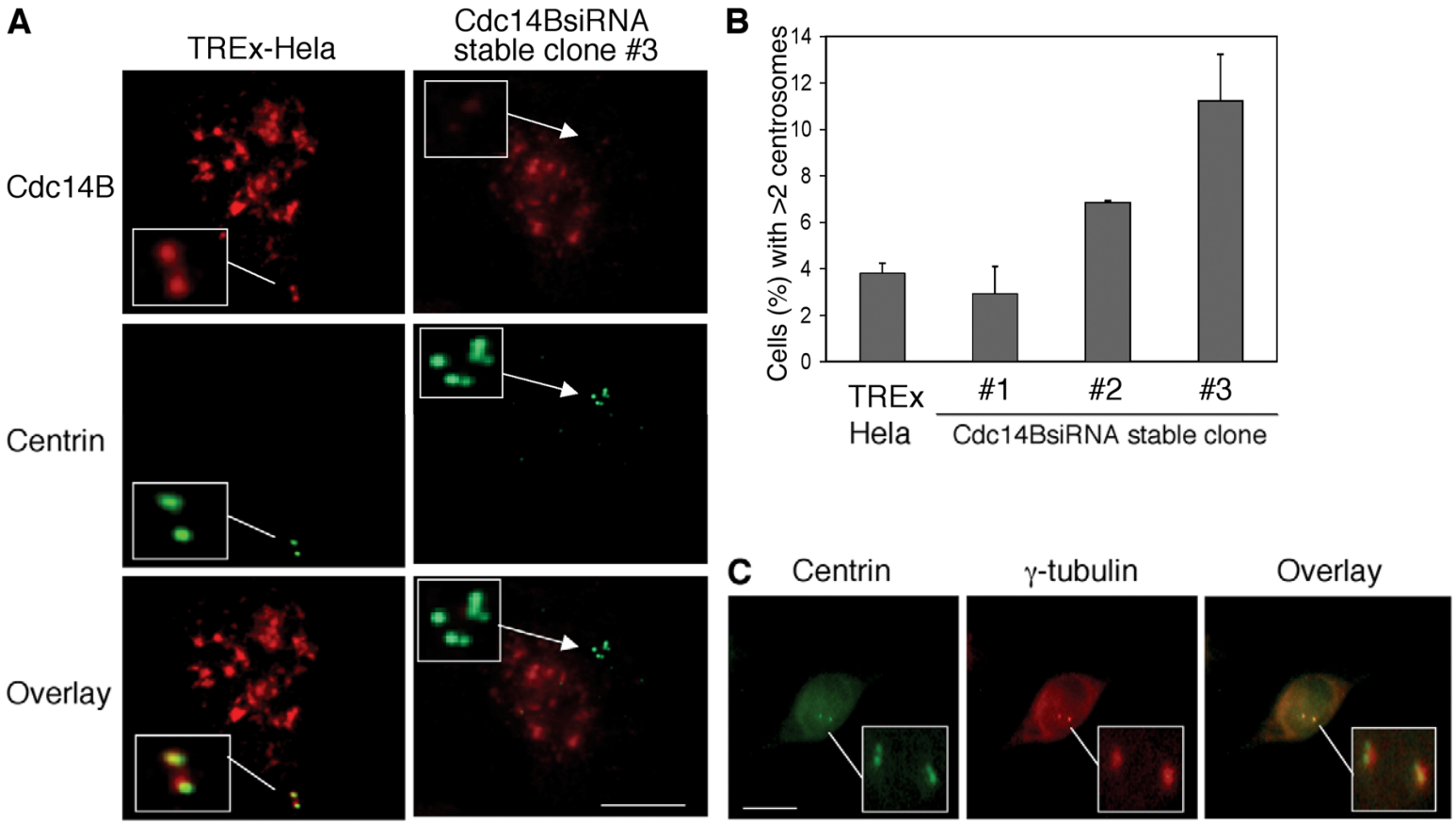

A.
Cdc14B-EGFP
U2OS
TRExHela
TREx-Hela Cdc14BsiRNA
#1
#2
#3
siRNA
Vector
Cdc14B
GFP-Cdc14B-
β-actin-
fold change:
1.00 1.30 0.48 0.54 0.37 1.00
B.
Centrin
TREx-Hela
Hela
Hela
Lower panel
pSuper
siGLO
pSuper
Cdc14BsiRNA clone#3
Cdc14BsiRNA SMARTpool
Cdc14BshRNA
C.
Cells (%) with > 4 centrioles
25
20
15
10
5
0
TREx Hela
#1
#2
#3
TREx-Hela Cdc14BsiRNA clone
D.
1 2 3 4
Cdc14B-
Cdc14A-
β-actin-
Fold change
Cdc14B: 1 0.48 1 0.53
Cdc14A: 1 1.08 1 0.90
Cells (%) with > 4 centrioles
16
12
8
4
0
1
2
Cells (%) with > 4 centrioles
14
12
10
8
6
4
2
0
3
4

Fig 4.3: Specificity of Chicken anti-Cdc14B antibody and centrosome amplification in Cdc14B knockdown cells using γ-tubulin as a centrosome marker. (A) Reduction of centriole Cdc14B expression by siRNA. TREx-HeLa and TREx-HeLa Cdc14BsiRNA stable clone No. 3 were briefly extracted with 0.5% Triton X-100, fixed in 100% cold methanol, and stained with chicken anti-Cdc14B and -Centrin-2 antibodies. Note that the extent of centriole Cdc14B knockdown varied in each TREx-HeLa Cdc14BsiRNA cell. Insets show magnified images of centrioles. Arrows indicate reduction of centriole Cdc14B expression and centriole amplification. Bar, 5 μm. (B) Depletion of Cdc14B leads to centrosome amplification. Percentage of cells with more than two centrosomes was calculated upon immunostaining with anti-γ-tubulin antibody in TREx-HeLa or TREx-HeLa-Cdc14BsiRNA stable lines (clones No. 1, 2, and 3). Data shown represent the means ± SD of three independent experiments. At least 300 cells were counted in each experiment.


A
TREx-Hela
Cdc14BsiRNA stable clone #3
Cdc14B
Centrin
Overlay
B
Cells (%) with >2 centrosomes
0
2
4
6
8
10
12
14
TREx Hela
#1
#2
#3
Cdc14BsiRNA stable clone
C
Centrin
γ-tubulin
Overlay

Careful examination of those clones with successful Cdc14B knockdown revealed a centriole amplification phenotype (Fig 4.2B [Left panel] & C). In comparison with the parental TREx-HeLa and clone #1 which did not exhibit any reduction of Cdc14B expression (Fig. 4.3A), clones #2 and #3 showed 2 to 3-fold increases in the number of cells with > 4 centrioles (Fig. 4.2B [Left panel]& C and Fig 4.3A) judged by immunostaining with anti-centrin antibody. The centriole number usually ranged from 5 to 20 with the majority around 6-8 centrioles per cell. Similar results were obtained when γ-tubulin was used as a centrosome marker (Fig 4.3B).

Ideally, siRNAs would be absolutely specific, regulating only the target gene of interest. However, a growing body of evidence suggests that this is not necessarily the case. Non-specific effects can be induced by siRNAs, both at the level of mRNA and protein (Jackson and Linsley, 2004). Therefore, to ascertain the specificity of Cdc14B knockdown phenotype and exclude the possibility of off-target effects of Cdc14B^{1234} siRNA, we tested a second small hairpin RNA duplex (989-1017, relative to Cdc14B start codon, designated as pSuper-Cdc14BshRNA-E9). After sub-cloning it into the pSuper-neo-GFP vector (OligoEngine), Stable transfectants pool were obtained by co-transfection with pBabe-puro vector into HeLa cells followed by selection with both G418 and puromycin up to a month. Compared with pSuperior-neo-GFP vector, pSuper-neo-GFP doesn't have binding sites for Tet repressor. Therefore, expression of pSuper-Cdc14BshRNA-E9 is not Tet regulatable. Western blot analysis confirmed that endogenous Cdc14B protein level was significantly reduced in these pooled HeLa stable transfectants constitutively expressing pSuper-Cdc14BshRNA-E9 (Fig 4.2D [Lanes 3

&4]).). As a control, when the same membrane was probed for Cdc14A, a paralog of Cdc14B in mammalian cells, the protein level of Cdc14A was almost the same across all the samples (Fig 4.2D [Lanes 3 &4]).). More importantly, knockdown of Cdc14B by this shRNA also led to a significant accumulation of cells with > 4 centrioles in these cells compared with the control HeLa stable pool with pSuper-neo-GFP vector backbone and pBabe-puro transfected (Fig 4.2B [Right panel] and Fig 4.2D [Lower panel 3 &4]).

In the third approach, we transiently transfected an *in vito* synthesized Cdc14B siRNA oligo pool (Dharmacon Smartpool containing four different Cdc14B siRNA oligos) each targeting different regions of Cdc14B coding sequences. As a nonspecific control, we used the siGLO control oligo (Dharmacon) that are designed in a way that no particular genes are targeted. Here, we are trying to exclude the possibility that the centrosome amplification phenotype observed in the two previous cases were due to stable clone selections. Also, a third siRNA approach will further confirm the specificity of knocking down endogenous Cdc14B. Transfection of Cdc14B smartpool, but not the siGLO control, resulted in a dramatic reduction of Cdc14B protein levels in HeLa cells as was revealed by western blot analysis (Fig 4.2D [Lanes 1 &2]). Also, probing the same membrane with anti-Cdc14A antibody showed the expression of Cdc14A was intact (Fig 4.2D [Lanes 1 &2]). Again, knockdown of Cdc14B by the Dharmacon siRNA pool resulted in a significant accumulation of cells with more than four centrioles (Fig 4.2B [Middle panel] and Fig 4.2D [Lower panel 1 &2]).

4.2.3 Ablation of Cdc14B leads to centriole amplification in normal human fibroblasts

Although HeLa cells have been widely used for centrosome studies, it is a cancer cell line which p53 pathway is frequently disrupted. HeLa is a transformed human epithelioid carcinoma cell line (CCL-2, ATCC). Although HeLa cells do not exhibit mutations in the p53 gene itself, they continuously express the E6 protein from human papillomavirus type 18 that targets the p53 tumor suppressor for degradation by the proteasome pathway. Therefore, in HeLa cells, the p53 protein level was reported to be low. Centrosome amplification phenotype is often found within cancer cells that don't have a functional p53 pathway. This is either due to direct loss of p53 function in centrosome cycle control or indirectly through absence of a p53-dependent checkpoint in the elimination of cells that arise from aborted divisions (Andreassen et al., 2001; Borel F, 2002; Meraldi P, 2002; Nigg, 2002). Indeed, in the control HeLa cells without Cdc14B knockdown, we did observe a small but significant percentage of cells harboring more than four centrioles. Thus, in order to study whether Cdc14B is involved in centrosome duplication in a normal situation, we used normal human diploid fibroblast BJ and MRC-5 cells. We transiently transfected both BJ and MRC-5 cells with the Dharmacon Cdc14BsiRNA smartpool to specifically knock down endogenous expression of Cdc14B in these cells. As a result shown in Fig. 4.4, the centriole amplification phenotype was faithfully reproduced in these normal fibroblast cells depleted of Cdc14B. These results demonstrate that depletion of Cdc14B leads to centrosome amplification in both normal and transformed cells.

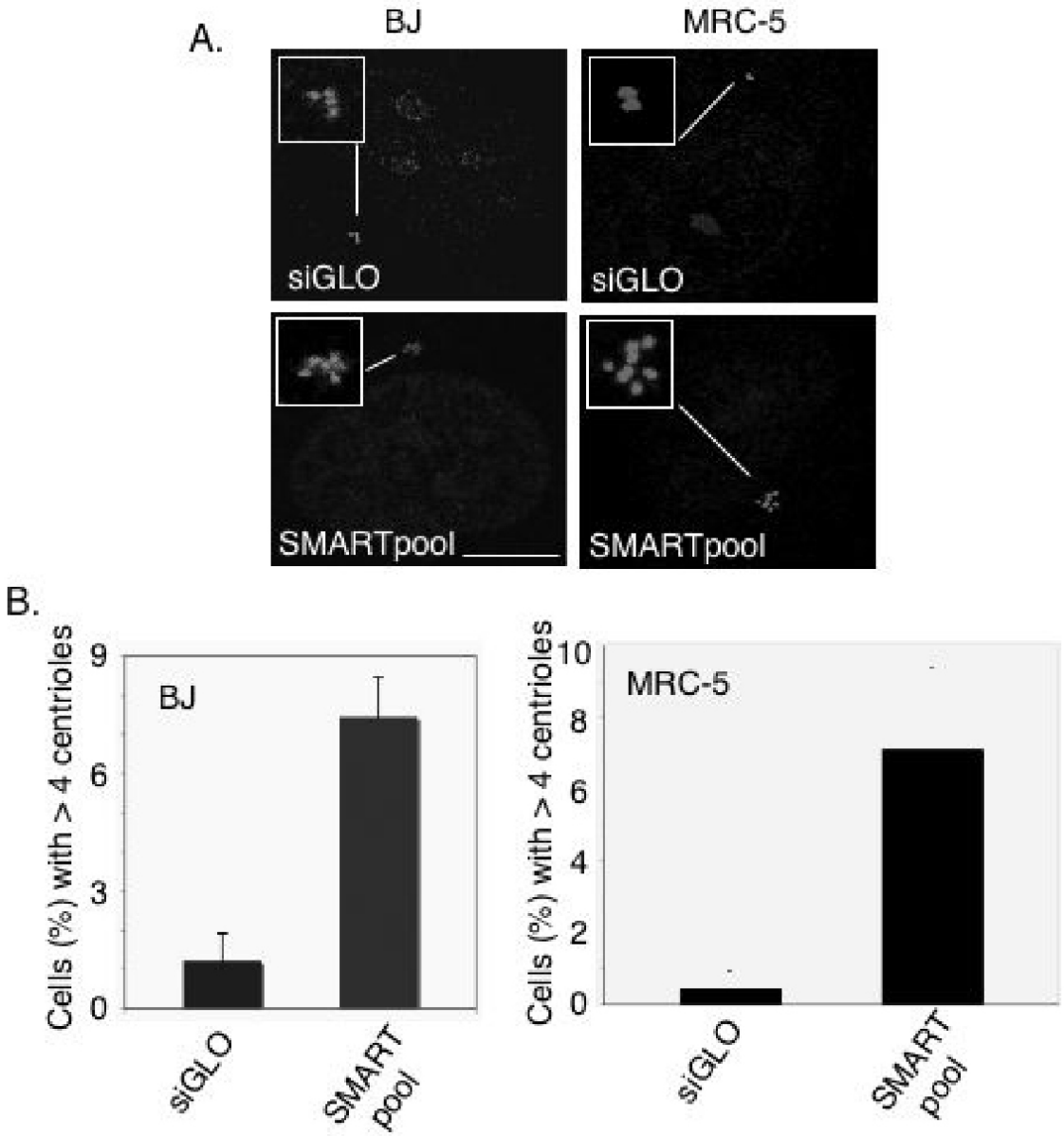
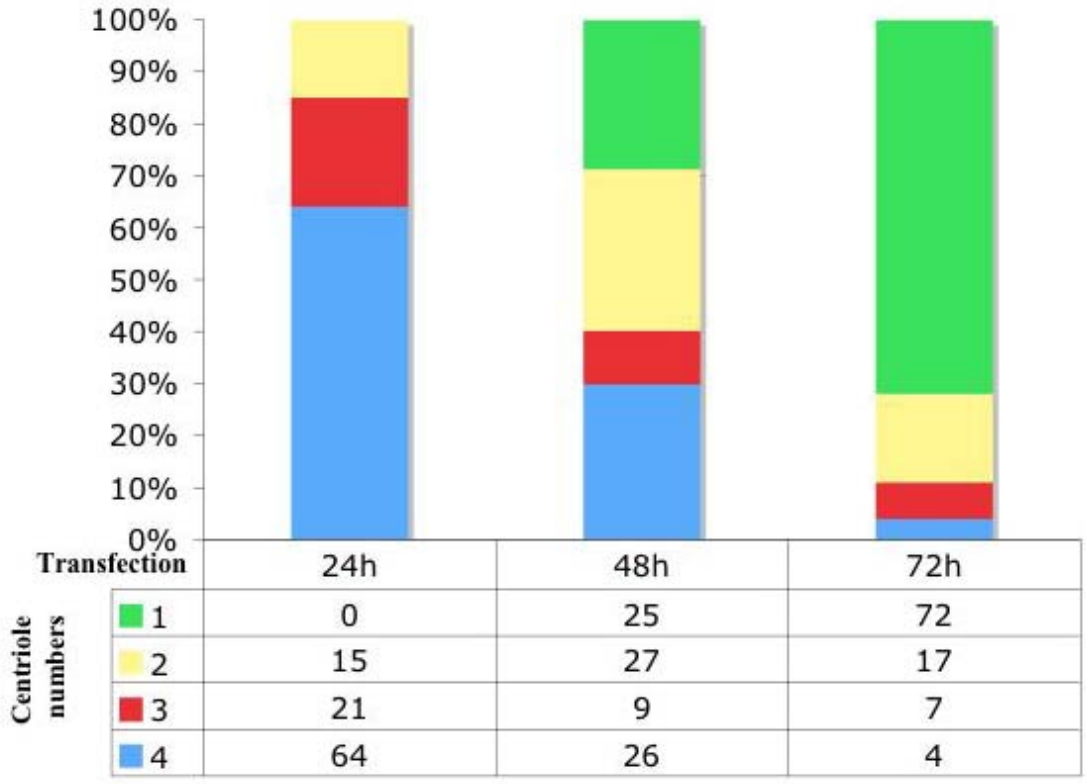


Figure 4.4: Depletion of Cdc14B causes centriole amplification in normal human fibroblast cells. (A) Representative confocal images of BJ and MRC-5 cells with clustered supernumerary centrioles are shown. Centrioles were labeled by anti-centrin antibody. Insets, magnified images of centrioles. Bar, 10 mm. (B) Percentage of cells with > 4 centrioles was calculated from the experiments shown in A. siGLO, control oligo; SMARTpool, Dharmacon SMARTpool Cdc14BsiRNAs. Data shown represent the means ± SD of three independent experiments. At least 300 cells were counted in each experiment.

4.2.4 Overexpression of Cdc14B^{WT}-EGFP leads to gradual loss of centrosomes

Our siRNA based loss-of-function experiments in both HeLa and normal human fibroblasts BJ and MRC5 cells have suggested Cdc14B's potential role in the negative regulation centrosome duplication. If this is true, we will be expecting a reduction in the number of centrioles as cells pass through successive cell cycles when excessive Cdc14B protein is present. To test this possibility, U2OS cells were transiently transfected with Cdc14B^{WT}-EGFP construct and centriole numbers were examined at 24h, 48h and 72h time points. We focused our analysis on mitotic population of the EGFP positive cells. To analyze centriole numbers, fixed cells were immunostained with an antibody against Centrin-2. Counting the number of centrioles revealed that virtually all mock transfected cells showed the expected number of centrioles, four, two at each pole, regardless of the length of incubation time (Data not shown). By contrast, examination of the Cdc14B^{WT}-EGFP positive mitotic cells revealed a progressive reduction in centriole numbers (Fig 4.5): After 24h transfection, the vast majority of all mitotic cells still appeared to form bipolar spindles haboring 4 centrioles (64 out of 100, Fig 4.5); after 48 h, however, many cells with only two or one centrioles were observed (27 and 25 out of 100, Fig 4.5); surprisingly, after 72 h of transfection, majority of mitotic cells observed only had one or no centriole (72 out of 100, Fig 4.5), and these cells appeared to be very small and seemingly was in the process of dying.



Transfection	24h	48h	72h
Centriole numbers: 1	0	25	72
2	15	27	17
3	21	9	7
4	64	26	4

Figure 4.5: Stepwise loss of centrioles in cells with overexpressed Cdc14B^{WT}-EGFP. U2OS cells were transiently transfected with Cdc14B^{WT}-EGFP and incubated for indicated times. Cells were then fixed for immunostaining with anti-Centrin-2 antibody. Cenriole numbers were counted for mitotic cells and divided into four groups: 4 centrioles (Blue), 3 centrioles (Red), 2 centrioles (Yellow) and 1 centriole (Green). Percentage of each group were calculated and plotted accordingly in the column. Totally 100 mitotic cells were counted for each indicated transfection time.

Taken together, the above results demonstrated that overexpression of Cdc14B^{WT}-EGFP in U2OS cells triggered a step-wise (4;2;1) loss of centrioles with continued passage through the cell cycle, further indicating that Cdc14B may control centriole duplication in a negative fashion.

4.2.5 Ectopic expression of centriole-associated Cdc14B inhibits centriole overduplication in prolonged S-phase-arrested cells

In certain transformed cells, such as U2OS cells, prolonged S-phase arrest by hydroxyurea (HU) or aphidicolin (APH) causes multiple rounds of centriole duplication in the absence of DNA replication and mitotic division (Balczon et al., 1995a; Chang et al., 2003). We therefore utilized this well-established centriole overduplication system to directly evaluate whether Cdc14B plays a negative role in the regulation of centriole duplication. For this purpose, we first tested if Cdc14B overexpression could inhibit HU-induced centriole overduplication in the stable doxycycline (DOX)-inducible Cdc14B^{WT}-EGFP U2OS$^{Tet\text{-}on}$ cells. Based on the localization of Cdc14B^{WT}-EGFP at centrioles (Fig. 3.1), the Cdc14B^{WT}-EGFP positive cells can be divided into two groups: Cdc14B^{WT}-EGFP at centrioles and not at centrioles. This provides an ideal system to directly examine whether localization of Cdc14B at centrioles conveys a critical function in centriole duplication. The number of Centrin-2-labeled centriole was counted in the inducible Cdc14B^{WT}-EGFP cells after cultivation in the presence of HU without DOX induction, and HU + DOX for 72 hrs. As expected, hydroxyurea treatment led to centriole amplification in un-induced Cdc14B^{WT}-EGFP cells or mock-transfected

U2OS$^{Tet\text{-}on}$ cells (Fig 4.6A). Remarkably, this centriole amplification phenotype was significantly attenuated in cells where Cdc14B^{WT}-EGFP was at centrioles, but not in cells where Cdc14B^{WT}-EGFP signal was invisible at centrioles (Fig 4.6A). This finding was confirmed in the same sets of cells treated with HU + DOX for 72 hrs using γ-tubulin as a centrosome marker (Fig 4.7A & B). Similar and yet more dramatic results were obtained in transient transfection experiments in which cells transfected with DOX-inducible Cdc14B^{WT}-EGFP exhibited an even more significant reduction of HU-induced centrosome amplification in comparison with the mock-transfected cells (Fig 4.6B). Together, these results suggest that Cdc14B overexpression suppresses abnormal centriole amplification and this inhibitory function requires the presence of Cdc14B-EGFP at centrioles in the HU-induced centriole overduplication system. Similar results were obtained with U2OS$^{Tet\text{-}on}$ cells treated with aphidicolin (Data not shown). Fluorescence-activated cell sorting (FACS) analysis revealed that induction of Cdc14B^{WT}-EGFP did not perturb cell cycle progression at the expression level set by the DOX-inducible promoter (Fig 4.6C), suggesting that the inhibition of centriole amplification in HU-arrested cells was not the result of a potential G1 cell cycle arrest.

4.2.5 Cdc14B phosphatase activity is essential to prevent centriole overduplication in prolonged S-phase arrested cells

In order to investigate whether inhibition of centriole overduplication in HU-arrested U2OS cells requires the intact phostatase activity of Cdc14B at centrioles, we took advantage of and tested the centriole-bound catalytic "dead" Cdc14B$^{K\&C}$-EGFP

Figure 4.6: Cdc14B phosphatase activity is required to prevent HU-induced centriole amplification. (A, top) Cdc14B-GFP fusion proteins were induced by 4 μg/ml DOX in the presence of 2 mM HU for 72 h in U2OS Tet-On stable cell lines carrying different Cdc14B-GFP constructs as indicated. Centrioles were visualized by anti-centrin staining (red) and overlaid with Cdc14B-GFP (green) and DAPI (blue). Note that Cdc14BC314S-GFP was not detectable at centrioles (arrow). Insets show magnified images of centrioles. Bar, 5 μm. (bottom) The percentage of cells with more than four centrioles was calculated from both induced (+DOX) and uninduced (–DOX) Cdc14B-GFP stable clones as indicated. Data shown represent the means ± SD of three independent experiments from two individual Cdc14B-GFP stable clones. At least 500 cells were counted in each experiment. (B, top) U2OS Tet-On cells were transfected as indicated. 16 h after transfection, cells were incubated with (+HU) or without (–HU) 2 mM HU and 4 μg/ml DOX for 72 h. Centrosomes were visualized by γ-tubulin staining (red). Representative centrosome amplification was detected in mock-transfected cells after HU treatment but not in pBI-tet-Cdc14BWT-GFP transfected cells where Cdc14BWT-GFP (green) associated with centrosomes. Insets show magnified images of centrosomes. DAPI (blue), DNA. Bar, 5 μm. (bottom) The percentage of cells with the indicated centrosome numbers was calculated from the experiments shown in the top panel. Centrosomes were counted in both mock and Cdc14B-GFP–transfected cells (Cdc14B-GFP–positive at centrosomes). All the data are shown as the means ± SD of three independent experiments. At least 500 cells were counted in each experiment. (C) Representative fluorescence-activated cell sorting profile on cell cycle distribution of DOX-inducible Cdc14B-GFP U2OS Tet-On stable clones. Cells were cultivated in the presence or absence of 4 μg/ml DOX for 72 h. Positions of cells with 2 N and 4 N DNA contents are labeled with arrowheads.

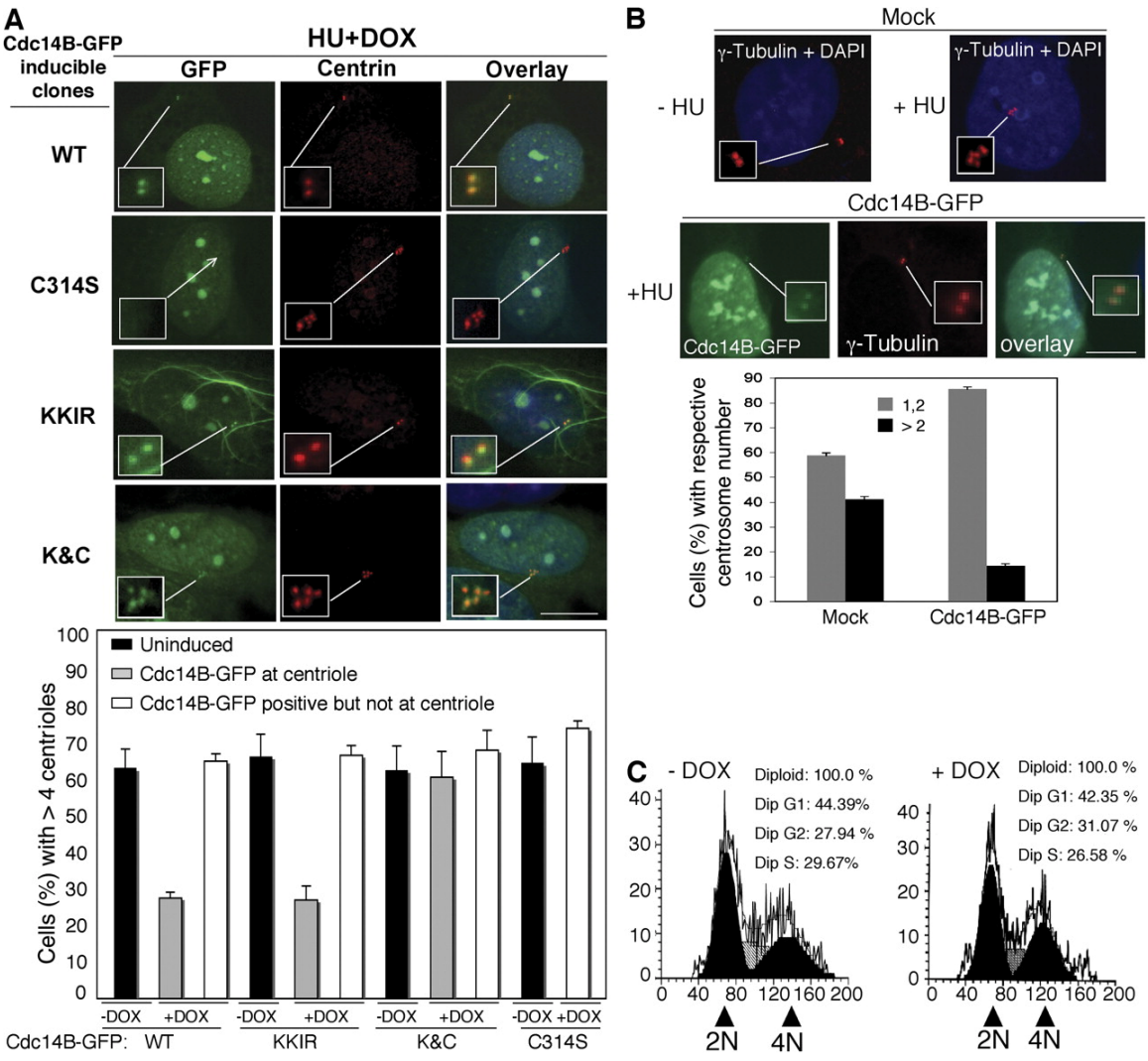
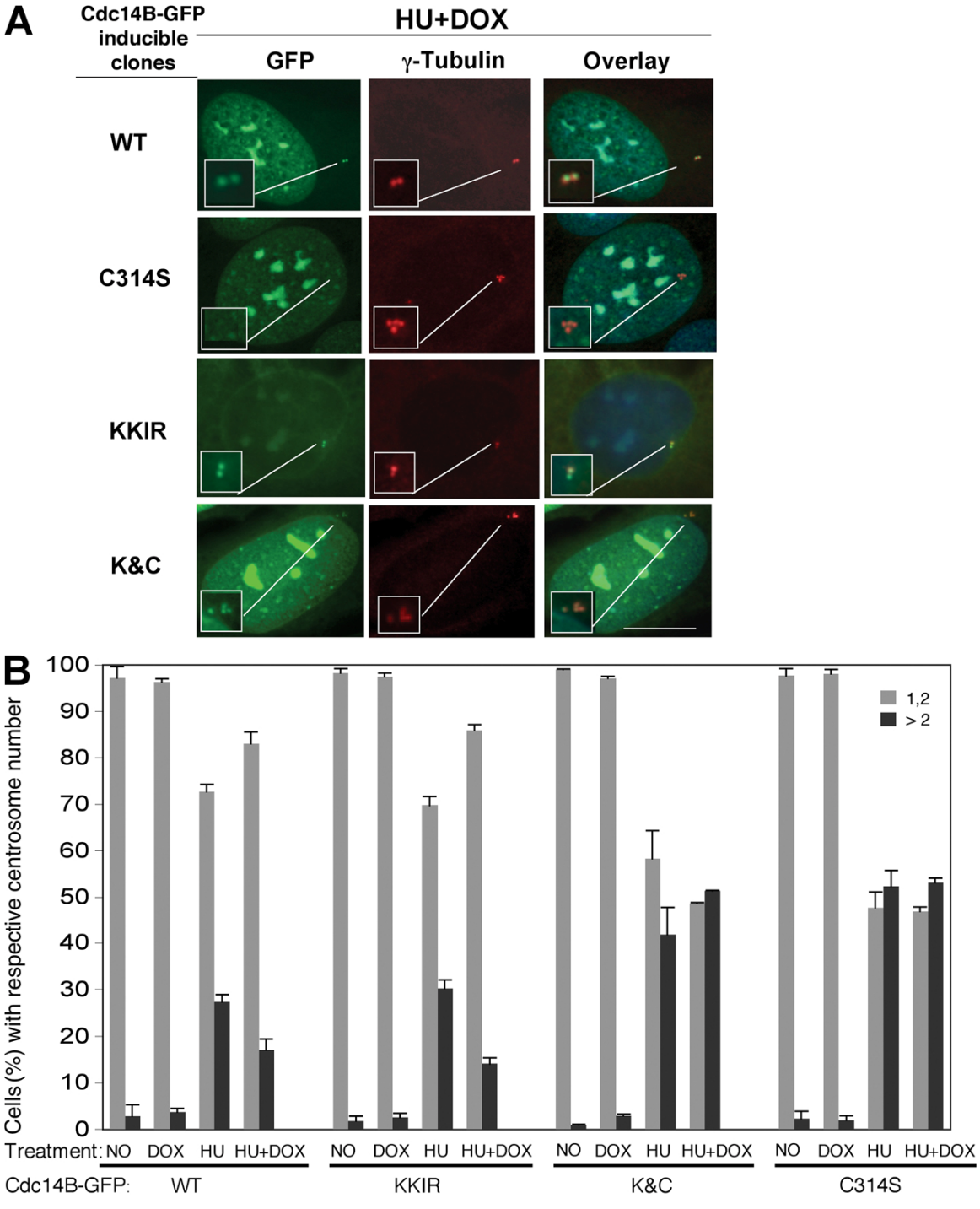

A
Cdc14B-GFP inducible clones
HU+DOX
GFP
Centrin
Overlay
WT
C314S
KKIR
K&C
Uninduced
Cdc14B-GFP at centriole
Cdc14B-GFP positive but not at centriole
Cells (%) with > 4 centrioles
-DOX
+DOX
Cdc14B-GFP: WT
KKIR
K&C
C314S
B
Mock
- HU
+ HU
γ-Tubulin + DAPI
Cdc14B-GFP
+HU
Cdc14B-GFP
γ-Tubulin
overlay
Cells (%) with respective centrosome number
1,2
> 2
Mock
Cdc14B-GFP
C
- DOX
Diploid: 100.0 %
Dip G1: 44.39%
Dip G2: 27.94 %
Dip S: 29.67%
+ DOX
Diploid: 100.0 %
Dip G1: 42.35 %
Dip G2: 31.07 %
Dip S: 26.58 %
2N
4N

Figure 4.7: Cdc14B catalytic activity is required to prevent HU-induced centrosome overduplication. (A) DOX-inducible U2OS Tet-On cell lines carrying the indicated Cdc14B-GFPs were induced by DOX in the presence of HU for 72 h. Centrosomes were visualized by γ-tubulin staining (red) and overlaid with Cdc14B-GFP (green) and DAPI (blue). Insets show magnified images of centrosomes. Bar, 5 μm. (B) The percentage of cells with normal (one or two) and overduplicated (more than two) centrosomes was calculated from the experiments shown in A and as indicated. At least 300 cells were counted in each experiment. Data shown represent the means ± SD of three independent experiments from at least two individual stable clones for each Cdc14B-GFP construct. C314S, Cdc14BC314S-GFP; DOX, 4 mg/ml doxycycline; HU, 2 mM HU; K&C, Cdc14BK&C-GFP; KKIR, Cdc14BKKIR-GFP; NO, no treatment; WT, wild-type Cdc14B-GFP. (C) Western blot analysis of Cdc14B-GFP expression in the U2OS Tet-On stable clones described in A.


A
Cdc14B-GFP inducible clones
HU+DOX
GFP
γ-Tubulin
Overlay
WT
C314S
KKIR
K&C
B
Cells (%) with respective centrosome number
100
90
80
70
60
50
40
30
20
10
0
1,2
> 2
Treatment: NO DOX HU HU+DOX NO DOX HU HU+DOX NO DOX HU HU+DOX NO DOX HU HU+DOX
Cdc14B-GFP: WT KKIR K&C C314S
C
Cdc14B-GFP: WT KKIR K&C C314S
DOX: - + - + - + - +
Cdc14B-GFP
-82 kD
β-actin
-43 kD
fold change: 1 0.93 0.98 1.09

mutant as described in chapter three (Fig 3.8). When the Cdc14B$^{K\&C}$-EGFP cells were exposed to HU, the DOX-induced Cdc14B$^{K\&C}$-EGFP was unable to block centriole overduplication, whereas the centriole-associated catalytic "active" Cdc14B^{KKIR}-EGFP mutant was as potent as its wild-type counterpart in inhibition of centriole overduplication (Fig. 4.6A). Similar results were obtained when the HU-treated cells were examined by γ-tubulin staining (Fig 4.7A & B). Western blot analysis revealed that all the Cdc14B-EGFPs expressed at the comparable level after DOX induction (Fig. 4.7C), indicating that failure of Cdc14B$^{K\&C}$-EGFP to inhibit centriole overduplication was not due to its expression level. This finding strongly argues that Cdc14B phosphatase activity is indispensable for the inhibition of centriole duplication and that docking a catalytically inactive Cdc14B-GFP to centrioles is not sufficient to prevent centriole overduplication.

4.2.6 Inhibition of Z-L_3VS - induced centriole overduplication requires Cdc14B phosphatase activity

It has been documented that treatment of U2OS cells with a proteasome inhibitor Z-L_3VS causes multiple daughter centriole growth from a single mother centriole template (Duensing et al., 2007a). This aberrant daughter centriole overduplication requires both Cdk2/Cyclin-E and Plk4 activities, the known positive regulators of centriole duplication (Nigg, 2007b). We thus used the Z-L_3VS induced centriole duplication system to evaluate the possibility of Cdc14B as a counterbalancing phosphatase of Cdk2/Cyclin-E and/or Plk4 in centriole duplication control. In the absence

of Cdc14B^{WT}-EGFP induction (DOX), treatment of U2OS$^{Tet\text{-}on}$-Cdc14B^{WT}-EGFP cells with Z-L_3VS evoked an aberrant centriole overgrowth, whereas in the presence of Cdc14B^{WT}-EGFP induction (+DOX), centriole-bound Cdc14B^{WT}-EGFP significantly attenuated the centriole overduplication phenotype (Fig 4.8). Moreover, similar to our observation in the HU experiment (Fig 4.6 A and 4.7 A & B), the centriole-bound catalytic dead Cdc14B$^{K\&C}$-EGFP mutant failed to prevent Z-L_3VS induced centriole overduplication (Fig 4.8), indicating that this inhibition also requires Cdc14B catalytic activity. Although additional experiments are required, our study supports the possibility that Cdc14B may counterbalance centrosomal kinases required for centriole overduplication in the Z-L_3VS induction system.

4.3 Discussion

RNA interference has provided biologists with a very powerful tool with which to turn off expression of a particular gene of interest. Previously, similar loss-of-function study using other approach such as knock out technology was proven to be very difficult, expensive and time-consuming in many organisms, especially mammals. RNA interference has made discovering the unknown function of a known gene which is also referred as 'reverse genetics' to be much more straightforward not only in mammals, but also in flies and worms.

Up to date, there are no Cdc14B knockout mice available. Therefore, RNAi approach maybe one of the few ways to perform loss-of-function studies on Cdc14B in mammals.

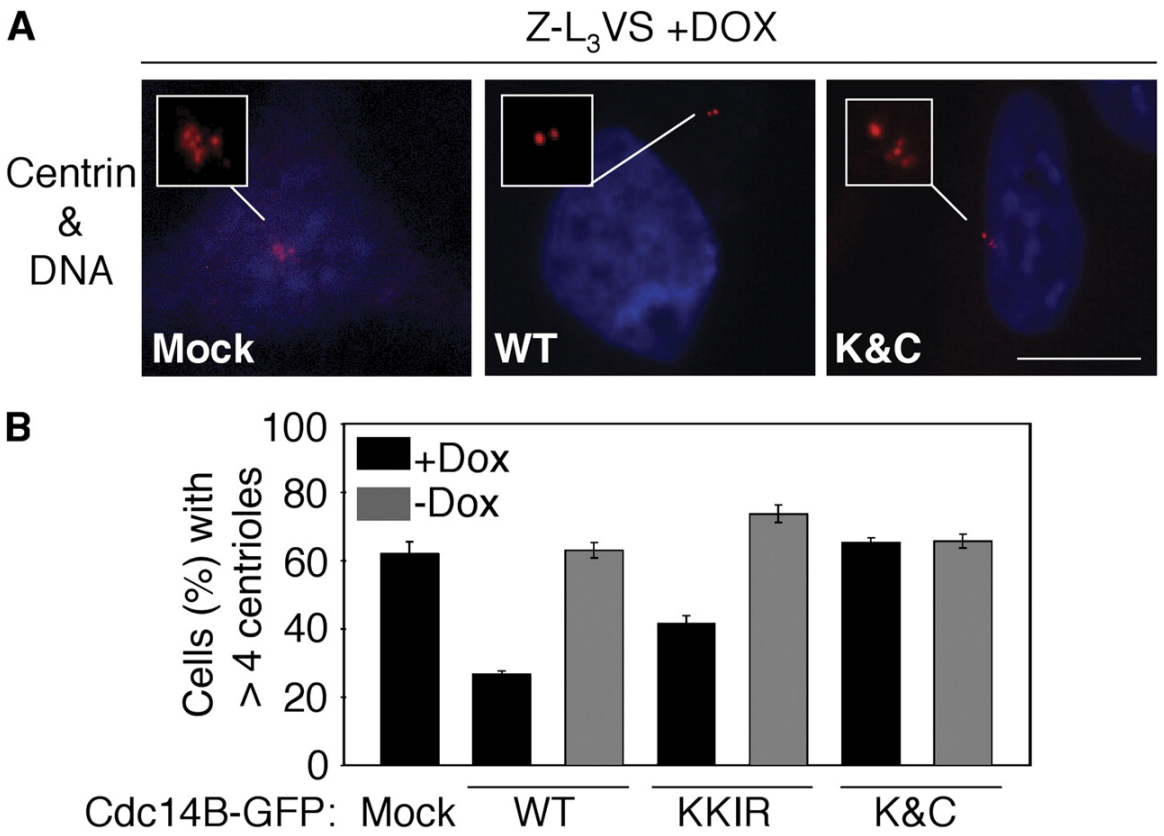


Figure 4.8: Cdc14B phosphatase activity is required to prevent Z-L3-VS–induced centriole overduplication. (A) Dox-inducible U2OS Tet-On cell lines carrying the indicated Cdc14B-GFPs and mock controls were treated with 1 μM Z-L3-VS in the presence or absence of 4 μg/ml Dox for 48 h. Representative centrioles (red) were visualized by an anti-centrin antibody and DNA (blue) was visualized by DAPI. Bar, 5 μM. (B) The percentage of cells with more than four centrioles was calculated from the experiments shown in A and as indicated. Centrioles were counted in mock, uninduced controls (–Dox) and Cdc14B-GFP–induced (+Dox) cells (Cdc14B-GFP–positive at centrioles). All the data are shown as the means ± SD of three independent experiments. At least 400 cells were counted in each experiment.

Here, we have used three independent RNAi approaches to switch off expression of Cdc14B in both transformed and non-transformed human cell lines. Despite the differences in knock down efficiencies, all three approaches confirm the same phenotype when Cdc14B is depleted in these cells, which is centrosome amplification. Centrosome amplification leads to aberrant mitotic spindle formation with more than two spindle poles, and subsequent chromosome segregation errors and genomic instability. Typically, normal cells either have two or four centrioles depend on the cell cycle. In our studies, when Cdc14B expression was down regulated in either stable siRNA clones or transient transfection, there was significant increase of the percentage of cells harboring more than four centrin dots (Fig 4.2), indicating unscheduled centriole duplication has occurred. This is the first study to dissect Cdc14B's novel function in centrosome cycle control even its localization on centrosomes has been suggested by several previous reports. Interesting, according to a recent study, when both Cdc14B loci were homozygously disrupted in human somatic cells, there were no evident lack of defects in spindle assembly, anaphase progression, mitotic exit, and cytokinesis, contrary to several previous studies (Berdougo E, 2008). It's tempting to know whether centrosome amplification phenotype exists in these Cdc14B null cells.

Supernumerary centrosomes we observed from Cdc14B depleteted cells, however, can arise from fundamentally distinct mechanisms:

The first possible mechanism is deregulation of centrosome duplication control. And we believe this is the case with our studies on Cdc14B. Normal centrosome duplication

involves proper timing for initiation of duplication and suppression of re-duplication of already duplicated centrosomes. If either or both of these controls are deregulated, centrosomes will duplicate more than once within a single cell cycle, also called centrosome re-duplication which results in supernumerary centrosomes (Balczon et al., 1995b; Meraldi et al., 1999).

A second plausible mechanism for the generation of cells with supernumerary centrosomes involves an aborted cell division. Interestingly, examination of TREx-HeLa Cdc14B^{1234}siRNA stable cells revealed a slight increase in the number of polyploid cells, such as multinucleate and macronuclear cells (Fig 4.9). Macronuclear and multinucleate cells were determined by visual judgment of the sizes (nuclear diameter and area) of DAPI-stained nuclei and the number of nuclei per cell respectively. It is important to note that among the TREx-HeLa Cdc14B^{1234}siRNA stable cells with centriole amplification phenotype, only about 1/5 (n= 300) were polyploid. Using the same criteria, however, we were unable to detect any significant increase of polyploid cells in HeLa cells transiently transfected with Dharmacon Cdc14BsiRNA smartpool (Fig. 4.9). In line with this finding, flow cytometry analysis did not reveal any significant increase of polyploidy in those Cdc14B knockdown cells (Fig. 4.9). Moreover, none of the Cdc14B-depleted BJ (0%; n=115) or MRC-5 (0%; n=74) cells with >4 centrioles were polyploid. Thus the centriole amplification phenotype in Cdc14B knockdown cells may not be the product of aborted cell division, and the slight increase in the number of polyploid cells in the TREx-HeLa Cdc14BsiRNA stable clones may have evolved during the course of stable clone selection.

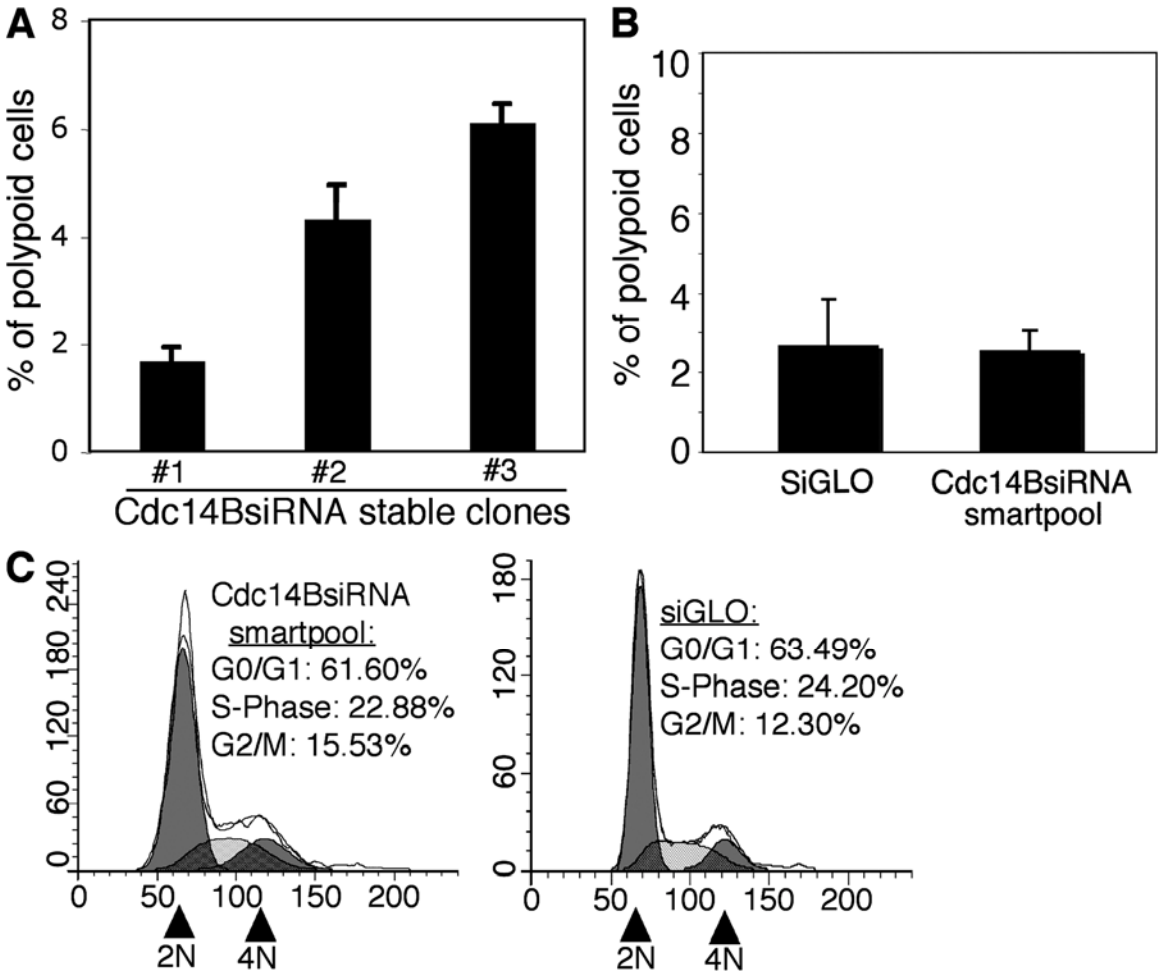


Figure 4.9: Polyploidy in Cdc14B knockdown cells. Polyploid (macronuclear and multinucleate) cells in TREx-HeLa Cdc14BsiRNA stable clones (A) and HeLa cells transiently transfected with SiGLO control oligo and Cdc14BsiRNA SMARTpool (B). Polyploid cells with more than four centrioles were determined by DAPI and centrin staining. All the data are presented as the means ± SD of three independent experiments. At least 900 cells were counted in each experiment. (C) Representative fluorescence-activated cell sorting profile on cell cycle distribution of Hela cells transfected with Cdc14BsiRNA SMARTpool and siGLO for 72 h. The positions of cells with 2 N and 4 N DNA contents were labeled with arrowheads. Note that no significant tetraploid peak (8 N) was observed in Cdc14BsiRNA SMARTpool transfected cells.

A third possiblility is that supernumerary centrioles as observed using Centrin-2 as centriole marker are caused by centrosome fragmentation. In this case, additional γ-tubulin or centrin dots may arise from fragments of PCM/Centriole instead of intact centrosomes. To exclude this possibility, we carefully evaluated the γ-tubullin-decorated centrosomes in HeLa cells transiently transfected with Cdc14BsiRNA smartpool. We found that a majority of the supernumerary centrosomes were clustered together and similar in size (Fig. 4.2B). All the supernumerary centrosomes (n=105) contained centrin-labeled centrioles and many of the centrioles were in pairs (Fig 4.3C). These data suggest that the supernumerary centrosomes may not arise from centrosome fragmentation.

Certain tumour-derived cell lines for example U2OS osteosarcoma cells can be induced to undergo several rounds of centrosome re-duplication when the DNA replication is arrested for an extended period of times by drugs such as hydroxyurea or aphidicolin (Balczon et al., 1995b; Meraldi et al., 1999). This in vito centrosome duplication system proves to be a good assay for studying proteins involved in centrosome duplication. The findings that overexpression of Cdc14B inhibits HU induced centriole overduplication directly confirmed Cdc14B's essential role in the regulation of centriole duplication cycle. In order to suppress the centrosome ampification phenotype caused by HU, $Cdc14B^{WT}$-EGFP has to localize to the centrosomes. As is shown in Fig 4.6 & 4.7, when $Cdc14B^{WT}$-EGFP was not found at centrosomes, centrosome amplification still persisted in these cells. Therefore the centriole-bound catalytic "dead" $Cdc14B^{K\&C}$-EGFP mutant came as convenient tool to study whether Cdc14B's phosphatase activity is also responsible for its function in centrosome duplication control beside its centrosomal localization. And

indeed, our findings supported the claim that catalytic activity of Cdc14B is required to harness centriole overduplication in HU experimental systems (Fig 4.6 & 4.7). Cdc14B may exert its effect through modulating the phosphorylation status of its substrates on centrosomes, in particular those involved in the control of centrosome duplication. It has been well-documented that the activities of centrosome-associated protein kinases, such as Cdk2/Cyclin-E/A, Plk2, Plk4, calcium-calmodulin kinase II, MpsI and etc are required for centriole duplication in various species and experimental settings (Duensing et al., 2007b; Fisk et al., 2003; Fisk and Winey, 2001; Habedanck et al., 2005b; Kleylein-Sohn et al., 2007b; Matsumoto et al., 1999b; Matsumoto and Maller, 2002; Meraldi et al., 1999; O'Connell et al., 2001b; Tsou and Stearns, 2006e; Warnke et al., 2004), suggesting that phosphorylation plays an important role for the precise reproduction of centrosomes during the cell cycle (Nigg, 2007a; Tsou and Stearns, 2006d). Phosphorylation of centrosome-associated proteins, such as nucleophosmin/B23, CP110 and Mps1 has been implicated in centrosome duplication control. In particular, phosphorylation of B23 Thr199 by Cdk2/Cyclin-E and of B23 Thr95 by an unknown kinase(s) dissociates B23 from centrosomes, which in turn allows centrosome duplication or re-duplication to occur (Budhu and Wang, 2005; Okuda et al., 2000b). Likewise, depletion of CP110 abolishes centrosome re-duplication in S-phase arrested cells (Chen et al., 2002b) and suppresses Plk4-induced procentriole re-duplication (Habedanck et al., 2005b), whereas inactivation of Cdk2 activity abolishes Mps1-dependent centrosome duplication (Fisk and Winey, 2001). Based on our observation in this study, it is possible that Cdc14B regulates centriole duplication cycle through counterbalancing centrosomal kinases through modulating the phosphorylation status of potential common centrosomal substrates, such

as B23, CP110 and Mps1.

A counter-balance mechanism of kinase and phosphatase has been proposed for governing centrosome splitting (Meraldi and Nigg, 2001). A similar mechanism may prevail in the regulation of centrosome duplication. Despite the significance of so many kinases characterized in the process, to date no phosphatase that may counterpoise the kinase effect has been identified to our knowledge.

Cdc14B has been implicated in the regulation of nuclear structure maintenance (Nalepa and Harper, 2004), microtubule dynamics (Cho et al., 2005a), mitotic exit control (Dryden et al., 2003b) and G1 progression (Rodier et al., 2008). The abnormal centrosome amplification observed in Cdc14B-depleted cells may arise from the combinatorial events involving failures in nuclear structure, cell and centrosome cycle regulation. However, because active Cdc14B can directly inhibit unscheduled centriole overduplication, and Cdc14B depletion leads to centriole amplification in the absence of obvious polyploidy in normal human fibroblast cells, it is possible one of Cdc14B functions is to serve as a centrosomal regulatory protein, and compromising centrosomal regulatory function of Cdc14B may account for at least part of the observed centriole amplification phenotype. Since it is frequently down-regulated in tumor cells (Ashida et al., 2004a; Martinez et al., 2003a; Neben et al., 2005a; Rubio-Moscardo et al., 2005; Wong et al., 1999; Yu et al., 2004b), Cdc14B may function as a tumor suppressor to maintain the fidelity of centrosome duplication cycle and genomic stability in human cells.

4.4 Summary

In summary, we have shown that: siRNA mediated depletion of Cdc14B expression led to centrosome duplication in both Hela cells and normal human fibroblasts (BJ and MRC-5); Overexpression of Cdc14B resulted in progressive loss of centrioles; overexpression Cdc14B could also suppress centriole overduplication in several established *in vitro* centriole duplication systems: HU/APH treated U2OS or CHO cells and Z-L_3VS treated U2OS cells. Taken together, these evidences support the negative role of Cdc14B in centrosome duplication control.

4.4 Experimental Procedures

4.4.1 Cell lines, Transfection and Drug treatment

U2OS$^{Tet\text{-}on}$ (BD Biosciences), U2OS (Invitrogen), HeLa, and TREx-HeLa (Invitrogen) cells were cultured as described previously (Cho et al., 2005a). Normal human fibroblasts BJ and MRC-5 cells were purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium supplied with 10% fetal bovine serum, 50 U/ml penicillin and 50 ug/ml streptomycin. To establish stable cell lines carrying DOX-inducible Cdc14B-EGFP, U2OS-TetON cells were co-transfected with pBI-tet-Cdc14B^{WT}-EGFP, pBI-tet-Cdc14B^{KKIR}-EGFP, pBI-tet-Cdc14B^{C314S}-EGFP, or pBI-tet-Cdc14B$^{K\&C}$ -EGFP plasmid (Cho et al., 2005a) and pBabe-puro vector (a kind gift from Dr. Gerald Evans) by FuGene6 (Roche) following the manufacture's protocol. Stable clones were obtained after selection with G418 (500 μg/ml) and puromycin (2 mg/ml) in the absence of DOX. For prolonged S-phase arrest experiment, the Cdc14B-EGFP stable

clones were treated with or without 4 mg/ml DOX, 2 mM hydroxyurea (HU, Sigma) alone, or 2 mM HU and 4 mg/ml DOX together for 72 hrs.

4.4.2 Cell cycle analysis

For cell cycle analysis, ~1x10^6 HeLa cells were collected and span down at 1,000 rpm for 3 minutes. Cell pellet was resuspended in 474ul of cold PBS and followed by fixation with 526ul of 95% ice-cold ethanol for 10 minutes. The fixed cells were then span down again and resuspended in 500ul PBS. Then 1 ul of 10mg/ml RNase A was added and incubated at 37C for 30 minutes. Finally, 5 ul of 1mg/ml propidium iodide were added to cells and then analyzed by a fluorescence-activated cell sorter (GUAVA). The percentage of cells in difference phases of the cell cycle was determined using ModFit LT software.

4.4.3 siRNA and shRNA experiments

Cdc14B protein was depleted using the following three approaches: First, small interfering RNA oligos that target the sequences 5'-GAACCCGAACCGTACAGTG-3' (1234-1254, relative to the start codon of Cdc14B) were annealed and cloned into the HindIII and BglII sites of pSuperior-neo-GFP vector (OligoEngine). Tetracycline-inducible cell lines expressing pSuperiorCdc14B^{1234}siRNA were generated by co-transfection with pBabe-puro (a kind gift from Dr. Gerald Evans) into TREx-HeLa cells that contain Tet repressors using Lipofectamine PLUS (Invitrogen) following the manufacture's recommended protocol. Stable clones were established by selection in a

growth medium containing 800 mg/ml G418 and 1 mg/ml puromycin in the absence of tetracycline. Before functional studies, pSuperiorCdc14B^{1234}siRNA stable clones were induced with 1 μg/ml tetracycline for 24-hrs and harvested.

Second, a small hairpin RNA expression vector carrying a Cdc14B shRNA oligo (989-1017, relative to Cdc14B start codon; cat#RHS-1271-8957575) was obtained from OpenBiosystems, and subcloned into the pSuper-neo-GFP vector (OligoEngine), which was designated as pSuper-Cdc14BshRNA-E9. Stable transfectants were obtained by co-transfection with pBabe-puro into HeLa cells followed by selection with 800 μg/ml G418 and 1μg/ml puromycin up to a month.

Third, Cdc14BsiRNA smartpool (targeting four different regions of Cdc14B mRNA sequences) and siGLO control oligo were obtained from the siGENOME collection (Dharmacon, Lafayette, CO) and transfected into HeLa, BJ and MRC-5 cells using siPORT NeoFX reagent (Ambion) or Dharmafect I transfect reagent (Dharmacon, Lafayette, CO) according to the manufacturer's recommendations followed by up to 96h incubation.

4.4.4 Western Analysis

Cultures were washed twice in ice-cold phosphate-buffered saline (PBS), lysed in lysis buffer containing 1% of SDS and Tris-Hcl at pH 7.4, samples were then boiled in 6X SDS sample buffer for 10 min, votexed and centrifuged at 13,000g for 10 min. For

western blotting, protein lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). Immunoblots were then probed with antibodies specific to Cdc14A (Zymed), Cdc14B (Zymed), GFP (BD Pharmingen) and β-actin (Cytoskeleton).

4.4.5 Immunofluorescence

For indirect immunofluorescence, cells were grown on glass coverslips, and fixed with either paraformaldehyde or cold 100% methanol. The cells were then permeabilized with PBS/0.5% Triton X-100 for 10 min followed by blocking with PBS/1% BSA for 30 min. Centrosomes or centrioles were visualized by immunostaining with antibodies against γ-tubulin (GTU-88, Sigma; C-20, Santa Cruz), pericentrin (Abcam) or centrin (20H5; a kind gift from Dr. J. Salisbury, Mayo Clinic Foundation). For visualization of centriole-associated endogenous Cdc14B, cells were treated without or with 10 μg/ml nocodazole (for better exposure of centrosomal Cdc14B) for 2 hrs, briefly extracted with 0.5% Triton X-100 on ice and fixed with cold 100% methanol and immunostained with chicken anti-Cdc14B antibody (GenWay Biotech). In our hands, methanol fixation preserved GFP signals and thus anti-GFP immunostaining was not employed to visualize Cdc14B-GFP fusion proteins in the fixed cells. Both GFP and chicken anti-Cdc14B antibodies recognized Cdc14B-GFP (data not shown). Secondary antibodies including Alexa Fluor 488 and 594 donkey-anti-mouse, -anti-goat, goat-anti-rabbit, and -anti-chicken IgY antibodies were purchased from Molecular Probes. DNA was counter-stained by DAPI. Cells were visualized with a 100 x Plan-Neofluar objective (1.30 oil;

∞/0.17) under an epi-fluorescence microscope (Axioskop 2, Carl Zeiss Inc). Images were acquired with a charge-coupled device camera (AxioCam HRC) controlled by Openlab software (version 3.5, Improvision Inc). For confocal microscopy, images were captured with a HCX-PL/APO 63 X 1.32 oil objective under a Leica SP2 laser scanning confocal microscope equipped with Leica confocal software (LCS Lite V. 2.0; Leica Microsystems Inc). The coverslips were mounted using PermaFlour Mountant media (Thermo Electron Inc) and imaging was performed at room temperature. Image processing was performed using Photoshop CS (V8.0).

Chapter 5

Potential counterbalance effect between Cdc14B phophatase and Plk4 kinase in regulating centrosome duplication

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5.1 Introduction

The C. elegans Zyg1 kinase is among the five proteins suggested to be important for centrosome duplication in embryogenesis (O'Connell et al., 2001a). Zyg1 was first identified in a genetic screen for temperature sensitive embryonic lethal mutants in the nematode C. elegans (Wood et al., 1980). Another genetic screen showed that Zyg1 activity is not only indispensable for embryonic development, but also requireed throughout development (O'Connell et al., 1998). Interestingly, in Zyg1 mutant embryos, the centrosomes failed to reproduce during the first or second round of division and resulted in blocked formation of a bipolar mitotic spindle, suggesting Zyg1's possible role in centrosome duplication (O'Connell et al., 1998). The first direct evidence of Zyg1's role in centrosome duplication came from a recent study in 2001. In this study, the authors demonstrated that Zyg1 was required for daughter centriole formation in early embryos with paternal Zyg1 activity required for centrosome duplication during the first cell cycle while maternal Zyg1 activity regulated centrosome duplication during later cell cycles (O'Connell et al., 2001a). Immunoflurescence analysis showed that Zyg1 appeared

on centrosomes from late anaphase till telophase but was absent from centrosome during interphase and prophase (O'Connell et al., 2001a). Since S phase starts right after telophase in early embryos, Zyg1 may be at the right place at the right time to promote centrosome duplication (Nigg, 2004).

The kinase Plk4 (also known as Sak) has unequivocally been identified as a positive regulator for centriole duplication in both human and Drosophila (Bettencourt-Dias et al., 2005; Habedanck et al., 2005a). Although Plk4 shares only low sequence similarity with *C.elegans* Zyg1, it is has been proposed to be the functional equivalent of *C. elegans* Zyg1 (Nigg, 2007b). Plk4 was originally cloned as a short-lived protein kinase subjected to cell cycle regulation. It is maximally expressed in testis and other actively dividing tissues (Fode C, 1994; Fode, 1997). $Plk4^{-/-}$ mouse embryos arrest after gastrulation at E7.5, displaying an increased incidence of apoptosis and anaphase arrest, indicating that Plk4 is essential for embryogenesis in mice (Hudson et al., 2001; Swallow et al., 2005). And, interestingly, adult $Plk4^{+/-}$ mice were prone for tumor formation, with spontaneous tumors developing in the liver and other organs, indicating that deregulation of Plk4 kinase can contribute to tumorigenesis (Ko et al., 2005). More importantly, when Plk4 activity is absent, both human and Drosophila cells will gradually lose centrioles through impaired centrosome duplication, resulting in severe abnormalities in spindle formation. On the contrary, overexpression of Plk4 in human cells resulted in centrosome overduplication, indicating a crucial role of Plk4 in regulating centriole duplication (Habedanck et al., 2005; Rodrigues-Martins et al., 2007). It is of particular interest that further electron microscopy analysis of Plk4 overexpressed cells revealed multiple

centriole precursors surrounded a single parental centriole, which often formed rosette-like structures in cross-sections (Habedanck et al., 2005a). No rosettes structures were formed, however, when a catalytically inactive Plk4 was overexpressed, demonstrating that their formation was strictly dependent on kinase activity (Habedanck et al., 2005a). In addition, this remarkable phenotype also relied on Plk4's association with centrioles. Pooled together, these evidences suggested that procentriole formation might critically depend on the phosphorylation of one or more substrates by Plk4 at the procentriole assembly site. Therefore, it's tempting to hypothesize that centrosomal Plk4 kinase activity is needed to phosphorylate a centrosomal substrate which in turn triggers the formation of a "seed" either through protein stabilization of recruitment that initiates the procentriole assembly process; When deregulated, unchecked phosphorylation of the substrate may promote the formation of multiple "grow" sites for the assembly of procentrioles which lead to multiple centriole progeny formation. If so, in normal cell cycle, Plk4 kinase activity will be expected to be closely balanced by phosphatases to prevent the simultaneous formation of multiple centrioles (Nigg, 2007b).

Multiple procentrioles formation around one maternal centriole is not limited only to Plk4 overexpression. Similarly, several procentrioles assembling from the base of one mother centriole has been previously observed in CHO or U2OS cells blocked in S phase (Balczon et al., 1995b; Meraldi et al., 1999); Also, as discussed in Chapter 4, treatment of human cells with a peptide vinyl sulfone proteasome inhibitor Z-L_3VS triggered more than one daughter formation at maternal centrioles in certain cells. And this effect requires Cdk2/Cyclin-E and more importantly, Plk4 activity (Duensing et al., 2007a);

Moroever, the same study showed human papillomavirus type 16 E7 oncoprotein could also promote the same phenotype (Duensing et al., 2007a); Most interestingly, a recent study by Strnad et al demonstrated that increased human Sas-6 levels also triggered formation of more than one procentriole per centriole (Strnad et al., 2007).

Like Zyg-1, Sas-6 is also among the five core components required for daughter centriole assembly in *C.elegans* (Dammermann et al., 2004; Leidel et al., 2005). Unlike Zyg-1 which was first discovered through forward genetic screens, the identification of Sas-6 as required for centriole formation in *C.elegans* benefited from comprehensive RNAi-based reverse genetic screens. In Sas-6 (RNAi) embryos, during the one-cell stage a biopolar spindle was formed, however, a monopolar spindle assembled in each blastomere at the two-cell stage, suggesting Sas-6 maybe required for normal centrosome duplication (Dammermann et al., 2004; Leidel et al., 2005; Leidel and Gˆnczy, 2005). Sas-6 is the founding member of an evolutionary conserved protein family with no obvious sequence module other than a coiled-coil motif in the middle of the coding region and a potential novel ~50 amino acid region of homology toward the N terminus, referred to as PISA motif. Sas-6 physically interacts with Sas-5, another core components for procentriole biogenesis in *C.elegans*. Sas-6/Sas5 is recruited to mother centrioles once per cell cycle at the onset of the centrosome duplication and this recruitment requires Zyg1 activity (Leidel et al., 2005). After been recruited, Sas-6/Sas-5 will in turn guide Sas-4 to procentriole and promote daughter centriole formation.

HsSas-6, the human homolog, was first identified as a potential centrosomal component by mass spectrometry based proteomic analysis (Andersen et al., 2003). Two later studies using GFP or YFP tagged full-length HsSas-6 confirmed its localization to centrosomes in cultured human cells (Dammermann et al., 2004; Leidel et al., 2005). Overexpression of HsSAS-6 resulted in an excess of centrioles; while siRNA mediated inactivation of HsSas-6 abrogated the centrosome re-duplication in aphidicolin treated U2OS cells. Also, depletion of HsSas-6 impaired normal centrosome duplication cycle as about half of mitotic cells depleted of HsSas-6 showed a monopolar spindle reminiscent of its *C.elegans* kin (Leidel et al., 2005). These data clearly suggests that HsSas-6 is required for progression through the centrosome duplication cycle in human cells. A recent study from Dr Pierre's lab confirmed HsSas-6's requirement for procentriole formation (Strnad et al., 2007). Immunofluorecence studies showed HsSas-6 localized to the proximal end of the procentrioles either in normal centrosome cycle or Plk4-induced "flowers" like multiple procentrioles generated around a single maternal centriole (Kleylein-Sohn et al., 2007a; Strnad et al., 2007). Similar as in *C.elegans*, HsSas-6 was recruited to the procentriole in a Plk4-dependent manner (Strnad et al., 2007) although another study concluded Plk4 is despensible for initial recruitment of HsSas-6 to the procentriole while instead is important for stabilizing centriolar HsSas-6 once recruited (Kleylein-Sohn et al., 2007a). Also, HsSas-6 protein levels oscillated during the cell cycle with its appearance on centrosomes during S, G2 until metaphase while absence from centrosomes in late mitosis and G1 phase (Strnad et al., 2007). Down regulation of HsSas-6 protein started in anaphase and was mediated by APC/C^{Cdh1} ubiqutination which targeted HsSas-6 for degradation by the 26S proteasome (Strnad et al., 2007).

When Cdc14B was knocked down in human cells, among the cells harboring more than 4 centrioles, we did observe "rosette" pattern of Centrin-2 staining in some cases, implying multiple procentriole generation around one maternal centriole. This piece of evidence, although needs to be confirmed by electron microscopy studies, has potentially placed Cdc14B phosphatase in the same pathway with both Plk4 kinase and HsSas-6 to establish the "copy number" control for procentriole biogenesis. In this chapter, we will be testing this hypothesis through a series of experiments.

5.2 Results

5.2.1 Overexpression of Cdc14B suppress centrosome amplification triggered by deregulated Plk4

When cells were treated with the peptide vinyl sulfone proteasome inhibitor Z-L_3VS for an extended period of time, multiple daughter centrioles simultaneously grew from a single mother centriole and this effect required the activities of both Cdk2/Cyclin-E and most importantly Plk4 (Duensing et al., 2007a). Similarly, overexpression of Plk4 kinase alone mimicked the centriole overduplication pheynotype in the absence of Z-L_3VS (Habedanck et al., 2005a; Kleylein-Sohn et al., 2007a). In Chapter three, we have demonstrated that Cdc14B may counterbalance centrosomal kinases required for centriole overduplication in the Z-L_3VS induction system. Thus, it's tempting to test whether Cdc14B phosphatase can directly counteract Plk4 kinase activity in centrosome duplication control. In the current study, we took advantage of the Plk4-induced centrosome amplification system to determine whether ectopic expression of Cdc14B

phosphatase can suppress the centriole overduplication phenotype caused by Plk4 overexpression. First we tried to confirm the phenotype caused by Plk4 overexpression. In our hand, when co-tranfected with Centrin-EGFP, ectopic expression of a wild type Plk4 in both U2OS and HeLa cells did lead to significant increase in the percentage of cells with excessive centriole formation (>4 Centrin-EGFP focuses) (Fig 5.1A [upper panel]). Notably, among the cells with overduplicated centrioles, many exhibited the phenotype with one mother centriole surrounded by more than one newly generated procentrioles arranged in a rosette-like structure (Fig 5.1A [upper panel]). Next, we co-transfected Cdc14B^{WT} - EGFP together with the same wild type Plk4 construct into U2OS or HeLa cells. After transfection, cells were incubated for additional 40 hours and fixed for immunofluorecence studies. γ-tubulin was used as a centrosome marker. As expected, in the control groups, transient Plk4 transfection triggered centrosome amplification (Fig 5.1A [middle panel]). When counted (Fig 5.1B), about 37% of Plk4/Centrin-EGFP transfected cells harboring more than two γ-tubulin focuses per cell, in agreement with previous studies (Duensing et al., 2007a; Habedanck et al., 2005a). Remarkably, this centrosome amplifcation phenotype was greatly attenuated in Cdc14B^{WT} - EGFP positive cells. The percentage of EGFP positive cells having more than two centrosomes significantly reduced to about 5% and majority of them showed either one or two γ-tubulin spots (Fig 5.1B). This result suggests that Cdc14B overexpression suppresses abnormal centrosome duplication caused by deregulated Plk4.

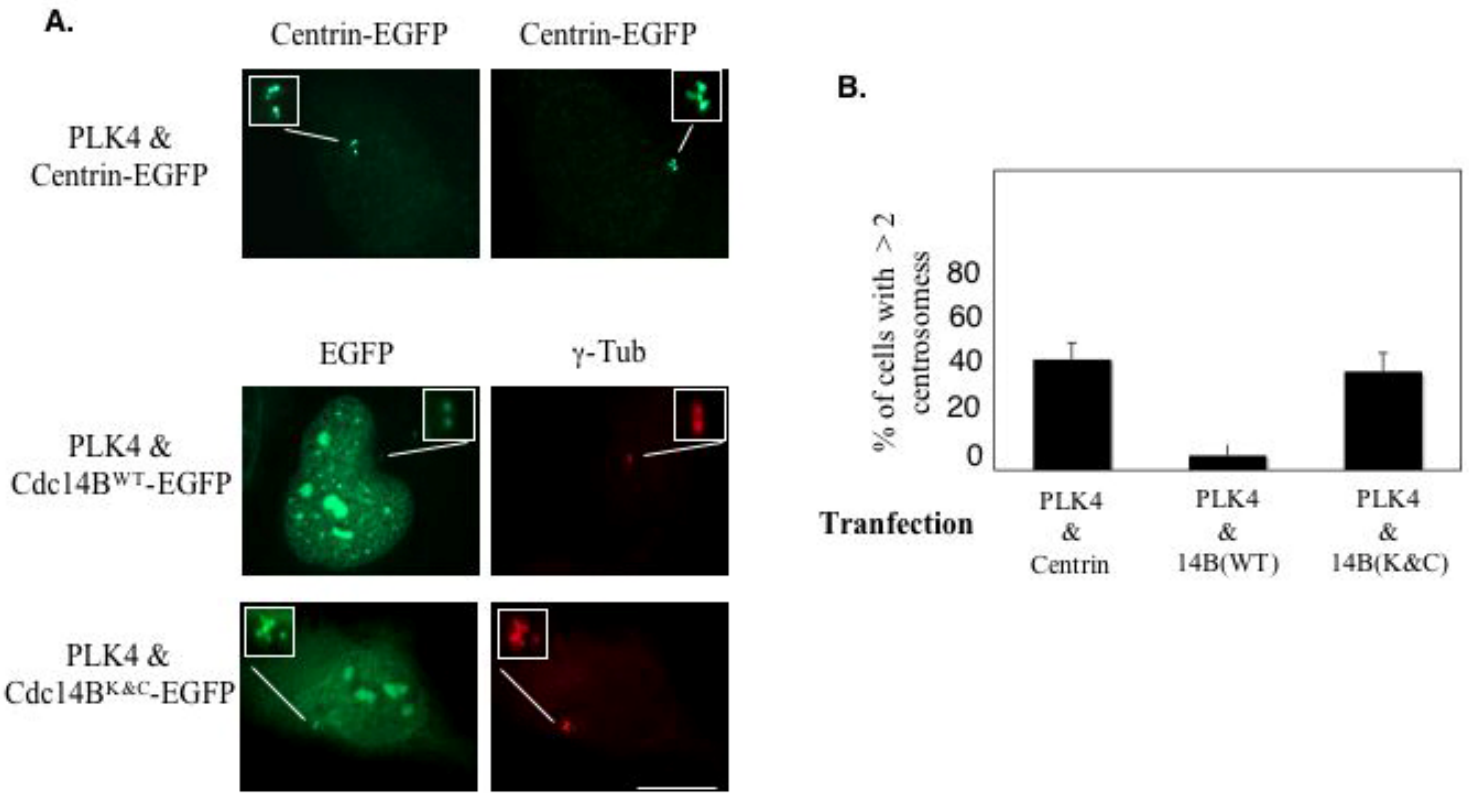

Figure 5.1: Cdc14B phosphatase suppresses centriole amplification triggered by deregulated Plk4 expression. (A) U2OS cells were transiently co-transfected with Plk4/Centrin-EGFP, Plk4/Cdc14B^{WT}-EGFP or Plk4/Cdc14B$^{K\&C}$-EGFP at a 4:1 ratio. Cells were fixed after 40 h transfection for immunostaining. Representative centrosomes (red) were visualized by anti-γ-tubulin antibody Bar, 5 μM. (B) Percentage of cells with > 2 centrosomes were calculated from the experiments shown in A and as indicated. Data shown represent three individual experiments. At least 100 cells were counted in each experiment.

Since catalytic-dead centrosome-bound mutant Cdc14B$^{K\&C}$ has been verified in our hydroxyurea and Z- L_3VS experiments, we therefore used the Cdc14B$^{K\&C}$ -EGFP construct to test whether Cdc14B's phosphates activity is directly involved. We then co-transfected the Cdc14B$^{K\&C}$-EGFP mutant together with wild type Plk4 into U2OS cells to examine the centrosome number in EGFP positive cell population. Interestingly, counting of the γ-tubulin spots in these EGFP positive cells revealed that the catalytic dead Cdc14B$^{K\&C}$-EGFP mutant failed to block centrosome amplifcation, with about 33% of cells with more than two centrosomes, a number comparable to the control (37%)(Fig 5.1B). Similar result was also obtained with HeLa cells and stable U2OS$^{Tet\text{-}on}$ clones expressing Cdc14B^{WT}-EGFP (clone 4D) or Cdc14B$^{K\&C}$-EGFP mutant (Clone #2) (Data not shown). Therefore, we concluded that Cdc14B phosphatase activity is indispensable for the inhibition of centrosome amplification in cells with ectopic Plk4 expression. Taken together, our data suggests that Cdc14B is a counterbalancing phosphatase of Plk4 kinase in the centrosome cycle control. It may act through opposing kinase phosphorylation on their common centrosomal substrate(s).

5.2.2 Depletion of both Plk4 and Cdc14B prevents centriole from overduplication caused by knocking down Cdc14B alone

We have demonstrated that when Cdc14B expression was ablated centriole overduplication occurred in both transformed and non-transformed human cells. Also we have shown that when both overexpressed, Cdc14B phosphatase may keep the Plk4 kinase activity in check in an *in vitro* centriole overduplication system. Thus, it's

reasonable to hypothesize that the centriole amplifcation phenotype observed in cells depleted of Cdc14B phosphatase might have arisen from the loss of control over endogenous Plk4 kinase. Therefore, in the next step, we further tested whether simultaneous depletion of both Cdc14B and Plk4 can restore the balance between endogenous phosphatase and kinase required for normal centriole duplication. For this purpose, HeLa cells were transiently transfected with either Cdc14B siRNA oligo pool (smartpool, Dharmacon) alone or together with a Plk4 siRNA oligo pool (on-target pool, Dharmacon). siGLO (a fluorescent, non-targeting control siRNA oligo pool) transfected cells were used as control. After transfection, cells were incubated for additional 60 hours and fixed for immunofluorescence studies. γ-tubulin was used as a centrosome marker. In cells with only Cdc14B knocked down, as expected, there was a significant increase of cells with more than two γ-tubulin labeled centrosomes compared with the siGLO siRNA oligo control transfected cells (23.78% vs 3.33%, Fig 5.2); Remarkably, when both Cdc14B and Plk4 were simultaneously depleted, the percentage of cells harboring more than two centrosomes greatly decreased (9.78%, Fig 5.2). Similar results were also obtained when U2OS cells was used (Data not shown). Taken together, these results further demonstrated that endogenous Plk4 and Cdc14B could also counterbalance with each other's activities in the control of centriole duplication. In situations such as ectopic Plk4 expression and Cdc14B depletion, disruption of this balance will lead to misregulation of normal centriole duplication cycle, and as a result unscheduled centriole overduplication occurs.

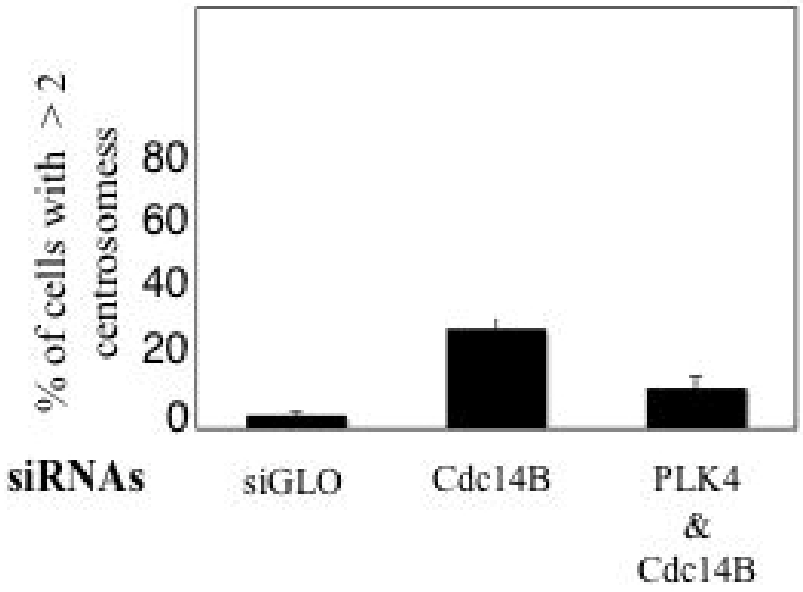

Figure 5.2: Counterbalance effect between Cdc14B and Plk4. Hela cells were transiently transfected with siRNA oligo pools (Dharmacon) targeting siGLO (control oligo), Cdc14B (smartpoo) or Plk4 (on-target pool) & Cdc14B (smartpool). Cells were fixed after 60 h transfection and immunostained with anti-γ-tubulin antibody as a centrosome marker. Percentage of cells with > 2 centrosomes were calculated in each transfection group and data shown represent three individual experiments. At least 300 cells were counted in each experiment.

5.2.3 Depletion of both HsSas-6 and Cdc14B prevents centriole from overduplication caused by knocking down Cdc14B alone

Plk4-induced centriole overduplication requires HsSas-6 since when HsSas-6 was depleted by siRNA, the percentage of cells bearing multiple centrosomes was greatly reduced (Habedanck et al., 2005a). Also, mitotic cells with inactivated HsSas-6 also showed increased number of monopolar cells similar to what was observed in Plk4 depleted mitotic cells (Leidel et al., 2005; Strnad et al., 2007). Further evidence showed Plk4 kinase activity might be required for initial recruitment of HsSas-6 to centrosome and the stability of centrosomal HsSas-6 (although another study found otherwise) (Kleylein-Sohn et al., 2007a; Strnad et al., 2007). More interestingly, careful examination of the HsSas-6 overexpressed cells revealed the same phenotype as expected with Plk4 overexpression, that is, multiple daughter centrioles surrounded one single mother centriole. This clearly raised the possibility that HsSas-6 may be the substrate of Plk4 kinase and act as the downstream effector of Plk4 in controlling centrosome duplication although further investigation is needed. Alternatively, HsSas-6 may constitute limiting building blocks that are recruited to a "site" marked by Plk4, thereby forming a "seed" for a nascent procentriole (Nigg, 2007b). Either case, based on our preliminary data for the counterbalancing effect between Plk4 and Cdc14B, it's interesting to test whether HsSas-6 also is a downstream effector or substrate for Cdc14B in regulating centrosome duplication.

In order to do so, we have used a similar scheme, that is, to ask whether simutanous inactivation of both HsSas-6 and Cdc14B using siRNA can abolish centriole overduplication phenotype as expected by depletion of Cdc14B alone. First, we sought to verify the siRNA oligo pool targeting HsSas-6 purchased from Dharmacon. Hela cells were transfected either with siGLO oligo control or HsSas-6 siRNA on-target pool. 66h post transfection, cell lysates were prepared and analyzed by immunoblot using antibodies against HsSas-6 (Fig 5.3A [top panel]) and β-actin (Fig 5.3A [bottom panel], loading control). As shown in Fig 5.3A, HsSas-6 protein level was greatly attenuated in cells transfected with HsSas-6 siRNA oligos, confirming the specificities of both HsSas-6 antibody (Abcam) and the siRNA pool (Dharmacon). Next we used this siRNA pool for double knock down experiment. HeLa cells were transiently transfected with either Cdc14B siRNA pool alone or together with the HsSas-6 siRNA pool. Non-targeting (NT) siRNA oligo transfected cells were used as control. 60 hours after transfection, cells were fixed and immunostained with anti-Centrin-2 antibody, a centriole marker. Interestingly, simultaneously knocking down both Cdc14B and HsSas-6 expression prevented the centriole amplification from occurring. As shown in Fig 5.3, when Cdc14B was depleted alone, about 28.33% of cells harboring more than four Centrin-2 labeled centrioles, significantly higher than non-targeting oligo transfected cells (~4%). However, when simultaneously knockdown both Cdc14B and HsSas-6 expression, only about 9.33% of cells showing more than four centrioles in their cytoplasms, indicating the requirement of HsSas-6 in Cdc14B's negative regulation of centrosome cycle. In summary, it is potential that HsSas-6 may also act downstream of Cdc14B and by eliminating HsSas-6 expression, Cdc14B's function in controlling centriole duplication is impaired.

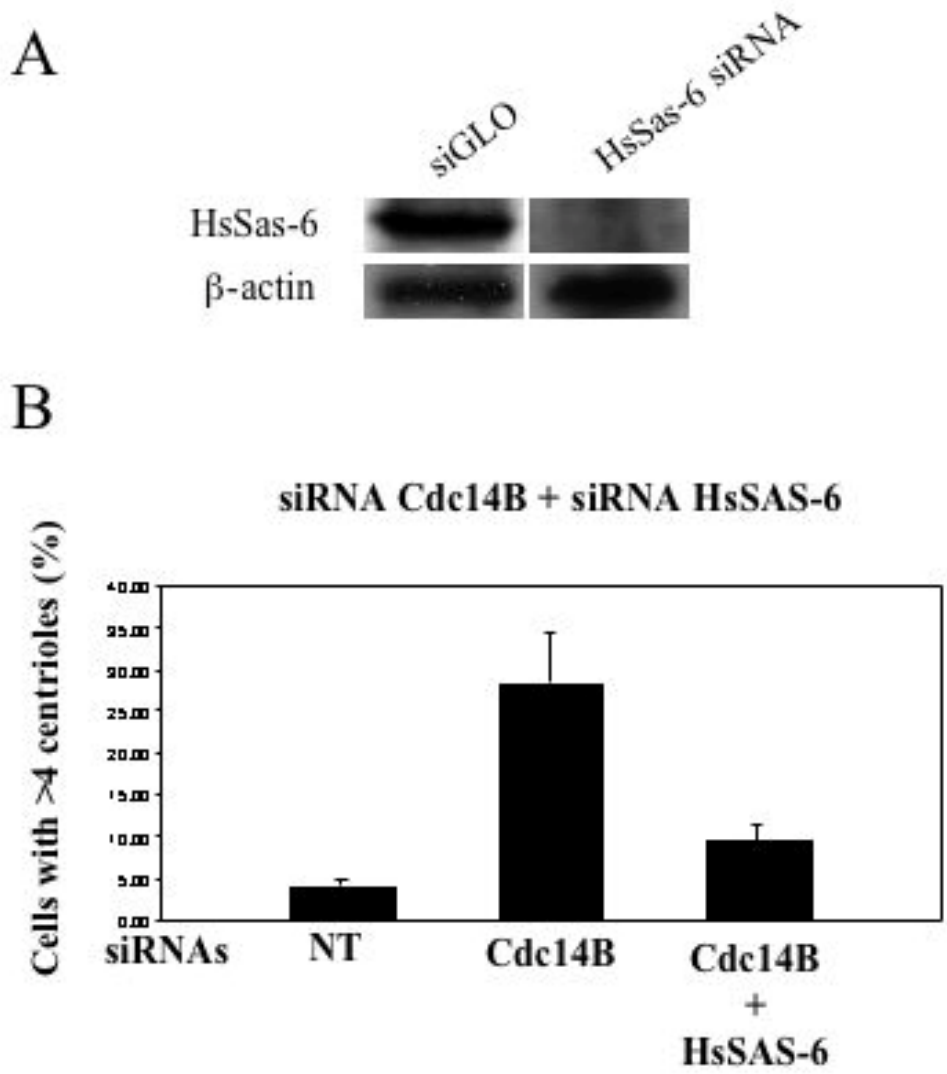

Figure 5.3: HsSas-6 as a potential downstream effector of Cdc14B in centriole duplication control. (A) Hela cells were transiently transfected with siGLO oligo control or siRNA on-target oligo pool targeting HsSas-6 (Dharmacon). 66h post transfection, cell lysates were analyzed by westerblot probed with antibodies against HsSas-6 (top) and β-actin (bottom, loading control). (B) Hela cells were transiently transfected with Non-targeting (NT) siRNA oligo, siRNA oligo pools targeting Cdc14B (smartpool, Dharmacon) or Cdc14B (smartpool, Dharmacon) & HsSAS-6 (on-target pool, Dharmacon). Cells were fixed after 60 h transfection and immunostained with anti-Centrin-2 antibody as a centriole marker. Percentage of cells with > 4 centrioles were calculated in each transfection group and data shown represent three individual experiments. At least 300 cells were counted in each experiment.

5.2.4 Depletion of Cdc14B stabilizes HsSas-6 in G1 phase

The presence of HsSas-6 at centrioles, and more importantly, the cellular HsSas-6 protein level is cell cycle regulated. Levels of HsSas-6 expression increase gradually from early S phase till late mitosis when the protein disappears suddenly. The disappearance of HsSas-6 expression is caused by the 26S proteasome mediated protein degradation since MG132, a specific 26S proteasome inihibitor could restore HsSas-6 appearance at centrioles in telophase cells(Strnad et al., 2007). Our double knockdown (Both Cdc14B and HsSas-6) experiment in Section 5.2.3 has shown Cdc14B's centrosomal function may rely on the presence of HsSas-6, next we want to further test whether Cdc14B can directly regulate endogenous HsSas-6 level. Since depletion of Cdc14B did not lead to obvious cell cycle defect, we can synchronize HeLa cells depleted of Cdc14B into different stages of the cell cycle and evaluate its effect on HsSas-6 level. First, we transiently transfected HeLa cells with siGLO control oligos or Cdc14B siRNA smartpool. After 24 h transfection, cells were blocked at G1/S boundary with double thymidine block. HeLa cells were then released from the block and cellular protein lysates were prepared at various timepoints after release. We focused our study on G1 cell lystates (14h post release) since during this time endogenous HsSas-6 level was minimal. We carried out western analysis using HsSAS-6 antibody (Fig 5.4A [top panel]), Cdc14B antibody (Fig 5.4A [middle panel]) and antibody against β-actin as a loading control (Fig 5.4A [bottom panel]). Compared with siGLO transfected cells, G1 cells with depleted Cdc14B showed significant increase of HsSas-6 level (Fig 5.4A).

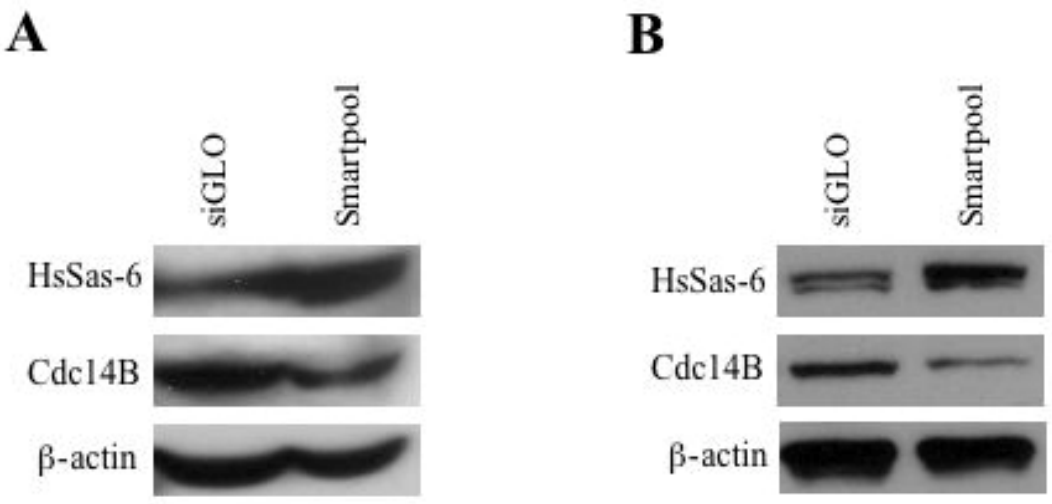

Figure 5.4: Downregulation of Cdc14B expression in G1 stabilizes HsSas-6. (A) HeLa cells transfected with either siGLO oligo control or Cdc14B smartpool siRNA pool. Shown are cells 14h (G1 phase) after released from a double thymidine block and analyzed by western blot using HsSAS-6 antibody (top), Cdc14B antibody (middle) and antibody against β-actin as a loading control (bottom). (B) Hela cells transfected with either siGLO oligo control or Cdc14B smartpool siRNA pool were blocked at G0/G1 phase by serum starvation for 48 h. After 2 h release into medium containing serum, cells were lysed and analyzed by western blot with HsSAS-6 antibody (top), Cdc14B antibody (middle) and antibody against β-actin as a loading control (bottom).

Similar results were obtained when HeLa cells were synchronized at G1 phase by 48h serum starvation and then released into medium containing serum for 2h (Fig 5.4B). Taken together, these results suggest that depletion of Cdc14B expression may help stabilize HsSas-6 protein level during G1 phase of the cell cycle. In another word, Cdc14B may be responsible for targeting HsSas-6 for degradation during G1 phase.

5.3 Discussion

How only one procentriole assembles at the base next to each maternal centriole has been baffling the cell and developmental biology community for a long time. This "copy number" control limits the formation of procentrioles to one per pre-existing mother centriole (Nigg, 2007b). Violation of this "one and only one" per centriole rule will result in the aberrant formation of more than one procentrioles per template. This interesting phenotype has been observed in a number of cases including CHO or U2OS cells blocked in S phase (Balczon et al., 1995b; Meraldi et al., 1999) as well as overexpression of Plk4, of HsSas-6, or of the human papillomavirus type 16 E7 oncoprotein and in cells treated with the proteasome inhibitor Z-L_3VS (Duensing et al., 2007a; Habedanck et al., 2005a; Kleylein-Sohn et al., 2007a; Strnad et al., 2007).

Although attractive, the "engagement-disengagement" licensing model discussed previously in chapter four alone does not readily account for this phenotype. One possible alternative explaination is that the duplication block imparted by the presence of a procentriole is regulatory in nature rather based solely on structural constrains (Strnad

and Gˆnczy, 2008). Studies of Zyg-1 kinase in *C.elegans* have raised the possibility that Zyg-1 might play such a regulatory role. Zyg-1 appeared on centrosome primarily at the onset of procentriole assembly (Telo/S phase transition) in wild type embryos and was absent from centrosome when centrosome duplication already started (Interphase till early anaphase). Interestingly, when procentriole assembly is prevented following Sas-6 depletion, Zyg-1 remains at centrosomes (Delattre et al., 2006; O'Connell et al., 2001a). This observation suggests that the disappearance of centriolar Zyg-1 upon procentriole assembly might normally serve as a mechanism to prevent ectopic multiple procentriole assembly events in *C.elegans* (Dammermann et al., 2008).

Although there is no obvious sequence homology of Zyg-1 in human genome, the similar requirement of Plk4 activity at centrosome has led to the proposal that Plk4 is the functional homolog of Zyg-1 in mammals. It is of particular interest that the overexpression of Plk4 in human cells leads to the concurrent formation of multiple procentriole around each mother template, arranged in a “rosette” conformation. More interestingly, multiple procentrioles generated remained tightly associated with the center mother centriole throughout the rest of interphase and early M phase while started to disassemble in late M phase, in agreement with the proposed disengagement model. This observation further supported the idea that structural constraints are not the only mechanism that governs centriole duplication. Also worth mentioning is that overexpression of Plk4 induced excess procentriole assembly in cells arrested in S phase when one procentriole has already assembled, implying an ectopic Plk4 expression could override the regulation normally imposed by the existing procentriole (Kleylein-Sohn et

al., 2007a). Therefore, tight regulation of either association of Plk4 with centrosomes or centrosomal Plk4 kinase activity is critical for controlling centriole number (copy number control). In *C.elegans*, Zyg-1 protein level and localization are cell cycle regulated and its appearance on centrosome corresponds well with new round of centrosome duplication. However, unlike its functional homology Zyg-1 in *C.elegans*, Plk4 was found to localize at centrioles throughout the cell cycle (Habedanck et al., 2005a). Thus, centrosomal Plk4 kinase activity is expected to be modulated and counterbalanced by a potential phosphatase upon procentriole assembly. If there is a loss of balance between kinase and phosphatase, disastrous consequences may follow.

In the current study, our data suggests Cdc14B may be one of the counterbalancing phosphatases of Plk4 in centrosome cycle control: First, overexpression of Cdc14B can suppress centriole amplification triggered by ectopic Plk4 expression; Second, concurrent ablation of both Plk4 and Cdc14B expression in human cells inhibited the centriole overduplication caused by knocking down Cdc14B alone; Third, it has been shown in chapter three that induction of $Cdc14B^{WT}$-EGFP in $U2OS^{Tet\text{-}on}$ cells led to a gradual loss of centrioles in mitotic cells with continued passage through the cell cycle, reminiscent of the phenotype observed in cells depleted of Plk4 (Habedanck et al., 2005a).

Also, it is interesting to mention that Cdk2 and Plk4 kinases may cooperate in controlling centrosome duplication since overexpression of Plk4 can't trigger centriole overduplication in the absence of Cdk2 activity and Cdk2 is not able to cause centriole amplification in the absence of Plk4 *vice versa* (Duensing et al., 2007a). Another study

showed overexpression of Cdk2/Cyclin-E together with Plk4 caused additional increase of abnormal centriole formation at maternal centriole when compared with overexpressing Plk4 alone, further reflecting their cooperative nature. Therefore, Plk4 clearly is not the sole regulator and further experiments to elucidate how Cdk2 and Plk4 may cooperate will provide important insights into the regulation of centriole duplication.

Beside Plk4, another distinct possibility responsible for the "copy number" control is that a centrosomal component needed for procentriole assembly is limiting around the G1 to S transition and is recruited to procentriole in a cooperative manner. This could favor procentriole assembly at just one site, and thus conceivably prevent concurrent growth at other sites (Strnad and Gˆnczy, 2008).

One of the most promising candidates of this limiting centrosomal component is HsSas-6. Strnad and co-workers have found recently HsSas-6 was indispensible for procentriole formation in human cells, and its protein levels were tightly regulated at the G1 to S transition to restrict procentriole formation (Strnad et al., 2007). The fluctuation of HsSas-6 level was not due to regulation at the transcription level since the HsSas-6 mRNA stayed relative the same across the cell cycle. Instead, protein degradation was shown to be responsible for HsSas-6 downregulation starting from anaphase till onset of S phase (Strnad et al., 2007). Treatment with 26S proteasome inhibitor MG132 restored HsSas-6 level in telophase cells suggesting E3 ubiquitin ligase APC/C coupled with adaptor protein Cdh1 is responsible for HsSas-6 degradation. A *priori,* the E3 ubiquitin ligase APC/C becomes active when coupled with its adaptor protein Cdh1 during late

mitosis and G1 phase. Cdh1 binds substrates containing a KEN box. Indeed, sequence analysis identified a KEN box in the C terminus of HsSas-6 and further mutagenesis studies confirmed the C terminal KEN box was required for targeting HsSas-6 for degradation by APC/C^{Cdh1}. When three critical residues within the KEN box on HsSas-6 were mutated to alanine (the HsSas-6-ΔKEN mutant with K589A, E590A, N591A), degradation of HsSas-6 was prevented. In addition, overexpression of Myc-tagged Cdh1, which resulted in constitutive activation of APC/C^{Cdh1}, decreased HsSas-6 protein level in S phase arrested cells. In summary, ubiquitination by APC/C^{Cdh1} from late anaphase till the end of G1 phase targets HsSas-6 protein for degradation by the 26S proteasome. As a result, HsSas-6 level is kept low at the onset of procentriole assembly possibly to ensure only one procentriole would form around a single mother template.

Cdc14B has been shown to specifically dephosphorylate Skp2 on Ser64 and render it more susceptible to APC/C^{Cdh1} degradation at the M to G1 transition (Rodier G, 2008). Therefore, it's reasonable to hypothesize that HsSas-6 level may also in part be regulated by Cdc14B phosphatase. And the observed low level of HsSas-6 duing G1 phase may due to dominant Cdc14B phosphatase activity. Although preliminary in nature, our study demonstrated that depletion of Cdc14B in G1 cells did lead to the increased expression of HsSas-6. This certainly raises the possibility that Cdc14B can target HsSas-6 for degradation through counterbalancing a kinase activity (Plk4?) whose action would stabilize HsSas-6. It'll be interesting to test whether overexpressing Plk4 kinase in G1 cells will achieve the same result. If true, Cdc14B may corporative with Plk4 in the pathway to limit HsSas-6 expression before centrosome duplication initiates. And this

regulated low level of HsSas-6 may ensure only one procentriole will be generated at the base of each mother centriole.

5.4 Summary

Taken together, as shown in Fig5.5, we propose that there is a balance between Plk4 kinase and Cdc14B phosphatase in the control of centriole duplication: In situations when there is excessive Plk4 expression or Cdc14B being depleted, Plk4 kinase activity predominates and the restriction for procentriole assembly (possibly imposed by limited expression of HsSas-6 during G1) is relaxed thereby allowing more than one procentriole generated per maternal centriole; On the other hand, siRNA mediated depletion of Plk4 or overexpression of wild type Cdc14B shift the balance toward the other direction where Cdc14B phosphatase activity prevails. And as a consequence, normal centrosome duplication cycle is disrupted and centrioles are gradually lost in successive passages.

5.5 Experimental Procedures

5.5.1 Plasmids and Antibodies

Transfection-ready plasmids encoding human Plk4 were purchased from OriGene. (Non-tagged [Cat. No SC115082] and C-terminal Myc/DDK-tagged [Cat. No RC206015]). HsCentrin-1 in pEGFP-C1 (Clontech) vector was kindly provided by Dr. Michel Bornens (Institut curie, Section De Recherche, France). $Cdc14B^{WT}$ and $Cdc14B^{K+C/S}$ were subcloned into pEGFP-N3 vector (Clontech) fused to a C-terminal EGFP tag whose expression was driven by the CMV promoters.

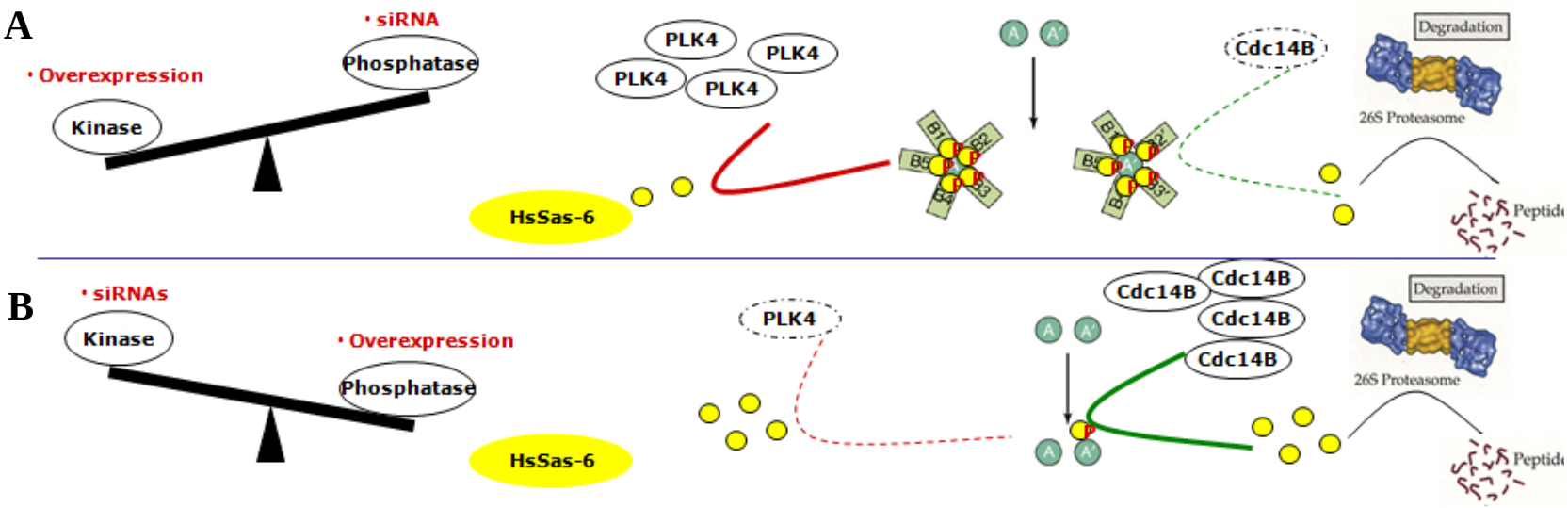

Figure 5.5: Cdc14B antagonizes Plk4 kinase activity in centriole duplication control. (A) Overexpression of Plk4 kinase or downregulation Cdc14B phosphatase through siRNA could lead to multiple procentrioles generated around a single mother centriole. HsSas-6 (Yellow circles) could be the common effector of both Plk4 and Cdc14B. When phosphorylated ("P"), HsSas-6 may be stabilized during G1, which resulted in unscheduled centriole duplication. (B) Overexpression of Cdc14B phosphatase or downregulation Plk4 kinase, on the other hand, could lead to gradual loss of centrioles. HsSas-6 may be targeted for degradation due to excessive Cdc14B phosphatase activity

Mouse polyclonal antibody against HsSas-6 was purchased from Abcam Inc (Cat No ab67395). Rabbit polyclonal antibody against human Cdc14B was bought from Zymed Labotories (Cat No 34-8900). Goat polyclonal antibody against human Plk4/Sak was purchased from Santa Cruz Biotechnology, Inc (Sak C-14, Cat No SC-49101). Anti-Centrin-2 antibody was purchased from Santa Cruz Biotechnology, Inc (Centrin-2 N-17, Cat No SC-27793-R). Monoclonal Anti-γ-tubulin antibody was obtained from Sigma-Aldrich (GTU-88, Cat No T6557).

5.5.2 Cell Culture and Transfections

U2OS (ATCC), U2OS^{Teton} (BD Bioscience) and HeLa cells were cultured under 5% CO_2 at 37°C in high-glucose Dulbecco's Modified Eagle's medium (DMEM, Invitrogen), 10% fetal bovine serum (FBS), and antibiotics (100ug/ml penicillin and 50ug/ml streptomycin sulfate). Cells were splited to 50% to 70% confluency and transfected with Fugene HD (Roche) using 2ug of DNA and 6ul of Fugene HD reagent per well in six-well plates. Co-transfection of Plk4 plasmids with Centrin-1-EGFP, Cdc14B^{WT}-EGFP and Cdc14B$^{K+C/S}$-EGFP were performed using a ratio of 4:1 (Plk4: Centrin-1/Cdc14B^{WT}/Cdc14B$^{K+C/S}$).

The doxycyclin-inducible cell lines stably expressing C-terminal EGFP-tagged Cdc14B^{WT} (Clone 4D) and Cdc14B$^{K+C/S}$ (Clone #2) were maintained in 100ug/ml of G418 (Invitrogen) and 2ug/ml of puromycin (Sigma-Aldrich). The expressions of constructs were induced by the addition of 4ug/ml of doxycyclin.

5.5.3 Indirect Immunofluorescence

For indirect immunofluorescence, cells grown on glass coverslips were fixed rapidly for 30 minutes in -20°C 100% methanol, washed in PBS, permeabilized with PBS/0.5% Triton X-100 for 10 min at room temperature, and followed by blocking with antibody dilution solution (1% bovine serum albumin in PBS) for 1 hour at room temperature. Cells were then incubated either 1 hour at room temperature or overnight at 4°C with primary antibodies, washed three times in PBS, and incubated for 1 hour at room temperature with secondary antibodies, washed, incubated with 0.1ug/ml of DAPI in PBS and mounted using PermaFlour Mountant media (Thermo Electron Inc) and imaging was performed at room temperature.

Primary antibodies were 1:2000 mouse anti-γ-tubulin (GTU-88, Sigma); 1:1500 goat anti-Centrin-2 (N-17, Santa Cruz Biotechnology, Inc). Secondary antibodies were 1:750 donkey anti-mouse coupled to Alexa 594, 1:750 donkey anti-goat coupled to Alexa 568 (Molecular Probes).

Cells were visualized with a 100 x Plan-Neofluar objective (1.30 oil; ∞/0.17) under an epi-fluorescence microscope (Axioskop 2, Carl Zeiss Inc). Images were acquired with a charge-coupled device camera (AxioCam HRC) controlled by Openlab software (version 3.5, Improvision Inc). Image processing was performed using Photoshop CS (V8.0).

5.5.4 RNA interference

Oligonucleotides targeting human Cdc14B were obtained from Dharmacon (siGENOME SMARTpool, Cat No M-003470-02-0010) which correspond to the flowing sequences: 5'GAUAAUACCAGACCGAUUUUU3', 5'GAUGCUACAUGGUUAUAUAUU3', 5'CAGUAUGGCUUCCUUAAUUUU3' and 5'CAACUCAUUUAACCUUGAUUU3' Oligonucleotides targeting human Plk4 were obtained from Dharmacon (On-TARGETplus SMARTpool, Cat No L-005036-00-0005) which correspond to the flowing sequences: 5'GAAGAUAGCAAUUAUGUGU3', 5'GUGGAAGACUCAAUUGAUA3', 5'GGACCUUAUUCACCAGUUA3' and 5'GGACUUGGUCUUACAACUA3'. Oligonucleotides targeting human Sas-6 were obtained from Dharmacon (On-TARGETplus SMARTpool, Cat No L-005036-00-0005) which correspond to the flowing sequences:

Oligos were transfected into HeLa or U2OS cells using DharmaFECT 1 transfection reagent (Dharmacon, Cat No T-2001-02). Briefly, cells were seeded at 40%-70% confluency in an antibiotic-free culture medium and transfected with siRNA oligo pools at a final concentration of 100nM for 60 hours (unless otherwise specified). Negative controls were cells transfected with 100nM siGLO RISC-Free Control siRNA (Dharmacon, Cat No D-001600-01-20). In co-transfection experiments, oligo pools were mixed at 1:1 ratio with 50mM final concentration of each.

5.5.5 Western Analysis

Cells were first lysed in a buffer containing 1% SDS in Tris-Hcl pH 7.4. Then 6xSDS sample buffer was added and samples were boiled for 10 minutes, votexed and cell debris spun down at 15,000 rpm for 5 minutes. An equal amount of proteins were loaded for SDS-PAGE and transferred onto nitrocellulose membrane (Biorad). Membranes were then blocked by 5% skim milk dissolved in PBST (0.01% Tween20 in PBS) for 1 hour at room temperature. After blocking, membranes were subjected to incubation with primary antibody diluted in 3% BSA in PBS for overnight at 4°C. After primary antibody incubation, membranes were washed three times with PBST and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (Sigma) for 1 hour at room temperature. Membranes were then washed again and specific signals were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Dilutions of primary antibodies used: Cdc14B (Zymed) 1:300; HsSas-6 (Abcam) 1:500; β-actin (Cytoskeleton, Inc) 1:5000.

5.5.6 Cell Synchronization

To study how Cdc14B can affect endogenous HsSas-6 level during the cell cycle, HeLa cells were synchronized by a double thymidine block. Briefly, 24 hours after transfection with siRNA oligos, HeLa cells were cultured in fresh medium containing 2mM thymidine for 19 hours. After first thymidine block, cells were washed three times with 1xPBS and incubated in fresh medium without thymidine for 9 hours to release

cells. After releasing, 2mM thymidine was added again to the culture medium and incubated for another 16 hours. After second block, cells were washed again and released into the fresh medium without thymidine, and samples of synchronized cells were collected and lysed at successive time points.

In a separate approach: For G0/G1 block, HeLa cells were washed and incubated in low serum medium (0.5% FBS in DMEM) for 48 hours; For S phase arrest, HeLa cells were treated with either 2mM hydroxyurea (HU,) or 10ug/ml aphidicolin (APH) for 48 hours. After incubation, lysates of cells blocked at each cell cyles were prepared and subjected to western analysis.

Chapter 6

Role of Cdc14B phosphatase in counterbalancing Cdk2/Cyclin-E/A in centrosome cycle control through regulation of its substrates

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6.1 Introduction

Cyclin-E, a regulatory subunit of cyclin dependent kinase 2 (Cdk2), is a known inducer of S phase entry in the mammalian cell cycle. In normal dividing cells, Cyclin-E expression increases at the G1/S phase boundary and is downregulated as cell progress through S phase (Duli*f*á et al., 1992; Koff et al., 1992; Spruck et al., 1999). When paired with Cyclin-E, Cdk2 is activated and plays a part in triggering the initiation of both DNA synthesis and centrosome duplication (Hinchcliffe EH, 2002).

The evidence for Cdk2/Cyclin-E's involvement in centrosome duplication control initially came from two separate in vitro studies using S-phase arrested Xenopus egg extract. Hinchcliffe *et al* developed an in vitro system by using S phase arrested frog egg extract. In this system, the investigators observed the repeated assembly of daughter centrosomes using polarized light microscopy (Hinchcliffe et al., 1999). When Cdk2 activity was selective inhibited using Δ34Xic-1, an NH3-terminal truncated form of Xenopus Cdk inhibitor Xic-1^{p27}, multiple rounds of centrosome reproduction was

blocked, indicating that Cdk2/Cyclin-E is required for repeated centrosome duplication (Hinchcliffe et al., 1999; Hinchcliffe EH, 2002). In a second study, Lacey and co-workers used a different system in which isolated mammalian centrosomes were added to Xenopus egg extract and centriole disengagement phenotype was observed (Lacey KR, 1999). Centriole disengagement or centriole disorientation was used as a measure of centrosome duplication because it is regarded as the leading morphological event in centrosome reproduction (Kuriyama and Borisy, 1981) and recently is suggested as a licensing step to release the centrosome-intrinsic block to re-duplication during a single cell cycle (Tsou and Stearns, 2006c). This study showed that centriole disorientation in the frog extract is dependent on active Cdk2/Cyclin-E as centriole disjunction didn't occur when Cdk2 kinase inhibitors (CKIs) p21 or p27 were added to the extract; In contrast, mother-daughter centriole pairs disjoined in the control extracts with intact Cdk2/Cyclin-E activity (Lacey KR, 1999). In summary, these in vitro experiments using Xenopus egg extracts demonstrated Cdk2/Cyclin-E activity is required for centrosome duplication in early embryo model systems (Hinchcliffe EH, 2002).

In mammalian somatic cells, in addition to Cyclin-E, a different Cdk2 cyclin, Cyclin-A, has also been shown to be important in promoting centrosome duplication, and in some cases is even more effective than Cyclin-E (Meraldi et al., 1999). Prolonged treatment of CHO cells with agents such as hydroxyurea (HU) or aphidicolin (APH) allows centrosome re-duplication to occur in the absence of DNA replication (Balczon et al., 1995b). Taking advantage of this in vitro centrosome duplication system, Matsumoto *et al* demonstrated that when Cdk2 activity was blocked by drugs such as butyrolactone-I

and roscovitine, or by expression of the Cdk inhibitor $p21^{Waf1/Cip1}$, centrosome re-duplication is inhibited (Matsumoto et al., 1999a), indicating the requirement of Cdk2 activity in centrosome duplication. Subsequent study using the same system led to the finding that Cdk2/Cyclin-A instead of Cdk2/Cyclin-E was mainly responsible for multiple rounds of centrosome duplication in CHO cells arrested at the G1/S boundary (Meraldi et al., 1999).

The above discrepancy between early embryo model system and mammalian somatic cells suggest the possibility that there may be functional redundancy for kinases that positively regulate centrosome duplication. Indeed, both Cyclin-E1 and Cyclin-E2 knockout mice developed normally and were viable, except that about half the Cyclin-E2 knockout male mice were sterile due to incomplete testis development (Geng et al., 2003). When these mice were crossed, the Cyclin-E1/E2 double knockout mice were embryonic lethal due to problems with placental development. Nevertheless, these embryos survived until the 10^{th} day of gestation and fibroblasts derived from double knockout mice embryos were able to undergo several rounds of division before becoming senescent. These observations make a strong argument that Cyclin-E is dispensable for cell proliferation and development in the mouse.

A recent study further showed the initiation of centrosome duplication is not significantly affected in cells derived from these mice, suggesting that the function of Cyclin-E can be readily replaced by other kinases (Fukasawa, 2008; Hanashiro et al., 2008). Hanashiro et al also showed that initiation of centrosome duplication was significantly delayed when

Cyclin-A expression was silenced in Cyclin E-deficient cells, implying that Cyclin-A is a primary candidate that compensates for the loss of Cyclin-E function for initiation of centrosome duplication (Hanashiro et al., 2008). However, as long as Cyclin-E is present, the role of cyclin A in the initiation of centrosome duplication appears to be minimal as initiation of centrosome duplication is noticeably delayed in Cyclin-E deficient cells compared with cells with normal level of Cyclin-E (Hanashiro et al., 2008).

Cdk2/Cyclin-E/A activity, although not essential, may nevertheless be important for the fidelity of centrosome duplication. Cdk2 complexed with either Cyclin-E or Cyclin-A are responsible for unscheduled centrosome re-duplication in a number of scenarios. It has been shown that cell cycle arrest in G1/S boundary by hydroxyurea (HU, a ribonucleotide reductase inhibitor) as well as aphidicolin (APH, a DNA polymerase inhibitor) and in late G2 phase by the G2/M checkpoint in response to DNA damage allowed centrosomes to regain the duplication competency and go through multiple rounds of re-duplication upon availability of the active Cdk2. However, Cyclin-A and Cyclin-E participated in centrosome re-duplication in a distinct manner depending on which cell cycle stage that cells are arrested: In cells arrested in early S phase, Cyclin-E is more important in centrosome re-duplication while Cyclin-A is a backup kinase; however, in contrast to the early S phase arrest, Cyclin-A is more important in the induction of centrosome re-duplication in cells arrested in late G2 due to DNA damage induced G2/M checkpoint. In addition, p53 also plays a part in centrosome re-duplication in these systems. In normal cells, upon treatment with inhibitors such as HU or APH, p53 is stabilized by the ARF-mediated inhibition of Mdm2 (Sherr, 2006), resulting in the up-regulation of $p21^{Waf1/Cip1}$,

a potent CDK inhibitor and major transactivation target of p53 . This leads to continuous inhibition of Cdk2, therefore blocking initiation of centrosome re-duplication; in cells lacking p53, however, Cdk2 activation is unchecked, causing centrosome to re-duplicate (Hanashiro et al., 2008).

It is known that phosphorylation of critical substrates by CDKs drives the cell cycle. When present, Cdk2/Cyclin-E/A appears to directly phosphorylate its centrosomal substrate(s) to regulate centrosome duplication. Up to date, however, only a few in vivo Cdk2/Cyclin-E/A centrosomal substrates have been identified. Therefore, the identification of physiological Cdk2/Cyclin-E/A centrosomal substrates is an important first step toward elucidation of the molecular mechanism underlying how Cdk2/Cyclin-E/A drives centrosome duplication.

B23 (also called nucleophosmin, NPM1, NO38, numatrin) is a multifunctional protein localized to the nucleolus and the centrosome, and is a substrate of Cdk2/Cyclin-E/A. It has been reported to play roles in ribosomal protein assembly, centrosome duplication, protein and histone chaperone activity and etc (Szebeni and Olson, 1999; Zou et al., 2008). Immunofluorescence evidence from Okuda and co-workers suggests that B23 controls centrosome duplication. Upon phosphorylation by Cdk2/Cyclin-E on Thr199 (threonine at position 199) at G1/S boundary, B23 dissociates from the centrosome allowing centrosome duplication to occur in S phase (Okuda et al., 2000a). Duplicated centrosomes remain free of B23 until mitosis when it re-associates with centrosomes to inhibit unscheduled centrosome duplication. Functional evidence for B23's involvement

in centrosome reproduction came from the finding that centrosome duplication was inhibited by the expression of non-phosphorylatable mutant of B23 (B23T199A, threonine 199 was mutated to alanine) that constitutively associated with centrosomes; in addition, microinjections of antibodies against B23 that sterically block its phosphorylation site by Cdk2/Cyclin-E also inhibited centrosome duplication. Together, these data led the authors to propose that Cdk2/CycE specifically phosphorylates B23 on T199 causing it to dissociate from the centrosome thereby allowing centrosome duplication to start (Okuda et al., 2000a; Tokuyama et al., 2001). Also, the authors argued that potentially B23 could be a licensing factor that limits centrosome duplication to once per cell cycle. Consistent with B23's inhibitory role in controlling centrosome duplication, Wang et al. further showed that the docking of B23 onto centrosome relied on the Ran/Crm1 complex (Wang et al., 2005), a nuclear-cytoplasmic shuttle system that, when disrupted, led to unrestricted centrosome duplication, similar to the phenotype observed in B23 deficient MEFs (Grisendi et al., 2005). However, if absence of B23 at centrosomes licenses them for duplication, this model cannot explain why centrosomes do not readily re-duplicate during S and G2 when B23 is not on centrosomes. Therefore, there seems to be more to the story for the molecular mechanisms underlying B23's role in centrosome duplication control.

A second possible centrosomal target of Cdk2/Cyclin-E is CP110, a protein localized to the centrioles. The expression of CP110 coincides with the activation of Cdk2/Cyclin-E as it's induced at the G1/S transition and its levels peaked around S phase, diminishing in mitosis (Chen et al., 2002a). Indeed, CP110 can be phosphorylated by by Cdk2/Cyclin-

E/A both in vitro and in vivo. Downregulation of CP110 expression by RNAi and blocking of Cdk2/Cyclin-E/A phosphorylation by mutations resulted in unscheduled centrosome separation. Also, RNAi-mediated reductions in CP110 inhibited centrosome reproduction in S phase arrested U2OS cells which otherwise exhibit centrosome overduplication (Chen et al., 2002a). More recently, Kleylein-Sohn *et al* identified CP110 is required for centriole biogenesis in human cells. In this study, they showed that CP110 associated with the distal ends of both parental centrioles and procentrioles. In particular, during centriole biogenesis, CP110 was recruited and decorated the distal tips of procentrioles and assembled into a cap-like structure underneath which α-/β- tubulin dimmers are inserted in support of procentrioles growth (Kleylein-Sohn et al., 2007a). Also, CP110 has been shown to suppress primary cilia formation (Spektor et al., 2007; Tsang et al., 2008).

Mps1 kinase was originally identified as essential for the duplication of the spindle pole body in budding yeast. Although it has been controversial, recent studies suggest that Mps1 plays a role in centrosome duplication. In mouse cells, Frisk and co-workers showed mMps1 localized to centrosomes throughout the cell cycle. Functional evidence for mMps1's involvement in centrosome duplication control came from the following findings: catalytically dead mMps1 mutant prevented centrosome duplication and suppress centrosome re-duplication in S-phase arrested cells; overexpression of active mMps1 directly caused centrosome re-duplication in a cell line that wouldn't readily undergo centrosome overduplication during S-phase arrest. Also, in the same study, mMps1 was identified as an in vitro substrate of Cdk2/Cyclin-E and Cdk2/Cyclin-E can

stabilize the protein levels of Mps1 kinase (Fisk and Winey, 2001). In contrast, a separate study by Stucke and co-workers did not find Mps1 at the centrosomes in U2OS cells by immunofluorescence and failed to collect functional evidence for an involvement of Mps1 in centrosome duplication by antibody injections, expression of kinase dead mutants, and siRNA mediated depletion (Stucke VM, 2002). These surprising differences between the two studies have led to a further investigation by Fisk and co-workers. And the new results confirmed their previous conclusions that Mps1 is present at centrosomes and its activity is required for centrosome duplication (Fisk HA, 2003). More recently, Kasbek *et al* from Fisk lab identified a single Cdk2 phosphorylation site and a degradation signal within Mps1 that regulate the accumulation of Mps1 at centrosomes. Also the new study showed Cdk2 coupled with Cyclin-A instead of Cyclin-E can phosphorylate Mps1 on T468 and prevent Mps1 degradation at centrosomes. And stabilized centrosomal Mps1 is sufficient enough to cause centrosome re-duplication in human cells (Kasbek et al., 2007).

In chapter four, we have shown that Cdc14B phosphatase can suppress unscheduled centrosome duplication in both HU and Z-L_3VS treated U2OS cells. It's important to note that in both of these settings Cdk2/Cyclin-E/A activity is in part responsible for the centrosome amplification phenotype. Thus, there is a potential counterbalancing effect between Cdc14B phosphatase and Cdk2/Cyclin-E/A kinase in the centrosome cycle control and here we will test this hypothesis in more details.

6.2 Results

6.2.1 Cdc14B phosphatase suppressed centrosome amplification caused by doxyrubisin treatment

When exposed to certain genotoxic drugs such as DNA intercalating drugs and topoisomerase II inhibitors (for example, doxorubicin, DXR), the majority of cells become arrested in late G2 due to the DNA damage activated G2/M checkpoint. Similar to the hydroxyurea and aphidicolin mediated early S-phase arrest, cells arrested in G2 is also believed to regain the centrosome duplication competency allowing newly duplicated centrosomes to re-duplicate upon availability of active Cdk2 (Hanashiro et al., 2008). Cyclin-E is unlikely to play a role here since it is only highly expressed specifically in late G1 to early S phase. Cyclin-A, on the other hand, is known to be expressed at high levels in S and G2/M and has been shown to be upregulated when cells were treated with DXR in both cyclin-$E^{-/-}$ and cyclin-$E^{+/+}$ MEFs (Desdouets C, 1995; Hanashiro et al., 2008). More importantly, RNAi mediated depletion of cyclin-A suppressed the centrosome amplifcation caused by DXR exposure regardless of the presence of cyclin-E (Hanashiro et al., 2008). Thus, cyclin-A, instead of cyclin-E, is critical for centrosome re-duplication in cells arrested in late G2 in response to DNA damage.

Therefore, treating cells with DXR becomes a perfect system for us to test whether Cdc14B phosphatase can also counterbalance Cdk2/cyclin-A during the DNA damaged induced centrosome amplifcation. This may further add to our pool of evidence that

Cdc14B may also be a negative regulator for abnormal centrosome duplication due to environmental cues. In order to do so, wild type U2OS$^{Tet\text{-}on}$ cells and doxycyclin-inducible cell lines stably expressing either C-terminal EGFP-tagged Cdc14B^{WT} (Clone 4D) or Cdc14B$^{K\&C}$ (Clone #2) were treated with DXR in presence of DOX induction for 48 h and then fixed for immunostaining with γ-tubulin antibody, a centrosome marker. In control U2OS$^{Tet\text{-}on}$ cells, centrosome amplification phenotype was faithfully reproduced, with about 48.67% of cells haboring more than two centrosomes (Fig 6.1A&B). Remarkably, when induced, only 23.33% of Cdc14B^{WT}-EGFP positive cells showed more than two γ-tubulin spots, indicating centrosome overduplication was significantly inhibited in the presence of Cdc14B^{WT}-EGFP (Fig 6.1A&B). When the Cdc14B$^{K+C/S}$ -EGFP (Clone #2) were examined for centrosome number instead, around 41.33% of EGFP positive cells had more than two centrosomes, at a level similar to what we observed in wild type U2OS$^{Tet\text{-}on}$ cells (Fig 6.1). This indicates that Cdc14B phosphatase activity is required for this action. Taken together, our data highly suggests that Cdc14B is also a counterbalancing phosphatase of Cdk2/Cyclin-A to ensure proper centrosome number in G2 arrested cells triggered by DNA damage.

6.2.2 Cdc14B interacts with B23 and dephosphorylates B23 on T199 *in vitro*

Previously in our lab during a search for potential Cdc14A substrates in mammalian cells, we created a Cdc14A mutant by changing Asp to Ala at position 251, designated as Cdc14A^{D251A} mutant. This mutant could be used as a “substrate trapping”

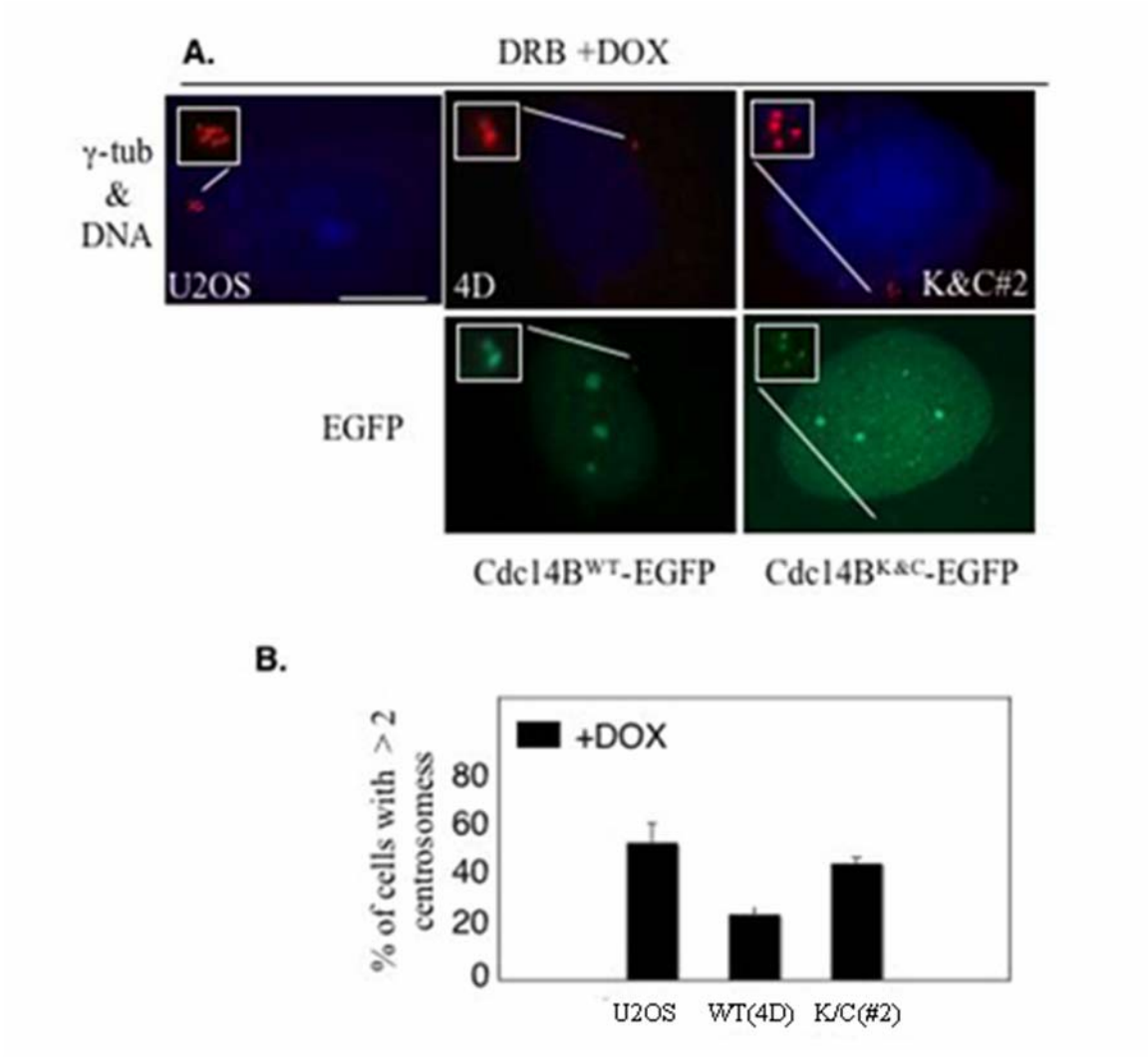


Figure 6.1: Cdc14B phosphatase activity is required to prevent DXR-induced centriole overduplication. (A) Wild type U2OS$^{Tet\text{-}ona}$ cell line as well as DOX-inducible U2OS$^{Tet\text{-}ona}$ cell lines carrying the indicated Cdc14B-EGFPs were treated with 1ug/ml doxyrubisin in the presence of 4μg/ml DOX for 48 hrs. Representative centrosomes (red) were visualized by anti-γ-tubulin antibody and DNA (blue) by DAPI. Bar, 5 μM. (B) Percentage of cells with >2 centrosomes were calculated accordingly. Data shown represent three individual experiments with (+DOX) induction of the indicated cell lines. At least 300 cells were counted in each experiment.

mutant given that it is catalytically inactive and potentially retains wild-type-like binding affinity toward its substrates. The construction of this Cdc14A mutant was based on its structure identity in Asp/PTP motif with a protein tyrosine phosphatase (PTP) "substrate trapping" mutant described previously (Flint et al., 1997; Gray et al., 2003). We took advantage of this "substrate trapping" Cdc14A^{D251A} mutant as bait for immunoprecipitation in combination with mass spectrometric analysis (Data not shown) to isolate potential Cdc14A interacting proteins or substrates. Interestingly, a gel-slice corresponding to ~ 33kDa band gave rise to the measured masses of three trypsin-digested peptides which matched theoretical isotopic masses of protonated tryptic peptides of nucleophosmin/B23 (NCBI entry 10835063) (Fig 6.2A). Further immunoprecipitation experiment confirmed this interaction between Cdc14A and B23 (Fig 6.2B). Since Cdc14A and Cdc14B shares high structural and sequence homology (Gray et al., 2003; Li et al., 1997a), we further tested whether Cdc14B could also physically interact with B23. We transiently transfected both Myc-tagged Cdc14B and Flag-tagged B23 into U2OS cells and found that anti-flag antibody also could pull down Myc-tagged Cdc14B (Fig 6.2A), indicating potential interaction between these two proteins. As discussed in the introduction section, dissociation of B23 from centrosomes correlates with the phosphorylation of B23 on Thr199 (Okuda et al., 2000b). Therefore, it's interesting to test whether Cdc14B phosphatase can dephosphorylate B23 at Thr199 both *in vitro* and *in vivo* based on its potential capability of counterbalancing Cdk2/Cyclin-E/A activity.

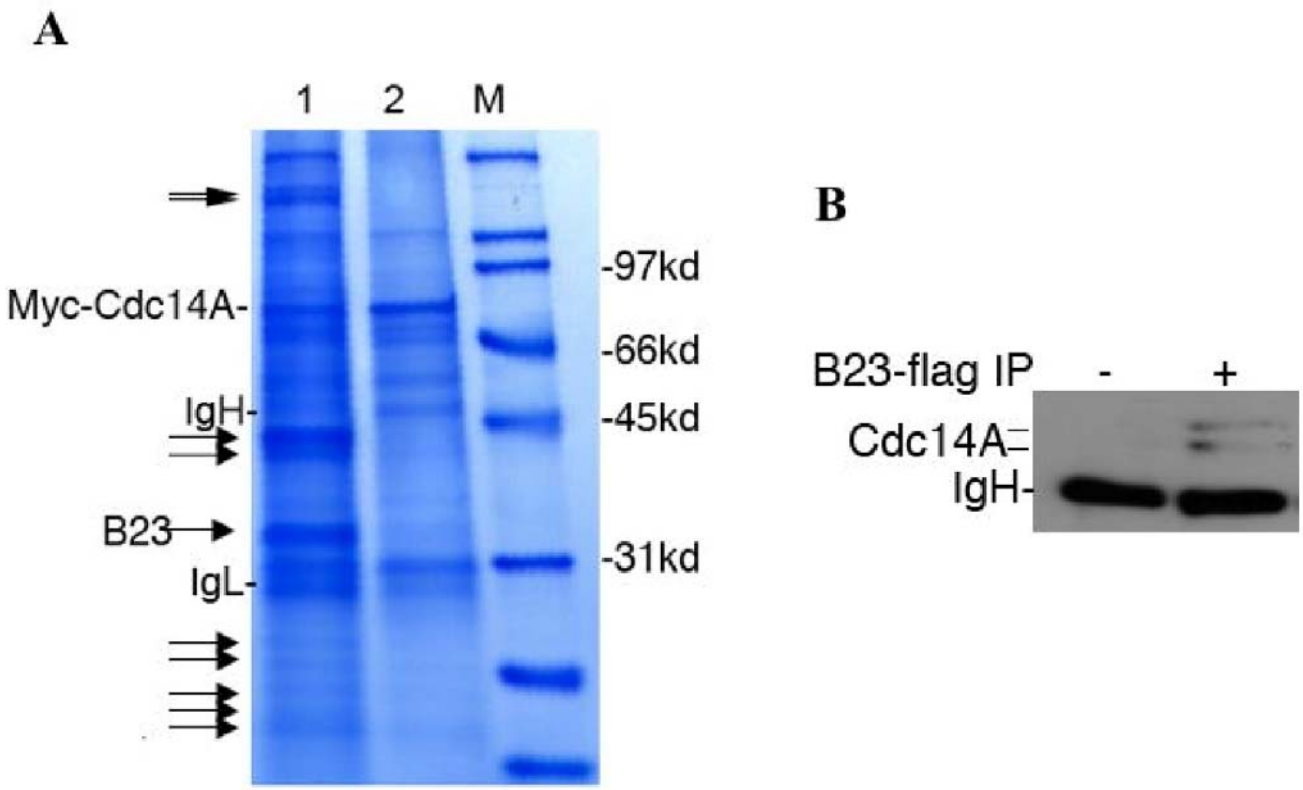
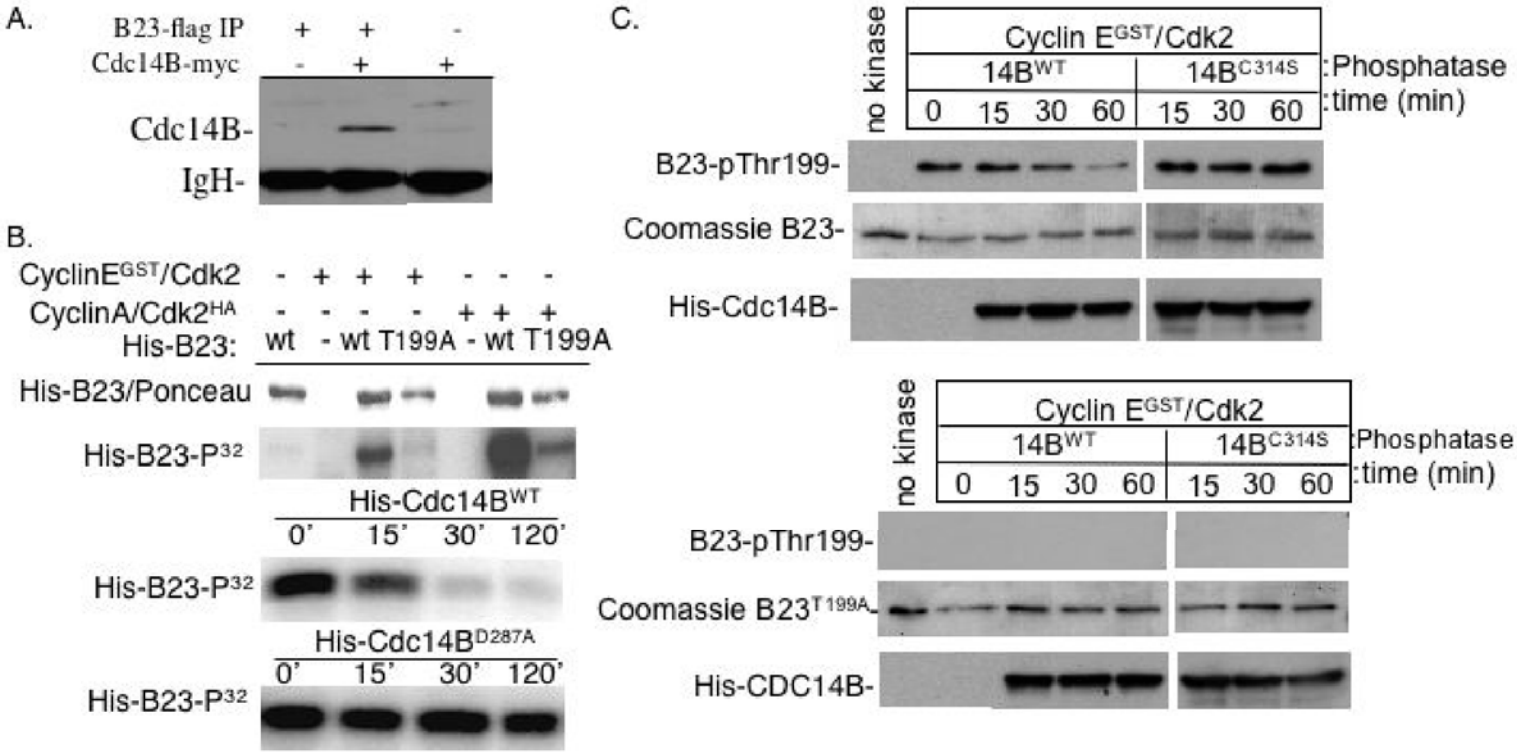


Figure 6.2: Cdc14A interacts with B23. (A) Coomassie stain of a representative immunoprecipitation experiment using myc-tagged Cdc14A^{D251A} mutant (lane 1) and WT Cdc14A (lane 2) as a bait in 293T cells. (B) Western analysis shows B23/Cdc14A co-immunoprecipitation. (Courtesy of Dr. Hyekyung P. Cho)

Figure 6.3: Cdc14B interacts with B23 and dephosphorylates phospho-Thr-199 of B23 *in vitro*. (A) Cdc14B/B23 co-immunoprecipitation. Flag-tagged B23 and myc-tagged Cdc14B were cotransfected into U2OS cells followed by immunoprecipitation with anti-Flag antibody and then immunoblot with anti-myc antibody. (B) In vitro dephosphorylation of CyclinE/Cdk2 γ-p32-ATP-labeled B23 by wild-type Cdc14B but not catalytic "dead" $Cdc14B^{D287A}$ mutant. (C) Upper panel: Dephosphorylation of Cyclin E/Cdk2-phosphorylated B23 by wild-type Cdc14B but not catalytic "dead" $Cdc14B^{C314S}$ mutant. B23Thr199 phosphorylation was detected by anti-phospho-B23-Thr199 (B23-pThr199, Cell Signaling Inc) antibody. Densitometry analysis showed that in comparison with 15 min rxn, 40% (30min) and 80 % (60min) reduction of phosho-B23 Thr199 was found. Lower panel: Cyclin E/Cdk2-phosphorylated B23Thr199A mutant was not detectable by anti-B23-Thr199 antibody showing the specificity of the phospho-B23-Thr199 antibody. Note for in vitro dephosphorylation experiments, CyclinE/A and Cdk2 were baculovirus-produced, whereas His-tagged B23 and Cdc14B were all produced in bacteria. (Courtesy of Dr. Hyekyung P. Cho)


A.
B23-flag IP + + -
Cdc14B-myc - + +
Cdc14B-
IgH-
B.
CyclinE^GST/Cdk2 - + + + - - -
CyclinA/Cdk2^HA - - - - + + +
His-B23: wt - wt T199A - wt T199A
His-B23/Ponceau
His-B23-P32
His-Cdc14B^WT
0' 15' 30' 120'
His-B23-P32
His-Cdc14B^D287A
0' 15' 30' 120'
His-B23-P32
C.
no kinase
Cyclin E^GST/Cdk2
14B^WT
14B^C314S
:Phosphatase
0 15 30 60 15 30 60
:time (min)
B23-pThr199-
Coomassie B23-
His-Cdc14B-
no kinase
Cyclin E^GST/Cdk2
14B^WT
14B^C314S
:Phosphatase
0 15 30 60 15 30 60
:time (min)
B23-pThr199-
Coomassie B23^T199A-
His-CDC14B-

As an initial step to study the relationship between Cdc14B and B23 in centrosome duplication control, we tested *in vitro* whether affinity purified Cdc14B protein could directly dephosphorylate B23Thr199 phosphorylated by baculovirus produced GST-Cdk2/Cyclin-E kinase in a phosphatase assay. First, we showed both *in vitro* baculovirus purified GST-Cdk2/Cyclin-E and HA-Cdk2/Cyclin-A successfully phophorylated baterial puried His-tagged B23 but not the His-B23T199A mutant (Fig 6.3B). Moreover, as shown in both Fig 6.3B and 6.3C, *in vitro* purified His-tagged wild type Cdc14B protein but not the phosphatase dead mutants: His-Cdc14B^{D287A} and His-Cdc14B^{C314S} respectively (Fig 6.3B and 6.3C), could dephosphorylate affinity-purified pre-phosphorylated wild type B23 (His-B23^{WT}). In Fig 6.3B we used γ-p32-ATP to label phosphorylated form of B23Thr199 by Cdk2/Cyclin-E/A and in Fig 6.3C we took advantage of a commercial antibody specifically recognizes phosphorylated B23Thr199. Both approaches led to the same conclusion. Before His-tagged Cdc14B was added to the reaction (0 min), the signals detected by both γ-p32-ATP and anti-Phospho-B23T199 were the highest. However, after addition of wild type His-tagged Cdc14B, the phosphorylation level on B23Thr199 gradually reduced with time. After 120 min and 60 min respectively, the phosphorylation on B23T199 was hardly detectable (Fig 6.3B & C). In contrast, when phosphatase dead Cdc14B mutants (His-Cdc14B^{D287A} and His-Cdc14B^{C314S}) were used instead, the phosphorylation level on B23T199 after addition His-Cdc14B$^{c/s}$ stayed relatively the same even after 120 min (Fig 6.3B and 6.3C). As a control, we created a B23 mutant His-B23^{T199A} by changing its Thr199 residue to Ala thereby eliminating its ability to be phosphorylated at this site. And when this His-B23^{T199A} mutant was used, there was no visible bands corresponding to phosphorylated

form of B23T199 indicating the specificity of anti-Phospho-B23T199 antibody (Fig 6.3C). In conclusion, our data shows Cdc14B physically interacts with B23 and can dephosplate B23 at Thr199 *in vitro*.

6.2.3 Cdc14B dephosphorylates B23Thr199 during early S phase

In the next step, we tested the possibility whether Cdc14B could dephosphorylate B23Thr199 *in vivo*. And if so, we would be expecting higher level of B23Thr199 phosphorylation in Cdc14B depleted cells. First, asynchronous HeLa cells were transfected either with siGLO control oligos or Cdc14B siRNA smartpool. 66h post-transfection, cells were lysed and subjected to western analysis probing for phosphorylated B23Thr199. Unfortunately, under this experiment condition, we didn't observe any inceased level of B23 phosphorylation on Thr199 in cells with significant reduction of Cdc14B expression due to siRNA transfection (Fig 6.4A), suggesting either B23 may not be targeted by Cdc14B or Cdc14B desphosphorylates B23 in specific cell cycle stage(s). Since depletion of Cdc14B did not lead to obvious cell cycle defect (Rodier G, 2008; Wu et al., 2008), we can synchronize HeLa cells depleted of Cdc14B into different stages of the cell cycle and evaluate its effect on B23Thr199 phosphorylation. Next, we again transiently transfected HeLa cells with siGLO control oligos or Cdc14B siRNA smartpool oligos, but this time, after 24 h transfection, we blocked cells at G1/S boundary using double thymidine block. HeLa cells were then released from the block and cellular protein lysates were prepared at indicated timepoints after release and levels of B23Thr199 phosphorylation was evaluated by western blot

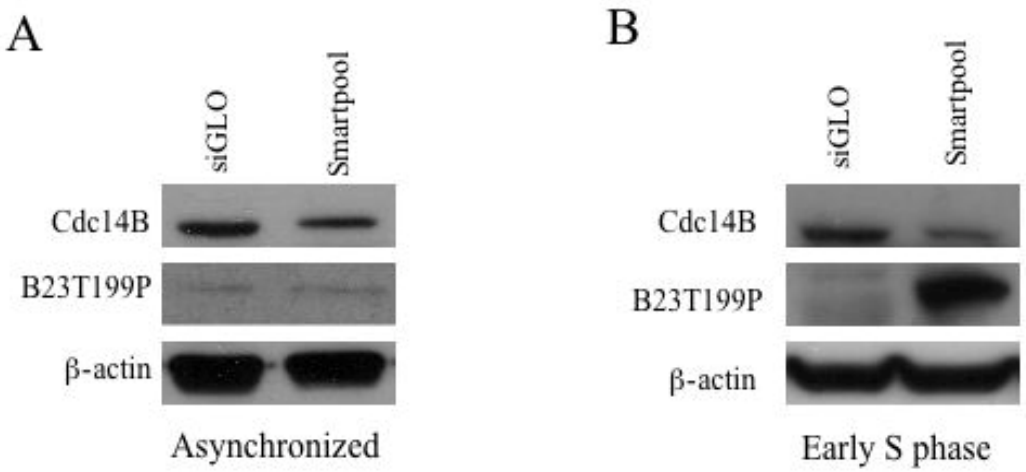


Figure 6.4: B23 as a potential substrate of Cdc14B. (A) Asynchoronized Hela cells were transfected with either siGLO oligo control or Cdc14B smartpool siRNA pool. Cell lysates were subjected to western analysis using antibodies against Cdc14B (top), Phospho-B23T199 (middle) and β-actin as a loading control (bottom). (B) HeLa cells transfected with either siGLO oligo control or Cdc14B smartpool siRNA pool. Shown are cells 3h (S phase) after released from a double thymidine block and analyzed by western blot using antibodies against Cdc14B (top), Phospho-B23T199 (middle) and β-actin as a loading control (bottom).

using a anti-phospho-B23Thr199 antibody. Interestingly, 3h after release when cells were in early S phase, Cdc14B depleted cells showed a significantly higher B23T199 phosphorylation signal compared with siGLO transfected cells (Fig 6.4B). This result suggests Cdc14B might regulate B23T199 phosphorylation during early S phase in the cell cycle when centrosome duplication events occur.

6.2.4 Requirement of B23 for Cdc14B-mediated centrosome duplication control.

Now we know Cdc14B can both dephosphorylate B23Thr199 *in vitro* and potentially *in vivo*. The next step will be to investigate whether B23 is required for Cdc14B's negative role in controlling centrosome duplication. And in chapter four we have demonstrated that Cdc14B suppressed centrosome amplification caused by prolonged treatment with hydroxyurea (HU) in U2OS cells. In the current study, we sought to knock down B23 expression in HU-treated U2OS cells in combination of overexpressing Cdc14B phosphatase. If B23 is indeed involved in Cdc14B's role in centrosome duplication, depletion of B23 will be expected to reverse Cdc14B's negative effect in inhibiting centrosome overduplication caused by HU treatment.

To knock down endogenous B23, we first used a vector-based RNAi approach. In search of the optimal siRNA target sequence to deplete the endogenous B23, we came across an oligo corresponding to nucleotide position 819-837 relative to the B23 start codon was most effective in ablating endogenous B23 expression (designated as $B23^{819}$ siRNA). We

cloned the $B23^{819}$ siRNA oligo into pSuper-neo-GFP vector purchased from Oligoengine and its expression was driven by the H1 promoter. After successful cloning, pSuper-$B23^{819}$ was transfected into $U2OS^{Tet\text{-}on}$ cells together with pBabe-puro (a kind gift from Dr. Gerald Evans) and selected for stable clones in a growth medium containing G418 and puromycin. After selection, several stable clones were isolated which constitutively expressed the $B23^{819}$ siRNA oligo. One of the clones (N1 clone) were tested for B23 expression, as shown in Fig 6.5A, when compared with wild type $U2OS^{Tet\text{-}on}$ cells, N1 cells showed significant reduction of B23 expression when probed using a monoclonal B23 antibody (Sigma).

Next, we transfected N1 cells with a plasmid expressing $Cdc14B^{WT}$-EGFP and subjected the transfected cells to 72 h of HU treatment. Surprisingly, compared with control (wild type $U2OS^{Tet\text{-}on}$ cells), $Cdc14B^{WT}$-EGFP positive N1 cells showed similar efficiency of suppressing of centrosome overduplication when compared with EGFP positive wild type $U2OS^{Tet\text{-}on}$ cells (Fig 6.5B). This came as a disspointment since it could mean B23 was dispensible for Cdc14B's function to suppress aberrant centrosome re-duplication in HU treated U2OS cells.

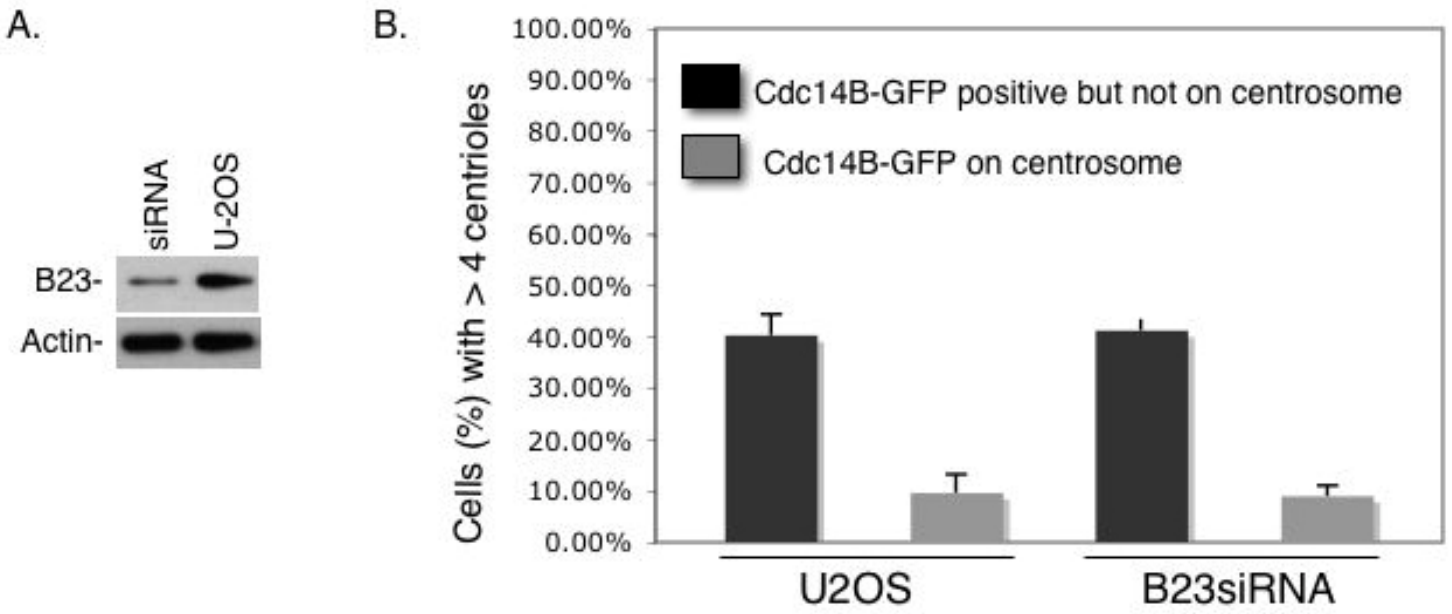
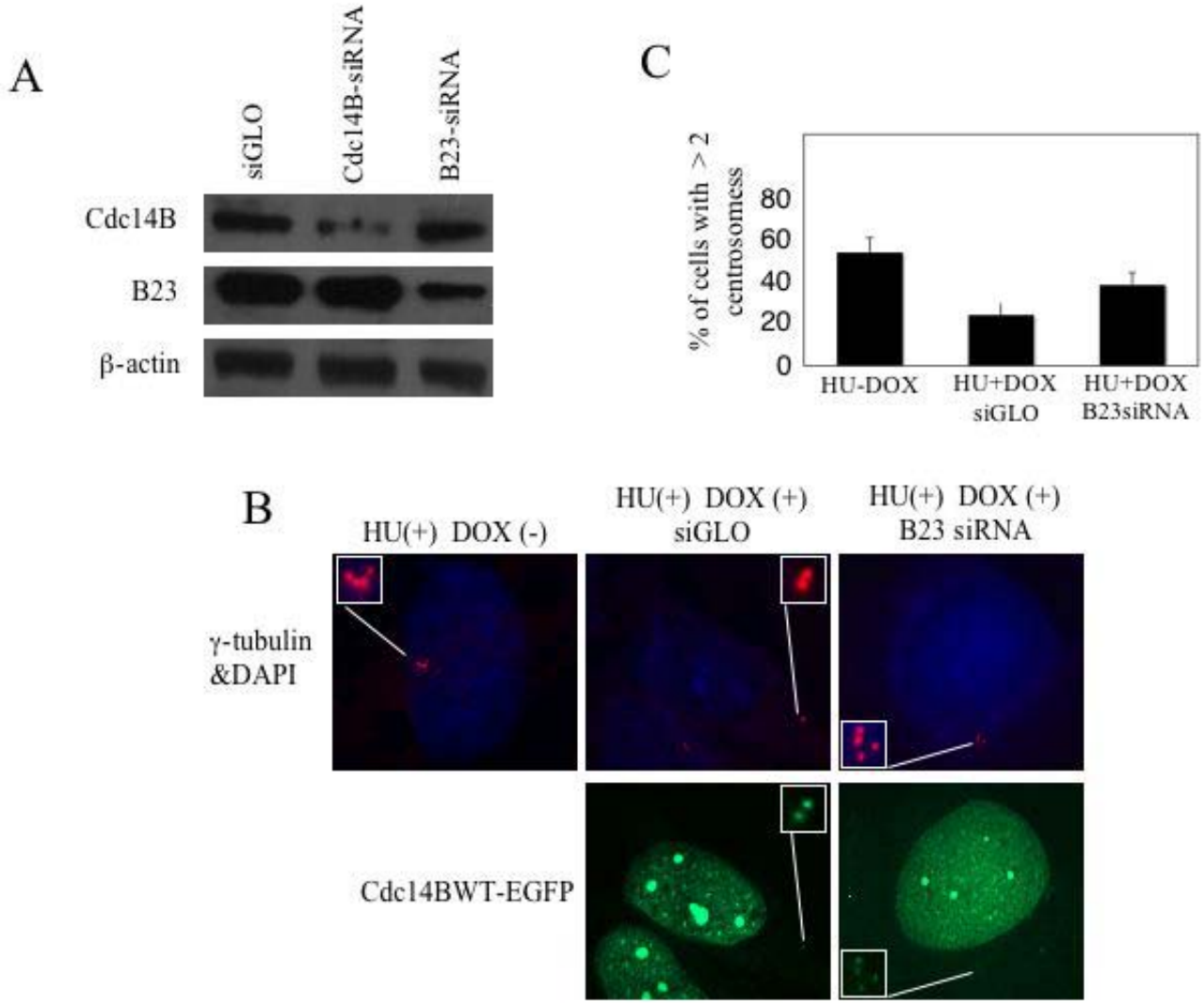


Figure 6.5: Cdc14B-mediated inhibition of centrosome overduplication does not depend on B23. (A) Western blot analysis using anti-B23 antibody (Sigma) revealed a significant knockdown of B23 in U2OS cells stably transfected with pSuper-B23 siRNA (targeting nucleotide position 819-837 relative to the start codon). (B) U2OS cells or B23siRNA cells were transiently transfected with Cdc14B-GFP expression vector for 24 hrs followed by hydroxyurea treatment for an additional 48 hrs. Cells were then fixed and stained with anti-Centrin-2 antibody. Percentage of cells with more than 4 centrioles was calculated from three independent experiments. At least 300 cells were counted in each experiment.

However, there are several weakness associated with this approach of knocking down B23. First, since the expression of siRNA oligo is constitutive, U2OS$^{Tet\text{-}on}$ cells under selection therefore were constantly having their endogenous B23 mRNA being targeted for degradation. Since B23 is essential for embryonic development and the maintenance of genomic stability and B23$^{-/-}$ mice is embryonic lethal, stable clone selection might have favored cells with the strongest survial potential with minimal levels of B23 present (Grisendi et al., 2005). In supporing this, we did observe a gradual loss of multipolar cells during selection (data not shown). These multiplepolar cells might have arised from centrosome overdupication as also observed in B23$^{-/-}$ / P53$^{-/-}$ MEFs (Grisendi et al., 2005); Second, the N1 clone didn't show any increase of centrosome number as expected with B23 depleted cells, further strengthing the idea that these stably clones generated were not representive of the most of cells when B23 is knocked down.

Therefore we decided to use an alternative approach. We tested transiently knocking down endogenous B23 using siRNA oligo pool purchased from Dharmacon. Compared with control siGLO oligo, when transfected into HeLa cells, Dharmacon B23 siRNA pool could efficiently knock down endogenous B23 expression as shown in Fig 6.6A. Next, we transfect this verified B23 siRNA oligo pool into the DOX-inducible U2OS$^{Tet\text{-}on}$ cell line stably expressing C-terminal EGFP-tagged Cdc14B^{WT} (Clone 4D). 24 h after transfection, 2mM of HU were added to the culture medium and cells were incubated for additional 48h and then fixed for immunostaining with γ-tubulin as a centrosome marker. As expected, in 4D cells transfected with siGLO oligo control, when induced with DOX,

Figure 6.6: B23 is a downstream mediator of Cdc14B in suppressing HU-induced centrosome amplification. (A) Hela cells were transiently transfected siGLO oligos control, siRNA oligo pools targeting endogenous Cdc14B (Dharmacon Smartpool) or B23 (Dharmacon On-target pool). 60h post transfection, cell lysates were analyzed by westernblot probing with antibodies against Cdc14B (top), B23 (middle) as well as β-actin (bottom) as a loading control. (B) Representative images showing γ-tubulin labeled centromes in 4D cells treated HU: uninduced (left), induced + siGLO (middle), induced + B23siRNA (right). (C) Percentage of cells with > 2 centrosomes were calculated in each experimental group and data shown represent three individual experiments. At least 100 cells were counted in each experiment.


A
siGLO
Cdc14B-siRNA
B23-siRNA
Cdc14B
B23
β-actin
C
% of cells with >2 centrosomess
80
60
40
20
0
HU-DOX
HU+DOX siGLO
HU+DOX B23siRNA
B
HU(+) DOX (-)
HU(+) DOX (+) siGLO
HU(+) DOX (+) B23 siRNA
γ-tubulin &DAPI
Cdc14BWT-EGFP

Cdc14B^{WT}-EGFP postive cells showed dramatic reduction of cells with more than two γ-tubulin spots (~22% as shown in Fig 6.6) compared with cells withouth DOX induction (~49%), confirming Cdc14B's negative effect on abberent centrosome duplication. In cells with B23 being targeted for depletion, when induced, Cdc14B^{WT}-EGFP postive cells showed ~35% percentage of cells harboring more than two centrosomes, significantly higher than siGLO control. This indiates that in the HU treated U2OS cells, B23 is potentially the downstream mediator of Cdc14B in suppressing centrosome amplification and when B23 expression is transiently knocked down, Cdc14B lost its ability to suppress centrosome amplification triggered by HU exposure.

6.3 Discussions

Without acquisition of duplication competency to re-duplicate the newly duplicated centrosomes do not re-duplicate readily (Wong and Stearns, 2003). It's been shown that cell cycle arrest in S phase by treatment with either hydroxyurea or aphidicolin and in late G2 phase by the G2/M checkpoint in response to DNA damage allow centrosomes to regain the duplication competency, and re-duplication occurs upon availability of the active Cdk2 (either coupled with Cyclin-E or Cyclin-A) (Hanashiro et al., 2008). It's commonly known that Cyclin-E and Cyclin-A are expressed at different stages during the cell cycle. The activation of Cdk2/Cyclin-E starts in late G1 to early S phase while Cdk2/Cyclin-A is activated in S phase through the rest of the cell cycle. Thus, in HU arrested U2OS cells, Cdk2/Cyclin-E is expected to be the major driving force for centrosome re-duplication. Our data in chapter four demonstrated that Cdc14B

phosphatase can suppress this centrosome overduplication phenotype, suggesting the counterbalancing effect between Cdc14B phosphatase and Cdk2/Cyclin-E kinase.In the current study, we confirmed that DXB-induced G2 arrest could cause centrosome to re-duplicate and further demonstrated this unscheduled centrosome duplication could also be inhibited by overexpression of Cdc14B phosphatase. In cells arrested in G2 due to DNA damage induced checkpoint, Cdk2 paired with Cyclin-A instead of Cyclin-E is responsible for the centrosome amplification. Thus, in addition to Cdk2/Cyclin-E, Cdc14B can also counterbalance Cdk2/Cyclin-A kinase activity at least in the DXB treated U2OS cells. Although circumstantial, these evidences further strenghthen the idea that Cdc14B is a negative regulator for centrosome duplication. In addition to its role in normal centrosome cycle control as we have discussed in chapter four, Cdc14B may also act as a "guardian" to protect centrosome from being overduplicated in cases when environmental cues such as drug exposure, radiation cause centrosomes to regain duplication potentials.

This idea is supported by several previous studies. Sugihara and co-workers showed that suppression of centrosome amplification after DNA damage depends on p27 accumulation (Sugihara et al., 2006). In their study, it was shown that when cells treated with DXR, p27 levels gradually increased and Skp2 levels declined. Skp2 (S-phase kinase associated protein 2, also known as Fbxl1) is one of the best characterized mammalian F-box proteins and it is an ubiquitin ligase receptor for p27 which targets p27 for degradation (Frescas and Pagano, 2008). During DNA damage induced G2/M checkpoint (γ-radiation, DXR, etc), Skp2 level is down regulated which results in the

reduction of p27 ubiquitination thereby allowing the accumulation of p27. Skp2 is degraded at the M to G1 transition and its level begins to accumulate again at late G1 and the abundance is maximal during S and G2 phases. Cdc14B has been shown to specifically dephosphorylate Skp2 on Ser64 and renders it more susceptible to APC/C^{Cdh1} degradation at the M to G1 transition (Rodier G, 2008). Also, it has been implicated that during the G2 DNA damage response checkpoint, Cdc14B is released from the nucleolus to the nucleoplasm, activating APC/C^{Cdh1}, which in turn, targets Skp2 for proteasomal degradation (Bassermann et al., 2008). Piece together, Cdc14B may indirectly stabilize p27 during G2 DNA damage checkpoint that leads to suppression of centrosome amplification. Indeed, our data support this hypothesis. When excessive Cdc14B expression was induced, the percentage of DXR treated U2OS cells harboring more than two centrosomes significantly reduced when compared with uninduced cells. Although further direct evidence supporting the Cdc14B- APC/C^{Cdh1} -Skp2-p27 pathway in suppressing centrosome overduplication upon DNA damage in G2 is needed, our observation combined with previous studies highly suggested Cdc14B might act at upstream in the DNA damage induced G2 checkpoint to ensure cells have normal copies of centrosome before enter mitosis.

The counterbalancing effect between Cdc14B phosphatase and Cdk2/Cyclin-E/A observed in our study, however, was not direct. When treated with HU or DXR, centrosome tends to overduplicate in U2OS cells. Even it's been extensively studied that under these conditions centrosome amplification phenotype was dependent on available Cdk2s' activity, it's not clear whether Cdk2 paired with either Cyclin-E or Cyclin-A were

the sole factors involved. Therefore, in order to confirm the counterbalancing effects, it's better to use simpler systems under which direct functional interaction in the context of centrosome duplication between Cdc14B and Cdk2/Cyclin-E/A can be assayed. It has been shown previously that Cyclin-E overexpression (constitutive activation of CDK2/Cyclin-E) in both mouse and human cells with defective 53 caused centrosome amplification (Kawamura et al., 2004; Mussman JG, 2000); Also, it's been reported that overexpression of Cyclin-A alone but not Cyclin-E triggered centrosome overduplication. Therefore, the next step is to use these characterized overpression systems to directly study the effect of Cdc14B phosphatase in counterbalancing these two kinases.

Although Cdc14 contains sequences in addition to its phosphatase domain, so far all of the Cdc14 known functions in yeast rely on its phosphatase activity (D'Amours and Amon, 2004). Previous in vitro experiments suggest that mammalian Cdc14A and Cdc14B may preferentially dephosphorylate proteins phosphorylated by MAPKs and CDKs (Kaiser et al., 2002b). Genetic and functional evidence on the relationship between Cdc14A&B and their substrates is lacking. We have identified a potential Cdc14A and Cdc14B substrate, namely nucleophosmin/B23 through coimmunoprecipitation/ mass spectrometry and in vitro phosphatase assay. Direct evidence for B23's role in centrosome cycle controls came from two studies using B23 knockout mice. Knocking out B23 caused embryonic lethal indicating B23's essential role in embryonic development. Also inactivation of B23 in MEFs led to unrestricted centrosome duplication and genomic instability (Colombo et al., 2005; Grisendi et al., 2005). Additonal experimental evidence suggests that B23 controls centrosome duplication at

G1/S boundary. B23 associates with unduplicated centrosomes in G1 and inhibits centrosome duplication. Upon phosphorylation by Cdk2/Cyclin-E at Thr199 (threonine of B23 at position 199) during G1/S transition, B23 dissociates from centrosomes allowing centrosome duplication to occur though some still remain at mother centrioles of the parental centriole pairs (Kazuya et al., 2005; Okuda et al., 2000a). From S to early mitosis, B23 remains separated from centrosomes. Starting from metaphase, B23 re-associates with the centrosomes (Okuda et al., 2000a; Zatsepina et al., 1999). The re-association of B23 with centrosome requires the dephosphorylated form. Therefore, a yet-to-be found phosphatase is needed to help remove the phospho group at Thr199 on B23 in late mitosis. Our data suggests that Cdc14B can be such a candidate phosphatase. The finding of Cdc14B interacts with B23 and dephosphorylates B23Thr199 *in vitro* rasied a possibility that the Cdc14B/B23 connection may be of importance in centrosome cycle regulation. Further *in vivo* evidence strenghthened this idea. When HeLa cells depleted of Cdc14B in each different cell cycle were collected after release from a double thymidine block and probed with a polyclonal anti-phospho-B23Thr199 antibody, cells at the G1/S boundary and early mitosis showed a significant increased level of B23Thr199 phophorylation compared with the control siGLO transfected cells. G1/S boundary is the time when phosphorylation of B23Thr199 occurs, a known Cdk2/Cyclin-E phosphorylation site involved in centrosome duplication (Tokuyama et al., 2001). In line with this, our data suggest Cdc14B phosphatase may directly antagonize Cdk2/Cyclin-E activity in phosphorylating B23Thr199 to maintain a physiological kinase-phosphatase balance required for new round of centrosome duplication.

In a separate approach, we have demonstrated in HU treated U2OS cells B23 could be the downstream mediator of Cdc14B in suppressing centrosome amplification. In this context, ectopic expression of Cdc14B could've shifted the kinase-phosphatase balance between Cdc14B and Cdk2/Cyclin-E. As a result, there may exisited more dephosphorylated B23Thr199 thereby causing unscheduled re-associateion of B23 to centrosomes.

Based on the recognized impact of Cdc14 on cell cycle regulation in yeast and on mammalian microtubule dynamics and centrosome fidelity, we conclude that a thorough study of Cdc14 phosphatases in centrosome regulation of mammalian cells must benefit to our understanding on the basic mechanism of centrosome duplication, potentially revealing a novel licensing mechanism to control centrosome duplication by the counter-regulation between the specific centrosomal kinases and Cdc14B phosphatase. By the same argument, we believe that an in-depth annotating the molecular mechanism of Cdc14B-B23 interaction will enable us to delineate potential protein signaling pathway(s) that regulate the centrosome duplication in coordination with cell cycle progression.

6.4 Summary

Taken together, it's tempting to propose that there is also kinase-phosphatase balances between Cdk2/Cyclin-E/A and Cdc14B in the control of centriole duplication. In HU/APH treated U2OS or CHO cells, Cdk2/Cyclin-E is majorly responsible for the centriole amplification phenotype observed, and our data in chapter four has shown

Cdc14B phosphatase activity is required to suppress this phenotype. Further, our data here suggests this counterbalancing effect may be through their common substrate, B23; In DRB treated U2OS cells, Cdk2/Cyclin-A was shown to be responsible for centriole overduplication and our data showed this phenotype could also be inhibited by Cdc14B phosphatase activity implying, albeit indirectly, a counterbalancing effect between Cdk2/Cyclin-A and Cdc14B.

6.4 Experimental Procedures

6.4.1 Plasmids and Antibodies

HsCentrin-1 in pEGFP-C1 (Clontech) vector was kindly provided by Dr. Michel Bornens (Institut curie, Section De Recherche, France). $Cdc14B^{WT}$ and $Cdc14B^{K+C/S}$ were subcloned into pEGFP-N3 vector (Clontech) fused to a C-terminal EGFP tag whose expression was driven by the CMV promoter.

Monoclonal antibody against human B23 was purchased from Zymed Laboratories (Cat No 32-5200). Rabbit polyclonal anti-phospho-$B23^{T199}$ antibody was purchased from Abcam (Cat No ab59353). Rabbit polyclonal antibody against human Cdc14B was bought from Zymed Laboratories (Cat No 34-8900). Goat polyclonal Anti-Centrin-2 antibody was purchased from Santa Cruz Biotechnology, Inc (N-17, Cat No SC-27793-R). Monoclonal Anti-γ-tubulin antibody was obtained from Sigma-Aldrich (GTU-88, Cat No T6557).

6.4.2 Cell Culture and Transfections

U2OS (ATCC), U2OS^{Teton} (BD Bioscience) and HeLa (ATCC) cells were cultured under 5% CO_2 at 37°C in high-glucose Dulbecco's Modified Eagle's medium (DMEM, Invitrogen), 10% fetal bovine serum (FBS), and antibiotics (100 U penicillin and 50ug/ml streptomycin sulfate). Chinese hamster ovary (CHO-K1) cells were obtained from ATCC and cultured in Ham's F-12 medium supplemented with 10% FBS and antibiotics.

For transfection, cells were split to 50% to 70% confluency and transfected with Fugene HD (Roche) using 2ug of DNA and 6ul of Fugene HD reagent respectively per well in six-well plates.

The doxycyclin-inducible cell lines stably expressing C-terminal EGFP-tagged Cdc14B^{WT} (Clone 4D) and Cdc14B$^{K+C/S}$ (Clone #2) were maintained in 100ug/ml of G418 (Invitrogen) and 2ug/ml of puromycin (Sigma-Aldrich). The expressions of both Cdc14B^{WT}-EGFP and Cdc14B$^{K+C/S}$-EGFP were induced by the addition of 4ug/ml of doxycyclin.

6.4.3 Indirect Immunofluorescence

For indirect immunofluorescence, cells grown on glass coverslips were fixed rapidly for 30 minutes in -20°C 100% methanol, washed in PBS, permeabilized with PBS/0.5% Triton X-100 for 10 min at room temperature, and followed by blocking with

antibody dilution solution (1% bovine serum albumin in PBS) for 1 hour at room temperature. Cells were then incubated either 1 hour at room temperature or overnight at 4°C with primary antibodies, washed three times in PBS, and incubated for 1 hour at room temperature with secondary antibodies, washed, incubated with 0.1ug/ml of DAPI in PBS and mounted using PermaFlour Mountant media (Thermo Electron Inc) and imaging was performed at room temperature.

Primary antibodies were 1:2000 mouse anti-γ-tubulin (GTU-88, Sigma); 1:1500 goat anti-Centrin-2 (N-17, Santa Cruz Biotechnology, Inc). Secondary antibodies were 1:750 donkey anti-mouse coupled to Alexa 594, 1:750 donkey anti-goat coupled to Alexa 568 (Molecular Probes).

Cells were visualized with a 100 x Plan-Neofluar objective (1.30 oil; ∞/0.17) under an epi-fluorescence microscope (Axioskop 2, Carl Zeiss Inc). Images were acquired with a charge-coupled device camera (AxioCam HRC) controlled by Openlab software (version 3.5, Improvision Inc). Image processing was performed using Photoshop CS (V8.0).

6.4.4 RNA interference

For transient siRNA knock down experiments, oligonucleotides targeting human Cdc14B were obtained from Dharmacon (siGENOME SMARTpool, Cat No M-003470-02-0010) which correspond to the flowing sequences:
5'GAUAAUACCAGACCGAUUUUU3', 5'GAUGCUACAUGGUUAUAUAUU3',

5’CAGUAUGGCUUCCUUAAUUUU3’ and 5’CAACUCAUUUAACCUUGAUUU3’; oligonucleotides targeting human B23 were obtained from Dharmacon (On-TARGETplus SMARTpool, Cat No L-015737-00-0005) which correspond to the flowing sequences: 5’GUAGAAGACAUUAAAGCAAUU3’, 5’AAUGCAAGCAAGUAUAGAAUU3’, 5’ACAAGAAUCCUUCAAGAAAUU3’ and 5’UAAAGGCCGACAAAGAUUAUU3’. siRNA oligo pools were transiently transfected into HeLa or U2OS cells with DharmaFECT 1 transfection reagent (Dharmacon, Cat No T-2001-02). Briefly, cells were seeded at 40%-70% confluency in an antibiotic-free culture medium and transfected with siRNA oligo pools at a final concentration of 100nM for 60 hours (unless otherwise specified). Negative controls were cells transfected with 100nM siGLO RISC-Free Control siRNA (Dharmacon, Cat No D-001600-01-20).

To generate B23 knockdown stable cell lines, siRNA oligo that target the sequences 5’-GAATTGCTTCCGGATGAC T-3’ (Korgaonkar et al., 2005) (819-837, relative to the start codon of B23, designated as B23siRNA819) was annealed and cloned into the XhoI and BglII sites of pSuper.retro.neo+GFP vector (Oligoengine). Stable clones constitutively expressing B23siRNA819 were established by cotransfection with pBabe-puro (a kind gift from Dr. Gerald Evans) into U-2OS$^{Tet\text{-}on}$ cells and selection in a growth medium containing 800 μg/ml G418 and 5μg/ml puromycin.

6.4.5 Western Analysis

Cells were first lysed in a buffer containing 1% SDS in Tris-Hcl pH 7.4. Then 6xSDS sample buffer was added and samples were boiled for 10 minutes, votexed and

cell debris spun down at 15,000 rpm for 5 minutes. An equal amount of proteins were loaded for SDS-PAGE and transferred onto nitrocellulose membrane (BioRad). Membranes were then blocked by 5% skim milk dissolved in PBST (0.01% Tween20 in PBS) for 1 hour at room temperature. After blocking, membranes subjected to incubation with primary antibody diluted in 3% BSA in PBS for overnight at 4°C. Then membranes were washed three times with PBST and incubated with horseradish peroxidase conjugated secondary antibodies (Sigma) for 1 hour at room temperature. Membranes were washed again and specific signals were detected using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

6.4.8 Cell Synchronization

For HeLa cell synchronization, double thymidine block approach was carried out. Briefly, cells were cultured in fresh medium containing 2mM thymidine for 19 hours. After first thymidine block, cells were washed three times with 1xPBS and incubated in fresh medium for 9 hours to release cells. After releasing, 2mM thymidine was added again to the culture medium and incubated for another 16 hours. After second block, cells were washed again and released into the fresh medium, and samples of synchronized cells were collected and lysed at successive time points.

In a separate approach, HeLa cells were also synchronized with drugs: For G0/G1 block, HeLa cells were washed and incubated in low serum medium (0.5% FBS in DMEM) for

48 hours; For S phase arrest, HeLa cells were treated with either 2mM hydroxyurea (HU) or 10ug/ml aphidicolin (APH) for 48 hours. After incubation, cells blocked at each cell cyles were lysed and subjected to western analysis.

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