O-H-I-O

Forever a Buckeye: It’s in your DNA
Mps1 and Plk4 Cooperate to Regulate Centriole Assembly

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree in Doctor of Philosophy in the Graduate School of The Ohio State University

By:

Amanda Nichole Bliemeister, B.S.
Graduate Program in Molecular, Cellular, Developmental Biology

*****

The Ohio State University
2014

Dissertation Committee:

Dr. Harold Fisk, Advisor
Dr. Jian-Qiu Wu
Dr. Paul Herman
Dr. Patrick Green
Abstract

The centrosome is comprised of two centrioles surrounded by a pericentriolar material. In dividing cells, centrosomes are the primary microtubule organizing centers, orchestrating mitotic spindle assembly. The centriole biogenesis pathway is the process by which a single centrosome is duplicated prior to cell division, resulting in the production of two mature centrosomes that are capable of organizing bipolar spindle assembly and orchestrating the microtubule networks. This ensures each daughter cell receives an identical copy of the genome and a single centrosome. Changes in centrosome number can lead to lack of mitotic fidelity and genome instability, leading to aneuploidy, a common hallmark of human tumors. Therefore, tight regulation of centrosomal replication is critical as it maintains the fidelity of chromosome segregation.

In human cells, the centriole biogenesis pathway is complex, and currently over 400 proteins have been indicated to participate in the localization, regulation, and duplication of centrosomes. Due to the complexity of this pathway, much of what is known about centriole biogenesis has been derived from model organisms. The protein kinase Zyg-1 is an essential regulator of centrosome duplication in *C. elegans*, which phosphorylates ceSas-6 and is required for its recruitment to the forming central tube. Centriole duplication in human cells is governed by Plk4, the initiator and master regulator of centriole assembly, and is the presumed ortholog of Zyg-1. However, Plk4
does not assume all of the functions of Zyg-1, as it has not been shown to phosphorylate hSas-6, and centriole amplification by overexpression of hSas-6 does not require Plk4. While overexpression of Plk4 induces centriole amplification, and loss of Plk4 leads to failure in centriole duplication, it remains unclear how Plk4 initiates centriole duplication. Combined, this suggests that the initiation and regulation of centriole assembly involves other players.

The hMps1 protein kinase is an intriguing and controversial player in centriole assembly. Originally shown to control duplication of the budding yeast spindle pole body, Mps1 is present in eukaryotes from yeast to humans, C. elegans being a notable exception, and has also been shown to regulate the spindle checkpoint and an increasing number of cellular functions relating to genomic stability. The role of hMps1 at the centrosome remains controversial, despite that expression of a non-degradable form of hMps1 leads to centriole amplification, and depletion of hMps1 leads to defects in proper centriole assembly. Combined, this indicates that hMps1 is a centrosomal kinase whose misregulation causes centrosome amplification.

Work described here shows centrosomal cooperation between the kinases hMps1 and Plk4. We have shown that the Mps1 inhibitors IN-1 and AZ3146 do not effectively inhibit centrosomal Mps1 activity both by in vitro kinase assays and an in vivo Centrin 2 recruitment assay, though AZ3146 is slightly more effective than IN-1. Overexpression of Plk4 or non-degradable hMps1 leads to centrosome reduplication in both cycling and S phase arrested cells. In each case, hMps1 driven centriole reduplication requires Plk4, and Plk4 driven centriole reduplication requires hMps1. Additionally, overexpression of
hMps1 can compensate for depletion of Plk4, which requires hMps1 kinase activity, and overexpression of Plk4 can compensate for hMps1. Furthermore, we confirmed that cycling cells are not entering S phase with extra centrioles. Overexpression of Mps1 reduces centrosomal levels of Plk4, and knockdown of Plk4 affects centrosomal levels of Mps1.

This is further supported evidence that each kinase is a substrate for the other, and use of centrosomal preparations has shown that hMps1 and Plk4 may share other centrosomal substrates. Dual knockdown of hMps1 and Plk4 leads to synergistic inhibition of proper recruitment of hSas-6 to the centriole. Additionally, hSas-6 is a substrate of both Plk4 and hMps1. We have shown that hMps1 primarily phosphorylates the C terminus of hSas-6 at multiple sites. It has been previously shown that overexpression of Sas-6 drives re-duplication. We have shown that overexpression of Sas-6 cannot compensate for the dual knockdown of hMps1 and Plk4, though Sas-6 can still drive centriole reduplication. Together, these results have a significant impact, providing evidence that the centrosomal protein kinases hMps1 and Plk4 cooperate to regulate the centriole biogenesis pathway.
This dissertation is dedicated to my family, especially to Peter Middaugh. This day and always, you are loved and missed beyond comprehension.
The cover of this dissertation has only my name. However, earning a Ph.D. is a long journey, and a great many individuals have contributed to its production throughout the years. I owe an overwhelming amount of gratitude to everyone that has made this dissertation possible.

First and foremost, I would like to thank my family. Thank you to my mother, Lisa Barton, and father, Marshal Bliemeister, for showing a constant support and willingness to listen, and especially for all of your sarcasm and sense of humor. Thank you to my grandparents Peter and Barbara Middaugh, you have always been a source of encouragement, comfort, and love. Thank you to my stepmother and friend, Rose Volkening. Thank you to Jeff Barton. Thank you to my grandmother, Donna Bliemeister. Thank you to my Uncle Matt, Aunt Julie, and cousins Becky, Ryan, Emily, Matt, and Steven. Thank you to my Uncle Dennis, Maureen, and my cousins LilyAnne and Sidney Peter. Thank you to my sister, Megan. All of you have shown me what it means to be a family, and words cannot express how grateful I am to each and every one of you. Thank you to Tesla Coomassie Bliemeister, my black Labrador retriever, who acted as my assistant in writing this dissertation. And last, but certainly not least, I am forever
friendship indebted and thankful to my best friend and brother, Sathiya Narayanan Manivannan.

I would like express profound gratitude and deep regards to my advisor, Dr. Harold Fisk, for all of his exemplary guidance, constant encouragement, and support through the past years. You have constructed a laboratory environment of collaboration and discussion, for which I cannot fully express my gratitude. In addition, I would like to thank all the original members of the Fisk lab, “Fiskies”, including Dr. Chris “The Stud” Kasbek, Dr. Ching-Hui Yang, Dr. Adlina Mohd Yosuf, and Dr. Shubhra Majumder, who provided an amazing laboratory environment of friendship, encouragement, discussion, providing a foundation for learning. Thank you to the current “Fiskies”, for their friendship, discussion, and sense of humor, including Dwitiya Sawant “Dritta”, for just being amazing, particularly her sensitivity and ability to listen, Joey Marquardt “Joe Joe Supreme” for his love of donuts and science – questioning and understanding, Sanh Tan “Fancy” Nguyen who will continue the Mps1/Plk4/Sas-6 project, Mitch Fasching “Lab Mitch” particularly for his sarcasm and addition of “pants” to proper PPE for radiation safety. Thank you to the previous “Fiskie” lab undergraduates for your friendship and for enhancing my graduate experience through teaching, particularly Lori Koch, for her exquisite contributions to the Sas-6 project and her astounding sense of humor and sarcasm, Ayla “Know That” Cash for encouraging me to follow my dreams and for reminding me of my love for community service, Kyle Beuoy who taught me that genuine kindness does still exist in the world, Tyler Brown who has an inspirational amount of motivation and drive, and Dominic Castanzo who I hope one day can make
“Berfers” without “Burburs” and that he finds “the girl in the yellow dress.” I would also like to thank my undergraduate advisor, Dr. Regina McClinton, especially for her encouragement, friendship, and for always believing in me.

I would not have completed this dissertation without the friendship of the following exceptional individuals, Skye Bochter, Kanu Wahi, Meriam Waqas, Kara Braunreiter, Katelyn Zac, Dr. Ilea Swinehart, Dr. Lindsey Richmond and Jeremy Pruneau, Dr. Sarah Hansen, and Justine Kramer for their support and friendship.
Vita

July 16th, 1985................................. Born – Columbus, Ohio
2003 – 2007...................................... Bachelor of Science
    Major: Cellular and Molecular Biology
    Minor: Chemistry
    Grand Valley State University
    Grand Rapids, Michigan
2008 – Present............................. Graduate Research Associate
    Molecular, Cellular, Developmental Biology
    Department of Molecular Genetics
    The Ohio State University
    Columbus, Ohio

Publications


Fields of Study

Major Field: Molecular, Cellular, Developmental Biology
Table of Contents

Abstract ..............................................................................................................ii
Dedications .......................................................................................................iii
Acknowledgements ..........................................................................................vi
Vita ..................................................................................................................viii
List of Figures ...................................................................................................xiii
List of Abbreviations .........................................................................................xvi
Chapter 1: Introduction ...................................................................................1
  1.1 Overview ..................................................................................................1
  1.2 Discovery of the Centrosome ...................................................................2
  1.3 The Centrosome Cycle – Intricately tied to the Eukaryotic Cell Cycle ......3
  1.4 Structure of the Spindle Pole Body and Centrosome .........................6
      1.4.1 Centriole Biogenesis in Caenorhabditis elegans .........................12
      1.4.2 Centriole Biogenesis in Human Cells ......................................13
      1.4.3 Spindle Pole Body Duplication Cycle ......................................14
      1.4.4 The Centrosome Duplication Cycle .......................................15
      1.4.5 Centriole Assembly: Canonical versus de novo pathways .........17
  1.5 Function of the Spindle Pole Body and Centrosome .........................18
List of Figures

Figure 1.1: Protein Composition of the Spindle Pole Body in *Saccharomyces cerevisiae* .................................................................34

Figure 1.2: Centrosome Assembly in *C. elegans* .........................................................35

Figure 1.3: Structure of the Mammalian Centrosome ..................................................36

Figure 1.4: Centrosome Assembly in Mammalian Cells ................................................37

Figure 1.5: The Mammalian Centrosome Duplication Cycle .........................................38

Figure 1.6: Regulation of Mps1, Plk4, and Sas-6 is Critical to Limiting Centrosome Duplication to once per cell cycle .................................39

Figure 1.7: Structural Comparison of the centrosomal kinases Plk4, Mps1, and Zyg-1 ...40

Figure 2.1: Cell cycle Profile of Mps1 Protein and Activity Levels and its control by Degradation .................................................................52

Figure 2.2: The Mps1 Inhibitor, IN-1, is not equally as effective against all Mps1 substrates *in vitro* .............................................................54

Figure 2.3: Mps1 inhibitors, IN-1 and AZ3146, are not as effective against all Mps1 substrates *in vitro* .............................................................56

Figure 2.4: Attenuation of Mps1 inhibits Centriole Biogenesis .......................................57

Figure 3.1: Simultaneous Knockdown of Mps1 and Plk4 Inhibits Recruitment of Sas-6 to the Centriole .........................................................80
Figure 3.2: Mps1 and Plk4 are Mutually dependent for Centriole Re-duplication in U2OS cells………………………………………………………………………………..82

Figure 3.3: Mps1 and Plk4 are Mutually dependent for Re-duplication in RPE-1 cells………………………………………………………………………………84

Figure 3.4: Plk4 Dependent Re-duplication Requires Mps1……………………………………………………………………………………………………85

Figure 3.5: Mps1 Δ12/13 Dependent Re-duplication Requires Plk4…………………………………………………………………………………………….86

Figure 3.6: Analysis of Centrioles in G1 Cells……………………………………………………………………………………………………………………..88

Figure 3.7: Confirmation that Cycling Cells are not entering S Phase with Extra Centrioles…………………………………………………………………..89

Figure 3.8: Knockdown of Plk4 affects Mps1 Centrosomal Levels……………………………………………………………………………………………91

Figure 3.9: Overexpression of Mps1 Reduces Centrosomal Plk4 Levels……………………………………………………………………………………92

Figure 4.1: Expression and Purification of SUMO-His-Plk4……………………………………………………………………………………………………..106

Figure 4.2: Expression and Purification of SUMO-His-MBP……………………………………………………………………………………………………108

Figure 4.3: In-Vitro Phosphorylation: Plk4 Phosphorylates Mps1 KD…………………..109

Figure 4.4: In-Vitro Phosphorylation: Mps1 Phosphorylates Plk4 KD…………………..110

Figure 4.5: Centrosomal Preparation…………………………………………………………..111

Figure 4.6: Mps1 and Plk4 may share Potential Substrates…………………………………………………………………………………………………….112

Figure 5.1: Expression and Purification of Sas-6………………………………………………………….124

Figure 5.2: Mps1 Phosphorylates Sas-6 in vitro………………………………………………………….125

Figure 5.3: Identification of Potential Mps1 Phosphorylation sites within Sas-6………….126

Figure 5.4: MBP-Sas-6 Truncations……………………………………………………………..127
Figure 5.5: The N- and C-terminus of MBP-Sas-6 contain potential in-vitro phosphorylation sites by GST-Mps1

Figure 5.6: Expression and Purification of Sas-6 Alanine Mutations in the N- or C-terminus of Sas-6

Figure 5.7: GST-Mps1 potentially phosphorylates MBP-Sas-6 in vitro at Multiple Sites

Figure 5.8: The C-terminus of MBP-Sas-6 contains Potential in-vitro phosphorylation sites by SUMO-His-Plk4

Figure 5.9: Overexpression of Sas-6 cannot compensate for dual depletion of Mps1 and Plk4

Figure 6.1: Preliminary Model that a Combination of Mps1 and Plk4 may be the true functional analogs of C. elegans Zyg-1

Figure 6.2: Preliminary Model for Potential Feedback Loop between Mps1 and Plk4
List of Abbreviations

Protein Abbreviations

Monopolar Spindle 1 (Mps1)
Mps1- Kinase Dead (KD)
Polo-like Kinase 4 (Plk4)
Spindle Assembly 6 (Sas-6)
Green Fluorescent Protein (GFP)
Anaphase Promoting Complex/Cyclosome (APC/C)
Cyclin Dependent Kinase (Cdk)
Cyclin Dependent Kinase 1 (Cdk1)
Cyclin Dependent Kinase 2 (Cdk2)
γ- Tubulin Ring Complex (γ-TuRC)
Centrin (Cetn)
SCL-Interrupting Locus Protein (STIL)
Snk Akin Kinase (SAK)

Technique/Reagent/Molecule Abbreviations

Indirect Immunofluorescence (IIF)
Hydroxyurea (HU)
Small Interfering Ribonucleic Acid (siRNA)

5- bromo-2-deoxyuridine (BrdU)

Deoxyribonucleic acid (DNA)

Complementary DNA (cDNA)

Dimethyl sulfoxide (DMSO)

Protein Correlation Profiling (PCP)

Electron Microscopy (EM)

Double Stranded RNA (dsRNA)

Dimethylsulfoxide (DMSO)

Fetal Bovine Serum (FBS)

Sodium Dodecyl Sulfate (SDS)

Other Abbreviations

Chromosomal Instability (CIN)

Microtubule Organizing Center (MTOC)

Spindle Pole Body (SPB)

kiloDalton (kDa)

Standard Deviation (SD)

Pericentrilolar matrix (PCM)

Retinal Pigment Epithelial Cells (RPE1)

Cervical Cancer Cells derived from Henrietta Lacks (HeLa)

Polo Box motif (PB)
Wild Type (Wt)

*Caenorhabditis elegans* (*C. elegans*)

Spindle Pole Body (SPB)
Chapter 1

Introduction: Structure and Function of the Centrosome

1.1 Overview

Centrosomes are cellular organelles best known as microtubule organizing centers (MTOCs) as they are directly involved in the generation, organization, and regulation of dynamic microtubules, mitotic spindles, and cilia. The core of the centrosome is comprised of a pair of centrioles, which function to recruit and organize pericentriolar material (PCM) which is responsible for microtubule nucleation. Despite the importance of centrosomes and their regulation, much remains unknown to the complicated and tightly regulated centriole biogenesis process. The maintenance of fidelity in centriole duplication is critical, as defects in either the number of centrioles or the assembly of centrioles, is capable of promoting aneuploidy, tumorigenesis, ciliopathies, birth defects, and primary microcephaly. This review will summarize the current understanding of centriole biogenesis and regulation, specifically highlighting the importance of the centrosomal proteins Sas-6, Mps1, and Plk4.
1.2 Discovery of the Centrosome

Walther Flemming illustrated the first glimpse of the centrosome in 1875 while describing cell division using the eggs of the freshwater mussel *Anodonta* [1][2]. In 1876, Edouard Van Beneden provided the first description of the centrosome in a family of parasitic nematodes, the Dicyemidae, as a subcellular structure involved in cell division [3][4]. Van Beneden went on to study fertilization in the roundworm species *Ascaris megalocenephala*, further describing the centrosome as a permanent “polar corpuscle” in 1887 [4]. In a simultaneous and independent study utilizing both *Ascaris* and artificially fertilized sea urchin eggs, Theodor Boveri discovered and published the same subcellular organelle in 1888, described as an “especial organ of cell division”. In this publication, Boveri demonstrated this organelle to be localized centrally, near the nucleus, and therefore was termed the “center body” or “centrosome” [4][5]. Both Van Beneden and Boveri observed permanent “rounded granules” with an autonomous self-replication behavior similar to the division of chromosomes, dividing when new asters were formed. Boveri demonstrated that the centrosome is the division center for the egg of *Ascaris*, and became one of the first scientists to postulate that a defective division center may lead to aneuploidy, the inheritance of too many or too few chromosomes, and further hypothesized that this may promote cancer [6][7][8]. This hypothesis was generated based on Boveri’s experiments in *Ascaris* eggs, demonstrating that cells with more than two centrosomes leads to the formation of multipolar spindles during mitosis, and this cell is not capable of distributing chromosomes equally to daughter cells [7].
The pioneering studies of Van Beneden and Boveri effectively demonstrated the potential importance of the centrosome in cell division. Though over 120 years has passed since the publication of Van Beneden and Boveri’s work, the centrosome remains clouded with mystery, as many secrets to the exact composition, structure, function, and how inappropriate regulation or construction of the centrosome can lead to human disease, remain fundamental open questions.

1.3 The Centrosome Cycle – Intricately tied to the Eukaryotic Cell Cycle

All organisms consist of cells that arise from pre-existing cells, and mitosis is the process by which new cells are generated. Van Beneden and Boveri were some of the first scientists to determine that the centrosome cycle is intricately tied to the eukaryotic cell cycle and cell division. While advancement through the cell cycle, specifically from G1 to S is the trigger for promoting centrosome duplication, centrosomes are not simply followers of the cell cycle. The centrosome serves as a catalytic center, a site for the concentration of proteins and catalyzing enzymatic reactions that are important for controlling cell cycle progression [9].

Cell division is the process by which a parental cell divides into two daughter cells, equally distributing identical genetic material, specifically deoxyribonucleic acid (DNA). In single celled organisms, each cell division produces a whole new organism, whereas in large multicellular organisms, such as mammals, cell division is required during development to produce many different cell types that make up the body from one fertilized egg cell. Cell division is also required in fully developed mammals for the
generation of tissues, as there is a high turnover rate of the body’s cells throughout life for tissue maintenance and repair after injury. Cell division occurs as part of a larger cell cycle, which is the entire process of duplication of the genetic information through to division into two new cells.

The main priority of cell division is the maintenance of genomic integrity. As the division center of the cell, centrosomes and other MTOCs organize this maintenance and hence are strictly regulated throughout the cell cycle. The cell cycle is an ordered series of events that leads to cell division and the production of two daughter cells, both of which contain chromosomes and a single centrosome, identical to those of the parental cell. Regulation of the cell cycle is critical, as any loss of control may lead to the generation of non-viable or aneuploid daughter cells. The cell cycle consists of two basic parts, mitosis (M) and interphase. Interphase is further divided into three stages including G1, S, and G2. In the synthesis (S) phase, replication of the parental DNA occurs and chromosomes are equally distributed to each daughter cell during mitosis (M). S phase is preceded by a gap called G1 during which the cell prepares for DNA synthesis and chromosome replication. S phase is followed by another gap, known as G2, during which the cell prepares for mitosis. The two gap phases are periods of time that allow the cell to monitor the external environment for growth factors or cell density and the internal environment for DNA damage or abnormal cellular structure. Under certain conditions, such as the absence of growth stimuli or terminal differentiation, cells can exit the cell cycle during early G1 phase and enter a quiescent phase called G0. In a typical mammalian cell, interphase is approximately 24 hours, as this is a period of cell growth.
increasing in mass and volume as the cell is preparing for mitosis, whereas passage through mitosis takes approximately 1 hour.

Mitosis (nuclear division) is further divided into five stages: prophase, metaphase, anaphase, telophase, and cytokinesis. Prophase is characterized by two key events. First, the chromatin becomes highly condensed, and second, the nuclear envelope breaks down. Metaphase marks alignment of chromosomes at the metaphase plate, and is easily visualized by microscopy, appearing as a line of chromosomes along the center of the cell (known as the metaphase plate) and at the center of the bipolar mitotic spindle. During anaphase, the chromatids begin to move to opposite poles of the spindle by microtubule attachments to chromosomal kinetochores. Telophase also has two key events. First, the chromosomes start to de-condense, and second, the nuclear envelope begins to reform. The final stage of mitosis is cytokinesis, when the mother cell divides into two daughter cells, each of which contain the same genetic information [10].

During mitosis, cells align their duplicated nuclear deoxyribonucleic acid (DNA), specifically chromosomes, along the center of the cell, as part of preparation for anaphase and subsequently cytokinesis. The human somatic cell contains of 23 pairs of chromosomes (46 chromosomes total), 22 pairs of numbered chromosomes, known as autosomes, and one pair of sex chromosomes, X and Y, localized within the nucleus. Chromosomes are made up of DNA that is tightly wrapped around histone proteins. These duplicated chromosomes are equally segregated to two daughter cells by generation of a bipolar mitotic spindle. All of this is coordinated by the centrosome, located at the heart of each mitotic spindle which localize to opposite sides of the cell
during mitosis which are then known as poles, when each formed spindle apparatus nucleates microtubules inwards toward the chromosomes. Microtubules are involved in the movement of materials, including cellular vesicles, organelles, and chromosomes, and have a role in the maintenance of overall cellular structure.

Cytokinesis is the final event in the cell cycle, and takes place once all sister chromatids have made a physical attachment with a microtubule and have been pulled toward opposite ends of the cell. The equal distribution of chromosomes ensures that the resulting daughter cells have the identical DNA content as the starting cell. Centrosomes are a critical component of this process, and hence understanding their structure, composition, and regulation is of utmost importance.

1.4 Structure of the SPB and Centrosome

Biologically speaking, centrioles are among the most beautiful structures, inspiring artistic renditions [11] and jewelry [12]. Centrioles are small cylindrical structures, approximately 450 nm in height [13][14], with an inner diameter of 130nm and an outer diameter of 250 nm [15], with an approximate diameter of 25 nm [16], surrounded by an amorphous pericentriolar material (PCM) [17]. Combined, these comprise the centrosome, the cellular organelle that is responsible for nucleating and organizing the microtubule cytoskeleton, commonly referred to as the microtubule organizing center (MTOC) [18]. This MTOC is a diverse structure, and different organisms have different types of MTOCs, from yeast spindle pole bodies (SPBs), plant asters, basal bodies, and centrosomes. Basal bodies and centrioles are found in higher
eukaryotes and are thought to be structurally and compositionally similar. In fact, the centrosome is composed of two centrioles, from which the primary cilium is formed from one of the centrioles in the centrosome, and this acts as a basal body.

The centrosome is a unique organelle in the fact that it does not have a distinct membranous boundary. Additionally, many centrosomal proteins are not unique or specific to the centrosome, and have other cellular roles. The centrosome also has a close association with the nuclear membrane and is relatively small in size. Together, this has made the accurate identification of centrosomal components difficult. However, many proteins are enriched at the centrosome, and this enrichment can be specific to the particular cell cycle phase. A variety of techniques have been utilized to identify true centrosomal components, including: yeast two-hybrid [19], genetic analysis [20], mass spectrometry-based proteomics [21][22][23][24] including multidimensional protein identification (MudPIT) [25], biotin identification (BioID) [26], and protein correlation profiling – stable isotope labeling by amino acids in cell culture (PCP-SILAC)/quantitative mass spectrometry [27].

A large-scale proteomics study identified the centrosome proteome with over 70 known proteins and 23 potential centrosome proteins utilizing PCP and mass spectrometry [21], and a second identified the major structural components of the centriole utilizing MudPIT [25]. These studies did not have a way to discriminate between contaminants and true centrosomal proteins, but an advanced follow up study utilized PCP-SILAC-MS combined with BAC transgeneOmics and an antibody screening attempted to address contaminants versus true centrosomal proteins [23]. This identified
170 centrosomal proteins, which included previously reported proteins and 61 new candidate proteins [23].

The most recent study utilized BioID to identify proximity interactions among centriole proteins known to be involved in centrosome duplication [26]. Proteins including Plk4, Cep63, Cep152, Cep192, and CPAP were labeled with a biotin ligase derived from *E. coli*. Cells expressing this protein were incubated with excess biotin, and these biotinylated proteins were affinity purified and identified by mass spectrometry. The strong affinity of the biotin-streptavidin interaction allows for preservation of proximal interactions, eliminating the need for physical interactions from traditional approaches such as standard immunoprecipitation, which requires denaturation of proteins from their native structure. Biotin labeled Plk4 identified many proteins within its vicinity including: Cep152 and Cep192 [28][29], both previously shown to physically interact with Plk4, PCNT, NEDD1, Plk1, STIL, as well as Mps1 (TTK) [26].

Combined, these studies have been critical in determining the composition and structure of the centrosome. Centrosomedb [30] is a database that is specific to the human centrosomal proteome. This database currently contains 464 genes encoding proteins that localize to the centrosome either stably or transiently [30].

In addition to a variety of techniques, numerous model systems are used to study the composition and structure of the centrosome, including the budding yeast *S. cerevisiae*. Yeast and other fungi contain a functional equivalent to the centrosome, known as the spindle pole body (SPB). Functionally, the SPB is very similar to the centrosome found in human cells, as it is duplicated once prior to mitosis, is involved in
the organization of microtubules for cell division, and is distributed to each daughter cell during mitosis. Structurally, the SPB is very different from the centrosome, though it does contain many proteins that have homologs in human cells. The most notable of these structural SPB components includes the core structural component Spc110, which has a human homolog, Kendrin, also known as pericentrin B [31][32]. Kendrin has recently been shown to be a novel substrate of separase, and is intimately involved in licensing of centriole duplication [33]. The SPB is a multi-layered organelle, composed of three plaques: An outer plaque (OP), an electrodense central plaque (CP) and an inner plaque (IP) [34][35] (Figure 1.1). Of the known 18 main centrosomal proteins in yeast, 10 have human homologs [24]. The nucleus is separated from the cytoplasm by the nuclear envelope (NE), which is where the yeast SPB is located; a prime location for contributing to microtubule organization, as the NE is contiguous with the central plaque, which is composed of several proteins, including: Spc29 [36], Spc42 [37], Spc110 (human homolog Kendrin) [38], and Cmd1 (human homolog Calmodulin) [24][39][40].

The inner plaque, which is localized within the nucleus, is linked to the central plaque by the spacer molecule Spc110 [41]. The inner plaque is responsible for the segregation of chromosomes and the maintenance of nuclear structure, as it is the nucleation site for nuclear microtubules. The inner plaque is composed of many proteins including: γ-TUSC (γ-tubulin small complex) [42], and a microtubule nucleation complex containing Spc97 (human homolog TUBGCP2) [31], Spc98 (human homolog TUBGCP3) [31], and Tub4 (human homolog TUBG1:γ-tubulin) [43]. The outer plaque also consists of the γ-TUSC [42], and orients the mitotic spindle by nucleating cytoplasmic microtubules to ensure
proper migration through the emerging bud of the daughter cell. The outer plaque is attached to the central plaque by Cnm67 [44], Nud1 (human homolog Centriolin) [45], and Spc72 (human homolog TACC) [46]. The insertion and linking of the SPB to the membrane is achieved by Nbp1, Ndc1, Bbp1, and Mps2 [47] for which human homologs do not exist. Also of interest, the SPB has an electron dense region embedded in the nuclear envelope that is termed the half-bridge which is the site of SPB duplication [48]. Mps3[49], Kar1 [50], Cdc31 (human homolog Centrin) [51], and Sfi1 (human homolog hSfi1) [52] are all components of this half bridge [48]. Yeast also have many protein kinases found in human cells, including Cdc28 (human homolog Cdk1) [53], Mps1 (human homolog TTK or hMps1) [54], and Cdc5 (human homolog – polo kinases) [55][56].

SPB duplication is well recognized as a model for centrosome duplication in human cells [48]. Centrioles are found across kingdoms: algae, fungi [57], plants, and even certain single-celled eukaryotes [57]. In human cells, the centrosome is located near the nucleus within the cytoplasm. It consists of two centrioles that are oriented orthogonally; with each centriole polarized along its long axis, with the tip of the centriole referred to as the distal end and the base as the proximal end. During G1, the two centrioles are connected by a flexible linker, which emanates from the base of the centriole. The centrosome is duplicated once per cell cycle, in a semi-conservative fashion, similar to DNA. The process of duplication begins at the proximal end of an existing centriole, with the recruitment of a cartwheel structure, which is composed of a central hub and spokes that act as a scaffold for the nine-fold symmetrical architecture of
triplet microtubules. The cartwheel is composed of several proteins including Sas-6 and Cep135. The formation and elongation of the microtubule triplets involves many proteins including: Cep152, Cep192, STIL, CPAP, and α-, β-, δ-, ε-tubulin. The distal end of the centriole is composed of Centrin 2 and CP110.

The centrosome contains two structurally distinct centrioles. The mother centrioles (MC) are the existing centrioles that were constructed during the previous cell cycle, and hence are older. The younger centriole is known as the daughter centriole (DC), and its construction occurs at the proximal end of the mother centriole during S phase in a process known as centriole biogenesis. The MC acquires two sets of appendages, distal and sub-distal in G2/M, which are involved in the anchoring of microtubules. These appendages are essential components of a centriole, and are involved in the conversion of the centriole to a structure known as a basal body, which acts as the scaffold for the production of cilia. Examples of appendage proteins include Cep164 [58], VDAC3 [59][60] and ODF2 [61]. The MC acts as a base or docking site for the construction of a daughter centriole every cell cycle, specifically during S phase. This suggests that the proximal portion of the mother centriole serves as a unique docking site serves as the position for recruitment of the cartwheel. Formation of the cartwheel is preceded by several proteins, including: Mono-polar Spindle 1 (Mps1), Cyclin-Dependent Kinase 2 (Cdk2), and Polo-like Kinase 4 (Plk4). The surrounding PCM contains several proteins, including pericentrin and γ-tubulin.
1.4.1 Centriole Biogenesis in *Caenorhabditis elegans*

Much of what is known about centriole biogenesis is derived from studies in model organisms [48][62]. Centrioles in *C. elegans* are 100nm in diameter and 150nm in length, and consist of a central tube surrounded by nine singlet microtubules [63].

*C. elegans* has proven instrumental for the discovery of centriole components and for determining their order of recruitment. Genome wide RNAi screens in *C. elegans* identified five essential centriole biogenesis proteins; SPD-2 [64][65], ZYG-1 [66][67], SAS-4 [68][69], SAS-5 [70], and SAS-6 [13][71] (Figure 1.2). Live cell imaging of *C. elegans* embryos placed these proteins into an ordered assembly pathway. Pro-centriole formation is initiated by the recruitment of SPD-2; The ZYG-1 protein kinase is recruited in an SPD-2 dependent manner; ZYG-1 recruits a complex containing SAS-5 and SAS-6, and this promotes the formation of a central tube which determines basic centriole structure; This is followed by SAS-4, which facilitates the assembly of microtubules and mediates pro-centriole elongation [64][72][63][71][73][74] (Figure 1.2).

Centrioles have an ancient evolutionary origin [4][75][76], as they are present in all major eukaryotic groups [57]. There is a conservation in the morphology of the centrosome and basal body, but this is a surprising finding, as the centriole and basal body are found in diverse contexts throughout eukaryotic life [76]. Analysis of eukaryotes has revealed proteins related to the *C. elegans* core centriole components [77][15][57]. Specific bioinformatics analysis of six proteins that are essential for centriole assembly in human cells, including Sas-6, Sas4/CPAP, Bld10/Cep135, Plks,
SPD2/Cep192, and CP110, revealed conserved regions and demonstrated that all of them generally contain coiled-coil domains [76].

1.4.2 Centriole Biogenesis in Human Cells

Centrioles are necessary for cilia and flagella formation, centrosome organization, spindle assembly, cytokinesis, and cell-cycle control. The canonical centriole assembly pathway in human cells follows the same core program displayed in C. elegans (Figure 1.3). Cep192 is the human ortholog of C. elegans SPD-2, and is required for both centriole biogenesis and centrosome maturation [78]. Cep152, which has no C. elegans ortholog, but is the ortholog of D. melanogaster Asterless, also binds to Plk4 [28], and serves as a scaffold for the onset of centriole assembly [79]. Plk4 requires the presence of both Cep152 [29] and Cep192 [78] for its recruitment to the centriole as they cooperate in the recruitment of Plk4 to the centriole, and depletion of both results in complete loss of Plk4 from centrosomes and impairs centriole duplication [77]. The centrosomal kinase Plk4 is the presumptive functional ortholog of Zyg-1 [80][81]. After Plk4, Sas-6 is recruited to the centriole [82] (ortholog of C. elegans Sas-6), followed by Cep135 (ortholog of D. melanogaster Bld10) [80][83] and CPAP/CENP-J (ortholog of C. elegans Sas4) [84][85] (Figure 1.4). Other major players in procentriole assembly, elongation, and maturation involves γ-tubulin and CP110 [86]. Centriole assembly in human cells also involves many other additional proteins not identified in C. elegans, including but not limited to Mps1 [87][88][89], Cep152 [90], Cetn2 [87][91], Poc proteins (hPoc5)[92], δ- and ε-tubulins [93][94], among others (Figure 1.3).
1.4.3 Spindle Pole Body Duplication Cycle

Duplication of the spindle pole body follows a specific cycle that can be divided into three discernable steps [95]. G1 phase marks the beginning of the first step, where satellite material from the central plaque deposits on the cytoplasmic tip of the elongating half-bridge [95]. Of particular interest to this study, the protein kinase Mps1, is a non-structural component of the SPB that is required early in SPB duplication [96]. The second phase involves expansion of the satellite material into a duplication plaque, which is the mature cytoplasmic half of the SPB, following retraction of the half-bridge. Again, Mps1 is required for completion of this second phase, along with another kinase, Cdc28 [39][54]. The third phase will complete SPB duplication and begins by the insertion of the duplication plaque into the nuclear envelope resulting in assembly of the inner plaque [39]. Formation of the inner plaque, specifically insertion of the duplication plaque into the inner envelope requires several proteins, including Mps1 [97], Mps2 [54], and Ndc1 [39][98] (Figure 1.1). Once membrane insertion is completed, Cmd1 and Spc110 localize to the central plaque, which is responsible for the assembly of the microtubule nucleation complex, comprised of Spe97 [99], Spe98, and Tub4 [100][32], which is the human ortholog of γ-tubulin (Figure 1.1). In yeast, duplication of the SPB is completed before the end of G1. Once the SPB is duplicated, the bridge connecting the SPBs is severed in S-phase, a process that requires Cdc28 [53]. Now the duplicated SPBs can separate and move to opposite sides of the nuclear envelope to form the bipolar mitotic spindle ensuring accurate chromosome segregation to the daughter cells in mitosis [95].
1.4.4 The Centrosome Duplication Cycle

The proper structure and function of centrosomes is dependent upon the strict doubling of these existing centrioles for formation of the bipolar mitotic spindle, making the molecular mechanisms underlying the centriole assembly cycle of particular importance to the maintenance of genomic integrity. Accordingly, the single centrosome that proliferating cells inherit must be precisely duplicated exactly once prior to mitosis. This process is ensured by the centrosome duplication cycle.

The canonical centrosome duplication cycle occurs in a very ordered manner concomitant with the cell cycle (Figure 1.4). The Centrosome duplication cycle begins in G1, as upon completion of mitosis in the previous cell cycle, the cell inherited a single centrosome that must be duplicated for the organization of a bipolar mitotic spindle. The first step of the centrosome duplication cycle is activation of centriole biogenesis which is triggered by centriole disengagement [101][102]. This is currently a hot topic in centrosome biology, as it remains unknown exactly how centriole biogenesis activation occurs [103]. The G1/S transition initiates the second step, recruitment of the cartwheel structure adjacent to the proximal portion of the mother centriole, which marks the beginning of procentriole formation. It is currently unknown the exact mechanism by which the cartwheel is recruited, but it does not require Plk4, though it does aid in the maintenance of Sas-6 at the centrosome [80][82]. Once the procentriole is formed, the process of microtubule nucleation and stabilization begins. This involves a variety of proteins, particularly γ-tubulin, Cetn2, and CP110. This results in the final step, centriole elongation and maturation. During metaphase of mitosis, Sas-6 is degraded in an APC
and SCF dependent manner, resulting in removal of the cartwheel in preparation for the next cell cycle [104]. G2/M also marks the addition of appendages to the daughter centrioles. There are two sets of appendages, distal and subdistal. Subdistal appendages have a role in anchoring microtubules [105], while distal appendages are thought to be critical for the recruitment of basal bodies to the membrane during cilia formation and assembly [106]. Upon mitotic exit, each cell has a single centrosome, comprised of two centrioles, one mother and one daughter. So upon entry into mitosis, there is a bipolar mitotic spindle, composed of two mature centrosomes, capable of organizing microtubules and facilitating proper DNA segregation in between two daughter cells.

As previously discussed, hundreds of proteins have been implicated to be involved in centriole biogenesis, duplication, and regulation of the centrosome duplication cycle. However, there are several proteins of particular interest to this study, all of which belong to families of proteins that are conserved from SPBs to centrosomes. These families include the Mps1 kinases, Calcium binding Centrins, and cyclin dependent kinases (Cdks). Similar to SPB duplication, cyclin-dependent kinase activity, specifically Cdk2, is important in centrosome duplication. Downstream targets of Cdk2 include Mps1 [107][108], NPM1 [109], and CP110 [110]. CP110 is a substrate of Cdk2 and is required for proper centriole duplication [110]. Failure of Cdk2 to phosphorylate CP110 leads to unscheduled centrosome separation and polyploidy [110]. Mps1 function in the centriole assembly pathway and centrosome duplication is a highly controversial topic and will be specifically covered in its own introductory section. Elongation and
maturation involves a variety of PCM components, including γ-tubulin, ant the procentriole is complete by the end of G2.

While cartwheels are thought to be dynamic in the fact that they are required to initiate procentriole assembly, they are degraded before entry into mitosis, meaning a cell in G1 will exhibit zero Sas-6 foci. After completion of mitosis, the cell cycle will begin again. At G1/S transition, the cartwheel structure is again recruited to the proximal end each of the mother centrioles at the site of procentriole assembly. So cartwheel proteins, particularly Sas-6, will demonstrate 2 foci in S phase, as there will be two cartwheels, each initiating procentriole formation, one at the base of each mother centriole. While proteins such as Cetn2 and CP110 that are actually structural components of the centriole, will not be degraded, and hence each mother and daughter centriole will have a single focus, for a total of 4 foci in S phase. In short, the centrosome duplication cycle is more complex than that of the SPB or centriole biogenesis in *C. elegans*, and involves a myriad of proteins and proper centriole assembly facilitates the formation of two centrosomes with a pair of resident centrioles at their core, providing the basis for bipolar spindle organization during mitosis.

1.4.5 Centriole Assembly: Canonical versus *de novo* pathways

The canonical centriole assembly is a conserved pathway, occurring in stages that ultimately culminate in the production of mature centrosomes. However, there is another centriole assembly pathway, known as the *de novo* formation pathway. *De novo* assembly was first observed in *Chlamydomonas reinhardtii* [111], and subsequently in flies
De novo formation also occurs in human cells, and begins with the formation of excess centrin aggregates in S phase [87]. The aggregates will not be functional until the next cell cycle, but are recognizable before mitosis occurs [114]. The \textit{de novo} pathway can be induced by the removal of existing centrioles by microsurgery or laser ablation [114][115][116]. However, the \textit{de novo} assembly pathway can be suppressed by the presence of single mature centriole [114][115]. The \textit{de novo} pathway isn’t necessarily subject to the same regulatory elements as the canonical centriole assembly pathway, which acts to strictly ensure the formation of one new daughter centriole at the base of each mother centriole. \textit{De novo} centrioles are constructed from the “top-down” and hence the number of centrioles formed is highly variable [87]. This “top-down” \textit{de novo} centriole construction has only been shown in a Cetn2-dependent pathway [87] and this construction is independent of Sas-6.

1.5 Function of the Centrosome and SPB

The centrosome has several functions. The central function is to serve as the major microtubule organizing center (MTOC) in proliferating animal cells. As the MTOC, it organizes the microtubules that form the mitotic spindle in dividing cells. The centrosome orchestrates a wide variety of cellular processes, from cell signaling, motility, polarity, adhesion, and the coordination of protein trafficking by the microtubule cytoskeleton. The centrosome is known as the cytoskeleton organizer, as it has critical links to the nucleus, the Golgi, cell to cell junctions, mitochondria, and the actin-myosin cytoskeleton, all of which are very important in positioning and shaping the microtubule
cytoskeleton in relation to the cell and whole organism. The centrosome also has a role in organizing the microtubules throughout the cell cycle, and this organization can vary from cell to cell and differs in the distinct phases of the life of a cell.

1.5.1 The Role of Centrosomes in Disease

Boveri first postulated the link between centrosomal abnormalities and the diseased state. As would be anticipated from the critical roles of the centrosome, aberrations in centriole structure or function is implicated in a number of disease conditions, including male sterility, dwarfism, mental disorders, primary microcephaly, ciliopathies, and cancer [117]. Most of these diseases are associated with defects in centrosome number, particularly the presence of excess centrosomes, which can arise due to the generation of multipolar spindles by a number of reasons including; failure of cytokinesis, misregulation of centrosome duplication, cell fusion, or PCM fragmentation [118]. Of extreme interest, the majority of cells that undergo multipolar division do not survive [119]. However, cells with excess centrosomes have been shown to have an intermediate structure, the multipolar spindle, that undergoes centrosome clustering, creating a pseudo-bipolar spindle [119][120]. This pseudo-bipolar spindle allows for normal mitosis, and these cells are prone to aneuploidy, the presence of chromosomal abnormalities. It is current theory that centrosomes are responsible for the generation of this aneuploidy through the generation multipolar spindles that are then resolved into a pseudo-bipolar spindle, as they are involved in regulation of chromosome movement.
Aneuploidy is a vastly complicated condition leading to defects in many cellular processes. Chromosomal abnormalities in the early development of the embryo are known to be the cause of many birth defects, which plagues approximately 3% of all live births in the United States [121]. Examples of such diseases include Down’s Syndrome [122], Klinefelter’s syndrome [123], and truncus arteriosus [124]. Unfortunately, the most common consequence of chromosomal abnormalities for an embryo is embryonic death [125].

Aneuploidy is the presence of a chromosomal abnormality; the presence of too many or too few chromosomes. Aneuploidy can add an extra chromosome or piece of a chromosome that may encode a gene encoding a positive regulator of cell growth. This can yield advantageous to a cell, allowing it to grow faster, or evade apoptosis – both trademarks of cancer. On the other end of the spectrum, an aneuploid cell may lack certain genes, like tumor suppressors, again allowing for growth and the evasion of apoptosis. One specific example is the knockdown of the tumor suppressor p53 by mutation or deletion, which has been statistically correlated with the predisposition of the cell to excess centrosomes. Aneuploidy is a trademark in human cancers, occurring in approximately 90% of all cases [126]. Centrosomal defects generating aneuploidy is thought to be of extreme importance in cancer, specifically bladder [127], breast [128] and prostate cancers [129]. To date, a causal relationship between excess centrosomes and aneuploidy has yet to be proven [130].

A prime case against excess centrosomes directly causing cancer is that no direct genetic evidence exists for mutations in human centrosomal genes and cancer, though
correlations exist. This is not true for other species. Flies are not normally affected by cancer [127]. In flies, overexpression of Plk4 leads to extra centrosomes in approximately 60% of cells, which initially form multipolar spindles that go on to form pseudo-bipolar spindles, leading to viable progeny. Though the flies appeared to be wild-type (WT), overexpression of Plk4 led to shortened adult life spans and a longer cell division cycle by a severe delay in mitosis, decreasing the number of cell divisions. However, transplantation of brain cells with extra centrosomes into the abdomen of WT flies caused lethal metastatic tumors [131]. These results were confirmed in a second study using transplantation assays of mutations showing defects in centrosome function in Drosophila [132]. Again, these flies showed centrosomal clustering forming a pseudo-bipolar spindle, which is strikingly similar to the mechanism in human cells [132][119].

Centrosome dysfunction also leads to abnormalities in the formation and maintenance of cilia and flagella. As most vertebrate cells are capable of the development of a cilium during their life cycle, it is not surprising that mutations in ciliary genes can affect developmental processes. Both motile and non-motile cilia are associated with several diseases, which are broadly classified as “ciliopathies.” Diseases are classified as Primary Ciliary Dyskinesias (PCDs) when they demonstrate defects in cilia structure. One example is Kartagener’s syndrome and results in defects in the typical arrangement of cilia structure. Bardet-Biedl Syndrome (BBS) is also caused by defects in primary cilia, specifically by a defect in intraflagellar transport (IFT). BBS is associated with a variety of phenotypic abnormalities such as brain malformation (associated with learning difficulties), blindness, polycystic kidney disease (renal dysfunction), malformation of
the genitals, obesity, cranio-facial abnormalities, and polydactyly [130]. It is also very common for defects in cilia to affect fertility, specifically by defects in the sperm flagellum (a modified ciliary structure) or immotile cilia in the fallopian tube failing to aid implantation by moving a fertilized ovum towards the uterus [133].

Autosomal recessive primary microcephaly (MCPH) could be classified as the most striking example of a ciliopathic disease. It is caused by a mutation in one of the MCPH loci, which encode centrosomal proteins including: CPAP/CENP-J [134], STIL [134][135], and Cep215 [136]. Individuals are born with brain defects and display mental retardation [135]. Additionally, mutations in pericentriolar matrix protein pericentrin, which is required for the formation of cilia, are associated with many diseases [117], including Majewski osteodysplastic primordial dwarfism type II (MOPD II) [137] and Seckel syndrome [138]. All of these diseases display impairment of brain function in some manner, ranging from the size of the brain to mental retardation, which implicates centrosomes as key players in proper neural development and neurogenesis.

1.5.2 Other Functions of the Centrosome

In addition to their role as the MTOC and in spindle formation, centrosomes have a variety of roles in animal cells. Though the centrosome serves to ensure faithful segregation of chromosomes during mitosis, centrosomes are not essential organelles in animal cells [139][140]. As previously mentioned, after the removal of centrosomes by microsurgery or laser ablation, cells are not only viable, but can still form bipolar mitotic
spindles [141]. The exact mechanisms of how bipolar spindles still form have not been
delineated, but it has been shown that microtubules form randomly within the cytoplasm
of acentrosomal cells and these microtubules form a functional bipolar spindle [141].
When a single centrosome is ablated during prophase, leaving one functional centrosome,
a functional acentrosomal pole forms, completing the bipolar mitotic spindle [141]. The
acentrosomal poles lack centrioles, γ-tubulin, and pericentrin, but the regions still contain
the structural protein NuMA. The fascinating observation from this single acentrosomal
pole occurs in the next cell cycle, as one daughter cell will inherit the functional
centrosome, while the other daughter cell is derived from the acentrosomal cell. The
daughter cell with the normal centrosome will proceed into S-phase, and that cell will
duplicate as normal. The acentrosomal daughter cell is unable to enter S phase and of
those that are capable of entering mitosis, over half fail in cytokinesis, clearly
demonstrating that the centrosome is involved in proper cell cycle progression
[142][143]. Many proteins that have roles in cytokinesis and cell cycle progression, such
as Aurora-A [144] and Aurora-B [145] kinase, Cyclin B/Cdk1 [146], CyclinE/Cdk2
[147], and Polo kinases (Plk1) [148], are also localized and involved in the regulation of
the centrosome, indicating the importance of the centrosome as more than the MTOC.

Yet another fascinating role of the centrosome involves cell cycle arrest as a
response to DNA damage [149]. A myriad of DNA damage checkpoint proteins have
been shown to localize the centrosome [150]. The first DNA damage protein shown to be
involved in centrosome amplification was Chk2 in Drosophila embryos [151]. Chk2 is a
tumor suppressor and DNA damage response protein that inactivates centrosomes by
uncoupling the spindle from damaged nuclei [151][152]. The current model in mammalian cells for centrosome amplification derived by the DNA damage response involves Chk1 and its inhibition of centriolar satellites. Centriolar satellites, previously known as “cytoplasmic granules” or “pericentriolar satellites,” serve as communication between the cytoplasm and the centrosome, functioning in protein targeting and exchange of centrosomal components [153]. Chk1 is a regulator of G2/M transition and prevents activation of CyclinB/Cdk1 [154]. Specifically Chk1 induces the formation of excess centriolar satellites, which in turn act as assembly platforms for the formation of centrosomal proteins, the construction of centrosomes, and hence, the presence of excess centrosomes [149]. Combined, this data supports the presence of DNA damage proteins in regulation of centrosomal function.

1.5.3 Regulation of Centriole Duplication

Proliferating cells must limit procentriole formation and centrosome duplication to once per cell cycle and as the centrosome is a highly dynamic structure, its components are subject to considerable regulation throughout the cell cycle.

Other groups have performed cell fusion assays suggesting an intrinsic control mechanism that prevents multiple duplication events within a cell cycle. In a normal cell, centrosomes will duplicate only once even if the cellular environment is permissive and can support further centrosome duplication. Centrosomes in G1 have a permissive cellular environment, as they are preparing for centriole biogenesis and centrosome duplication, while cells in G2 do not have a permissive environment for centriole
biogenesis/duplication as they already have fully functional centrosomes. The cell fusion assay set out to test if there is a cytoplasmic difference between these two cells and their corresponding centrosomes. The cell fusion assay involved cells in G1 or G2 fused with S phase cells. This was an elegant experiment, demonstrating that the G1 centrosome has the ability to duplicate, but the G2 centrosome does not [155]. Fusion of a G1 and G2 phase cell showed that only the G1 centrosome was capable of duplication. This strongly suggests the existence of an intrinsic mechanism the regulation of centrosome duplication [155], e.g. there is some component intrinsic to the centrosome itself that regulates its ability to duplicate.

Another mechanism of regulation is thought to be temporal and is based on the location of the centrosomes in vicinity to one another. This switch, from tightly associated centrioles or centriole engagement, to centriole disengagement is a factor that determines whether a cell is allowed to proceed through centriole duplication and mitosis [156]. Centriole disengagement occurs in late mitosis or early G1 before entry into S phase, but are again tightly associated relative to the formation of the new centriole during S phase when the centrioles are duplicated [157]. In fact, centriole disengagement at the G1/S transition is required for centrosome duplication, and the centrioles will only disengage by a process requiring activity of the protein kinase Plk1 and the cysteine protease separase in late mitosis [158].

While the regulation of centrosome duplication and centriole biogenesis are highly regulated processes, centrosome amplification still occurs. Overexpression of a multitude of centrosomal proteins can lead to centrosome amplification. These include all of the
main proteins of interest to this study, for which this dissertation provides evidence of cooperation between, Sas-6 [82][13], Plk4 [80][81], and non-degradable versions of Mps1, specifically used in this thesis Mps1Δ12/13 [107]. Mps1 is capable of causing centrosome amplification in a cell type specific manner [108][159]. Additionally, several cell lines are capable of escaping the intrinsic block of centrosome amplification, including U2OS [159][160] and CHO [161] cells, which are known to reduplicate centrosomes upon prolonged S phase arrest, while normal mammalian somatic cells and cell lines with normal chromosome numbers, such as RPE1, do not.

1.6 The Role of Mps1 in the SAC

Mps1 was originally identified for its role in SPB duplication in budding yeast [54]. Mps1 is required for every step of SPB duplication and was named for the phenotype observed in mutant yeast cells, which fail in SPB duplication and form Monopolar spindles upon entry into mitosis [54]. Yeast Msp1 was subsequently shown to regulate the mitotic spindle assembly checkpoint (SAC), preventing mitosis until the chromosomes are properly aligned by attachment to the bipolar spindle [162][163]. Mps1 is an essential component of the SAC in other organisms, including zebrafish [164], human [165], and Xenopus [166]. In human cells, the involvement of Mps1 is also required for the recruitment of several SAC proteins, including Mad1/Mad2 and CENP-E [165]. Additionally, the Mps1 substrate Borealin is involved in the SAC, and its phosphorylation is necessary for correcting improper chromosome attachments [167].
1.7 The Role of Mps1 at the Centrosome

Mps1 is a dual specificity protein kinase that is conserved from yeast to mammals [168]. Mps1 has held several names in vertebrates, including Esk [169], Esk1[108], TTK [170][171], and PYT [172], though the accepted and used names are Mps1 and TTK. Centrosome duplication requires Mps1 in mouse [108], and may be required in humans [173], but this is a current debate in the centrosome field.

While the centrosome field contains several publications supporting the requirement of Mps1 only in the SAC [160][174], these studies fail to address the role of Mps1 at the centrosome. Evidence, including this document, prove that while Mps1 may be dispensable for centriole biogenesis and duplication, it definitely has a role at the centrosome [87][88][175]. The studies that argue against a centrosomal role for Mps1 have severe flaws that would bring interpretation of any lack of centrosome function into question. For example, Mps1 is a strictly regulated kinase that demonstrates two peaks during the cell cycle. A prominent increase in Mps1 protein levels during the SAC, and a prominent increase in Mps1 kinase activity levels during centriole duplication (S phase) [171] (Figure 2.1). This peak in kinase activity may be small, but it is large in relation to the total levels of Mps1 protein, as this reflects suppression of degradation and localized accumulation, specific to the centrosome [107]. As Mps1 protein levels at the centrosome during G1/S are much lower than that in the SAC, but the kinase activity of Mps1 is high, it is necessary to deplete Mps1 levels beyond a threshold to visualize abnormal centrosomal phenotypes, which previous studies have failed to do, as only minor depletion of Mps1 causes aberrations with the SAC, but may show no centrosomal
phenotype [107]. Other studies have used chemical genetics [176] or Mps1 specific inhibitors [174], which “inactivate” the kinase activity of Mps1, but do not deplete the Mps1 protein from the centrosome.

Mps1 has several centrosomal substrates, including Cetn2 [177], Mortalin [89], and Cep215 [178]. Mps1 has been identified as a centrosomal protein by PCP-SILAC [23], and was recently confirmed two fold by MudPIT, confirming Mps1 is a centrosomal protein and has a potential substrate, Cep215 [27]. Additionally, Mps1 has been shown to be regulated by antizyme (OAZ), and it has been shown that overexpression of OAZ or Mps1KD causes a defect in the recruitment of Sas-6 [88].

1.8 The Role of Plk4 at the Centrosome

Plk4, initially identified in the mouse, is a kinase that shares homology with the murine Snk (As was subsequently named Snk akin kinase or SAK), Drosophila Polo kinase, and S. cereisiae CDC5 [179]. Plk4 is a member of the Polo-like kinases (Plks) family of serine-threonine kinases, which play key roles in the regulation of cell cycle, mitosis, and cytokinesis. Eukaryotes typically contain four Plk family members (Plk1, Plk2, Plk3, Plk4). Plk4 is the most divergent and last member of this family to be identified [180].

Plk4 is not present in C. elegans, which has no direct homolog of Plk4, though it has been proposed and the majority of the centrosomal field believes that the kinase zyg-1 is a functional equivalent, as it is required for centriole duplication. Of particular interest, the kinase domain of zyg-1 shares closer homology to Mps1 than the C. elegans Plks (1-
3), though the structural organization of zyg-1 is more closely related to Plk4 [76] (Figure 1.7). Plk4 knockout mice have demonstrated that Plk4 is essential for embryonic development beyond gastrulation and is required for mitotic progression [181]. Plk4 homozygous null embryos arrest at stage E7.5 and have increased numbers of late mitotic and apoptotic cells, while Plk4 +/- embryos develop normally, but have increased cases of spontaneous lung and liver cancers [182]. Furthermore, fibroblasts derived from these mice are more aneuploid, which is thought to be derived from the presence of extra centrosomes and their frequent aberrant cell division.

Plk4 has an essential role in the centrosome duplication cycle. It localizes to the outer wall of centrioles, enriched at the proximal end, precisely where procentriole biogenesis is initiated. It is well known that overexpression of Plk4 in somatic cells leads to centrosome amplification. Additionally, Plk4 RNAi (RNA interference) in somatic cells prevents centriole duplication. At present, two mitotic Plk4 substrates have been identified: the phosphatase CDC25C [183] and the RhoA guanine exchange factor (GEF), Ect2 [184].

1.9 The Role of Sas-6 at the Centrosome

The centriole is a cylindrical, barrel shaped organelle that displays an outer wall with a nine-fold symmetrical structure constructed of peripheral triplet microtubules. The scaffold for accurate centriole construction is the cartwheel structure (Figure 1.4), which collectively refers to the nine spokes and the central hub from which they radiate. Sas-6,
spindle assembly abnormal 6, is a highly conserved and essential component of the central hub of the cartwheel structure [185].

Sas-6 is the founding member of the conserved PISA-containing (Present In Sas-6) protein family [13]. The PISA motif is located near the N-terminus, followed by the coiled-coil domain. The overall protein localization of Sas-6 was assessed \textit{in vitro} by EM, revealing an N-terminal globular head that is immediately followed by a coiled-coil domain [186]. The C-terminal domain is predicted to be unstructured [187], but contains a KEN box. HsSas-6 also contains three D boxes, two in the N terminal domain and one within the coiled-coil. Sas-6 uses its long coiled-coil domain interaction to dimerize [186]. Sas-6 forms rod-shaped homodimers that interact through their N-terminal head domains to form oligomers[16][186]. Recombinant Sas-6 self-associates \textit{in-vitro}, assembling into structures that resemble cartwheel centers [188][189][190].

The current model of the cartwheel structure shows the globular N-terminal heads form into the ring like structures at the base of the central hub, while the coiled-coil domains form the spokes that radiate from the central hub [186]. This model is strongly supported by a study in \textit{Trichonympha sp.}, which demonstrates cartwheel formation by electron tomography [191]. However, in \textit{C. elegans}, EM and crystallographic studies show Sas-6 self-assembly into spiral formations, but not ring-like structures [190]. However, the data supporting \textit{C. elegans} conformation of Sas-6 into spiral formations does not exclude the formation of a ninefold structure, which strongly suggests that additional components are required for ring formation and symmetry [190]. Furthermore, in human cells, oligomerization is essential for centriole formation [16] and point
mutations support that centriole formation in vivo is dependent upon the self-assembly of Sas-6 [188]. Of interest with these findings, nine-fold symmetry is conserved in virtually all centrioles, but certain species and cell types display only doublet or singlet microtubule arrangements [186].

In C. elegans, Sas-6 interacts physically with Sas5 [13] and centriolar localization of Sas-6 is greatly diminished in the absence of either Sas5 or Zyg-1 [13]. Zyg-1 phosphorylates Sas-6 at serine 123 and this ensures the maintenance of Sas-6 at centrioles [192]. It was subsequently shown that this phosphorylation is not required for centriole assembly [187]. The Zyg-1 protein kinase directly binds to the coiled-coil domain of Sas-6 [187]. Interestingly, this interaction does not require the kinase activity of Zyg-1, but kinase activity is required for cartwheel assembly [187]. Prevention of Sas-6 interaction with either Sas5 or Zyg-1 leads to failure of cartwheel assembly due to loss of Sas-6 recruitment [187]. The combination of this data is intriguing, as it is the first evidence that cartwheel formation occurs in two separable steps. First, Sas-6 is recruited to the base of the mother centriole through interaction with Zyg-1 and Sas5. Second, the cartwheel is formed, which requires Zyg-1 kinase activity.

Sas-6 overexpression yields excess foci containing centriolar proteins in human cells [13][82][193] and causes the formation of irregular tube like structures in Drosophila [194] and C. elegans [195]. Sas-6 RNAi in C. elegans and EM analysis of Sas-6 RNAi treated human cells shows partially formed centrioles and failure to form pro-centrioles [68][82]. Absence of Drosophila Sas-6 displays a loss of centriole cohesion and nine-fold symmetry [194]. Sas-6 is required for proper cartwheel assembly and is required for
centrosome duplication in both *C. elegans* and human cells [13]. It is a tightly regulated protein, recruited to the centriole only once per cell cycle at the onset of S phase [13][82]. HsSas-6 is degraded in anaphase by the 26S proteasome and this degradation is dependent on the C terminal KEN box [82].

1.10 Overview

Combined, this introduction suggests that the centriole biogenesis pathway in human cells is complex, and recruitment and maintenance of hSas-6 at the centrosome can’t be ascribed to Plk4 kinase activity alone. The data within this thesis was derived from work in human cells, and hence, from this point forward, unless otherwise specified, are *Homo sapiens* versions of all proteins; e.g. Sas-6 (hSas-6), Mps1, Plk4.

This thesis aims to demonstrate that Mps1 not only has a role in centriole biogenesis, but that role involves the master regulator of centriole biogenesis and centrosome duplication, Plk4. In addition, we have identified another centrosomal substrate of Mps1, which happens to be one of the earliest proteins recruited to the procentriole, Sas-6.

Chapter 2 will discuss Mps1 inhibitors, specifically that Mps1 inhibition or depletion attenuates centriole biogenesis, and that Mps1 may be dispensable for centriole assembly and duplication.

Chapter 3 will discuss the interplay of Mps1 and Plk4. Specifically, the intriguing result that the roles of Plk4 and Mps1 are mutually dependent in both cycling and S-phase arrested cells. Additionally, Mps1 and Plk4 co-regulate their centrosomal levels,
and simultaneous knockdown of Mps1 and Plk4 inhibits recruitment of Sas-6 to the procentriole, while supporting that Mps1 may be dispensable for centriole assembly and duplication.

Chapter 4 shows the expression and purification of Plk4, Plk4KD (kinase dead), and the control Sumo-His-MBP (Maltose Binding Protein) which was used to demonstrate by in vitro kinase assay that Mps1 and Plk4 are mutual substrates of one another. Additionally, centrosomes were purified and used for in vitro kinase assays with Mps1 and Plk4, revealing that these centrosomal kinases may phosphorylate a similar subset of centrosomal proteins.

Chapter 5 will lay the foundation for Sas-6 as a substrate of Mps1, and determines this phosphorylation is due to multiple phosphorylation sites, though exact phosphorylation sites have yet to be identified. It also reveals that Sas-6 may be a substrate of Plk4. Of particular interest and surprise, dual knockdown of Mps1 and Plk4 does not prevent centriole amplification due to Sas-6 overexpression.
Figure 1.1: Protein Composition of the Spindle Pole Body in *Saccharomyces cerevisiae*.

Indicates the proteins that compose the SPB and their positions relative to the nucleus and cytoplasm, including the nuclear microtubules (nMTs) and cytoplasmic microtubules (cMTs). Also indicates the positions of the Outer plaque, central plaque, and inner plaque.
Figure 1.2: Centrosome Assembly in *C. elegans*.

Schematic representation of centrosome assembly in *C. elegans*. Centriole assembly can be divided into three steps: Initiation of Duplication, Central Tube Formation, and Assembly of Centriolar Microtubules. Initiation occurs at the parental centriole requires SPD2 and the protein kinase Zyg-1. Central tube formation involves the complex of Sas5/Sas-6 (red) and the assembly of microtubules requires Sas4 (green).
Figure 1.3. Structure of the Mammalian Centrosome.

Schematic representation of the mammalian centrosome structure. Centrosomes are composed of a maternal (mother) and daughter centrioles. The formation of this complex structure involves an array of proteins, listed with their particular area of involvement in centrosome duplication.
Figure 1.4. Centrosome Assembly in Mammalian Cells.

Schematic representation of mammalian cell centriole biogenesis. During S phase, the two centrioles in a cell each duplicate (for simplicity, a single centriole is shown here). The new cartwheel structure forms at the base of the existing mother centriole at a right angle, and is an important early step in centriole biogenesis. During S and G2 phases, the new centrioles elongate until they are approximately the same size as the original mother centriole (not shown). Inset: An end-on view of a centriole showing the central hub (gold) and spokes (red) of the cartwheel structure and the surrounding “blades” of triplet microtubules (gray).
Figure 1.5. The Mammalian Centrosome Duplication Cycle.

Schematic representation of the duplication cycle of the mammalian centrosome. The canonical centriole assembly pathway occurs in a very ordered manner with the cell cycle and ensures that the centrosome is duplicated exactly once prior to mitosis. The duplication cycle can be broken into four stages. Upon mitotic exit, each cell has a single centrosome, which act to template the initiation of procentriole formation in S phase by the recruitment of the cartwheel. Elongation and maturation of the centriole occurs in G2, resulting in two mature centrosomes, capable of forming the bipolar spindle during mitosis for accurate DNA segregation for cell division.
Figure 1.6. Regulation of Mps1, Plk4, and Sas-6 is critical to limiting centrosome duplication to once per cell cycle.

Centrosome duplication. Mps1, Plk4, and Sas-6 are regulated, allowing for daughter centriole (green) formation from mother centrioles (gray). This leads to a cell with two centrosomes and a proper bipolar spindle in mitosis (M). A mouse 3T3 mitotic cell is shown with DNA in blue, microtubules in green, kinetochores in purple, and centrosomes in red. Cytology picture reprinted with permission from Harold A. Fisk of The Ohio State University. (B) In the centrosome reduplication pathway, misregulation, particularly overexpression, leads to the production of more than two centrosomes and a multipolar spindle. A mouse 3T3 mitotic cell is shown overexpressing Mps1 with DNA in blue, microtubules in green, kinetochores in purple, and centrosomes in red.
Figure 1.7. Structural comparison of the centrosomal kinases Plk4, Mps1, and Zyg-1.

Structural comparison of the human centrosomal kinases, Mps1 and Plk4 to the C. elegans centrosomal kinase, zyg-1, with the kinase domains (KD) in red, Proline/Glutamic Acid/Serine/Threonine (PEST) motifs in gray, Cryptic Polo Box (CPB) in black, Polo Box (PB) in blue, Centrosome Localization Domain (CLD) in green and the degradation sequence (DS) in orange. Note: In Plk4, both the CPB and PB are capable of centrosomal targeting.
Chapter 2

Investigation of Mps1 Inhibitors in Centriole Assembly


Derived from: Amanda N. Bliemeister, Shubhra Majumder and Harold A. Fisk, et. al. Mps1 and Plk4 Cooperate to Regulate Centriole Biogenesis. Under Review

*Subsequent name change to Bliemeister

2.1 Abstract

Successful mitosis, defined as faithful segregation of genetic material, is dependent upon two precise duplication events, that of the genome and that of the centrosome. The requirement of Mps1 and its kinase activity in centrosome duplication is currently a controversy in the centrosome field. Much of the Mps1 controversy is based on the use of chemical genetics and small molecule inhibitors. In an attempt to address this controversy, we obtained these Mps1 inhibitors, AZ3146 and IN-1. We have shown that neither Mps1 inhibitor inhibits Mps1-dependent phosphorylation of a known Mps1 substrate, Cetn2, to the same degree as that of a generic substrate, MBP, by in-vitro kinase assay, suggesting that at least some inhibitors may not completely inhibit Mps1
kinase activity. Additionally, AZ3146 inhibits Cetn2 phosphorylation more effectively than IN-1, though the increased effect was less than two-fold. Additionally, we have confirmed that Mps1 is likely dispensable for centriole assembly. While depletion or inhibition of Mps1 does affect the recruitment of Cetn2 to the centriole, the majority of cells complete centriole biogenesis.

2.2 Introduction

Mps1 is a dual specificity kinase originally identified in budding yeast, based on the phenotype of the mps1-1 mutant, demonstrating monopolar spindles due to a failed spindle pole body (SPB) duplication event. Subsequently, it was shown to regulate the mitotic spindle assembly checkpoint (SAC) [196]. While it is undisputed that Mps1 is essential for the SAC in higher eukaryotes, whether Mps1 functions within the centriole biogenesis pathway or has a role in centrosome amplification in vertebrate cells remains controversial.

Experiments using the small molecule Mps1 inhibitor, IN-1, support the suggestion that at least some kinase activity is dispensable for centrosome duplication [197]. However, there are several caveats to the use of small molecule inhibitors, in addition to the caveat of temporal and spatial regulation and localization of Mps1. Kwiatkowski et. al. [197] concluded that Mps1 IN-1 has no effect on centrosome duplication, but that study was limited to the examination of centrosome number in mitotic cells. Hence, while this does show that cells can ultimately assemble two new centrioles and functional mitotic spindle poles in the presence of IN-1, no information
can be obtained about the kinetics of assembly from analyzing the end point of mitosis, as this analysis does not actually monitor centrosome duplication. Additionally, this does not account for the potential non-catalytic role of Mps1, as Mps1 IN-1 only inhibits the ability of Mps1 to phosphorylate its substrates, but does not eliminate its localization from the centrosome. To our knowledge, no study of an Mps1 inhibitor has reported direct analysis of centrosome duplication.

Mps1 has its maximal kinase activity and protein level in mitosis (Figure 2.1). While Mps1 protein levels do not peak at the G1/S transition, precisely when centriole biogenesis is initiated, protein levels gradually rise through S phase. Of particular interest, there is a sharp peak of kinase activity, specifically when centrosome duplication is initiated at the G1/S transition (Figure 2.1). We suggest that Mps1’s protein levels and its kinase activity have important roles in centriole biogenesis and this suggestion is supported by a myriad of previous results validating that Mps1 is a centrosomal protein with centrosomal function: (1) Depletion of Mps1 with small interfering RNA (siRNA) attenuates centrosome duplication in human cells [173]; (2) Compared to the spindle checkpoint, centrosome duplication requires significantly less Mps1 [173] (Figure 2.1); (3) Cetn2, a well-known and early recruited centriole protein, is a robust centrosomal substrate of Mps1 [87]; (4) Centrosomal levels of Msp1 are precisely controlled – Antizyme restrains the assembly of centrioles by controlling Mps1 levels at the centrosome, and this requires the Cdk2-regulated degradation signal [88] (Figure 2.1). Additional evidence supporting the role of Mps1 at the centrosome continues to accumulate.
Published centrosomal substrates of Mps1, include Cetn2 [177], Mortalin [89], and a peptide of Cep215 [178]. Mps1 has been identified as a centrosomal protein by both PCP-SILAC [23], and MudPIT [27]. Additionally, Mps1 centrosomal regulation by degradation involves antizyme (OAZ), and of particular interest, defects in the recruitment of Sas-6 is caused by overexpression of Mps1KD or OAZ [88].

However, overexpression of Mps1 is not enough to cause centrosome reduplication in human cells [173][165], although it is sufficient in mouse cells [108]. This could be viewed as contradictory to the hypothesis that a local concentration of Mps1 is spatially and temporally regulated to cause centrosome reduplication. However, overexpression of non-degradable Mps1 (Mps1 Δ12/13) leads to the formation of extra centrosomes, suggesting this event results from persistent phosphorylation of its substrates [107]. As the role of Mps1 at the centrosome remains controversial, our first goal was to directly examine centrosome duplication in cells treated with the recently developed small molecular inhibitors of Mps1, IN-1 and AZ3146.

2.3 Materials and Methods

Cells and Cell Culture

Rpe1 cells were cultured in a 1:1 mixture of DMEM and Ham’s F12 supplemented with 10% FBS (Atlanta Biologicals, Atlanta GA), 100U/mL penicillin (Hyclone), and 100µg/mL streptomycin (Hyclone). Cells were cultured at 37°C in a humidified chamber in the presence of 5% CO₂.
Plasmids

Previously described plasmids used for this study include, pHF64 GST-Mps1 in the pGEX-6p vector, pHF188 6-His-Cetn2. Recombinant Myelin Basic Protein (MBP) was ordered from Sigma Aldrich M2941-1MG.

siRNA Transfections, Inhibitors, and BrdU Incorporation Assay

Stealth siRNAs directed against Mps1 (siMps1: nucleotides 1360-1384) obtained from Invitrogen and SiGLO Lamin A/C siRNA (siCon) obtained from Dharmacon (Lafayette, CO) were used at a final concentration using Lipofectamine RNAiMAX (Invitrogen), and cells were analyzed 48 hours after transfection. Efficiency of siRNA depletion for Mps1 was determined by quantitative dual color immunoblot using the Odyssey imaging system (LiCor, Lincoln, NE) as previously described [173]. IN-1 was the kind gift of Dr. Nathaniel Gray (Harvard, Cambridge MA), and AZ3146 was obtained from Tocris Biosciences (Minneapolis, MN). Both inhibitors, IN-1 and AZ3146 were added at a concentration of 10µM simultaneously with siRNA transfection. To limit analysis to S phase cells, BrdU (40µM) was added 44 hours post transfection, and both BrdU incorporation and centriole number were then assessed by IIF at 48 hours post transfection, known as a BrdU pulse.

Antibodies and Indirect Immunofluorescence (IIF) Analysis

Primary antibodies for IIF were as follows: GTU-88 mouse anti-gamma-tubulin, 1:200 (Sigma-Aldrich, St. Louis, MO), Rabbit anti-Cetn2,1:4000 [87], Rat anti-BrdU,
Primary antibodies were Alexa 488-, 594-, or 750-conjugated donkey anti-rabbit and donkey anti-mouse (Invitrogen) and Alexa-350-conjugated goat anti-rat. Cells were fixed in ice cold -20°C methanol for 10 minutes at -20°C, washed four times with PBS, and processed for IIF as previously described. Cells were then washed four times with PBS-T (0.05% Triton X-100 in PBS), and incubated in a humidified chamber with primary antibodies overnight at 4°C. Cells were then washed three times, and incubated with secondary antibodies for one hour at room temperature and washed three times, before mounting with Citifluor. All images were acquired at ambient temperature using an Olympus IX-81 microscope, with a 100X Plan Apo oil immersion objective (1.4 numerical aperture) and a QCAM Retiga Exi FAST 1394 camera and analyzed using the Slidebook software package (Intelligent Imaging Innovations, Denver CO).

**In vitro Kinase Assays**

Kinase assays were performed as previously described [108][107]. Briefly, a 5X kinase assay buffer (KAB) consisted of: 250mM Tris-HCl pH 7.5, 25mM DTT, 5X Protease Inhibitor, 10µM AEBSF, and 50mM MgCl₂. Additionally, a final concentration of 10µM ATP and 10µCi gamma-³²P (GE Healthcare) were added per reaction. Kinase assays consisted of 10mM of recombinant protein as substrate and 0.4mM of GST-Mps1 protein kinase [87]. Inhibitors were added at the indicated concentrations (µM). Reactions were incubated for 30 minutes at 30°C. Kinase reactions were analyzed by SDS-PAGE, Coomassie stained, and followed by autoradiography of dried gels.
2.4 Results

2.4.1 Mps1 Inhibitors are not Equally Effective against Substrates

To address the caveat that small molecule inhibitors may not be equally effective against all substrates, we obtained the published Mps1 IN-1, a very gracious gift from Dr. Nathaniel Gray (Harvard; Dana-Farber Cancer Institute, Boston MA). At 10µM IN-1 abolishes in-vitro phosphorylation of MBP by hMps1 (Figure 2.2) and causes mitotic defects *in vivo* [197], but it requires significantly higher concentrations of IN-1 to completely block *in vitro* Cetn2 phosphorylation [198]. While this *in vitro* result was obtained under different conditions than used by Kwiatkowski et al. [197], even at an IN-1 concentration of 100µM, ten times more inhibitor than was used by Kwiatkowski et al., Mps1 can still phosphorylate Cetn2 *in vitro*.

Another inhibitor of Mps1, AZ3146, was found to inhibit phosphorylation of MBP by Mps1 at a concentration of 2µM by *in vitro* kinase assay of recombinant proteins [199]. We assessed the ability of IN-1 and AZ3146 to phosphorylate MBP or Cetn2 by *in vitro* kinase assay (Figure 2.3). AZ3146 inhibits Cetn2 phosphorylation more effectively than IN-1 (Figure 2.3). Again, Mps1 IN-1 failed to inhibit phosphorylation of Cetn2 even at 10µM, while AZ3146 does show a slight inhibition of phosphorylation at 10µM, but not at the published 2µM concentration used for the previous AZ3146 *in vivo* studies [199]. This is yet another *in vitro* result suggesting that Mps1 can still phosphorylate Cetn2 in the presence of the Mps1 specific inhibitors AZ3146 at concentrations five times greater than were used previously [199].
While these results cannot be directly compared to the use of IN-1 or AZ3146 \textit{in vivo}, it supports the possibility that despite causing mitotic failures, hMps1 inhibitors might leave sufficient residual hMps1 activity to support centrosome duplication.

\subsection*{2.4.2 Centriole Biogenesis is Attenuated upon Depletion or Inhibition of Mps1}

To directly examine centriole biogenesis, we used a BrdU pulse assay \cite{87} in RPE1 cells to analyze the effect inhibitors have on the incorporation of Cetn2 during S-phase in an unperturbed cell cycle, as determined by examining centriole markers in cells that incorporate BrdU during a four hour pulse (Figure 2.4-A). As centrioles are rapidly replicated upon entry into S-phase, the majority of BrdU positive cells have four Cetn2 foci. Cells were transfected with Control (siCon) or Mps1-specific (siMps1) siRNAs, or treated with 10µM IN-1 or AZ3146 \cite{199}. Depletion of Mps1 inhibits centriole assembly, as indicated by the observation that nearly 34\% of BrdU positive Mps1 depleted cells have just two Cetn2 foci (Figure 2.4-B) and these centriole effects are reversed by an siRNA resistant Mps1 cDNA \cite{107}. This is compared to >90\% of BrdU positive, siCon-transfected cells (siCon) that had undergone centriole biogenesis presenting with four Cetn2 foci and 9\% of cells that had not, presenting with two Cetn2 foci (Figure 2.4-B).

Neither IN-1 nor AZ3146 blocked centriole biogenesis as effectively as siMps1 (Figure 2.4-B), as expected from the \textit{in vitro} kinase data (Figure 2.3), though both IN-1 and AZ3146 inhibit recruitment of Cetn2 to the centriole. There is a near two fold reduction in the ability of IN-1 or AZ3146 to block centriole biogenesis in comparison to Mps1. As 34\% of siMps1 cells show inhibition of Cetn2 recruitment compared to 18\% of
IN-1 and 21% of AZ3146 treated cells. Representative images of these results are shown in Figure 2.4-C.

2.5 Discussion

Here, we have utilized two inhibitors of Mps1, IN-1 and AZ3146, which both show inhibition of the recruitment of Cetn2, a known Mps1 substrate, to the centriole. However, neither inhibits Cent2 recruitment as effectively as knockdown of Mps1 using siRNA, suggesting that neither of these inhibitors effectively prevent Mps1 from recruiting Cetn2 to the procentriole. This strongly suggests that these inhibitors do not effectively inhibit centrosomal Mps1 activity, which is corroborated by in vitro data that neither IN-1 or AZ3146 prevents Mps1 from phosphorylating Cetn2, even at levels 100 fold greater than utilized for in vivo cell culture experiments [197][199].

It is not surprising that either of the Mps1 inhibitors are less effective in vivo than in vitro in regard to analyzing centrosome duplication. First and foremost, it is common for inhibitors to behave differently in vitro versus in vivo. The in vitro environment is substantially different compared to in vivo, as proteins are regulated temporally, spatially, and are surrounded by a unique cellular environment full of interacting components and post-translational modifications that could potentially block the effectiveness of inhibitors. Additionally, the in vitro kinase assays utilize recombinant proteins, both substrate and kinase, which both may act differently in the cellular environment. This is in addition to the fact that the effectiveness of inhibitors was tested against a generic substrate, MBP, and not a known centrosomal substrate of Mps1, such as Cetn2.
Secondly, typical assays have utilized end point analysis (mitosis) rather than actually monitoring centrosome duplication. By analyzing mitosis, centrosomal defects could easily miss detection, as the vast majority of Mps1 is not centrosomal, and centrosome duplication requires less Mps1 than mitosis (Figure 2.1) [171][173][198], such that not even the presence of mitotic defects is a reliable indication of depletion/inhibition of Mps1 at the centrosome. Additionally, partial depletion of Mps1 causes mitotic catastrophe despite the presence of residual Mps1[159], and therefore it is likely that partial inhibition would produce the same effect. Furthermore, Mps1 siRNA shows that cells with reduced Mps1 undergo mitotic and cytokinetic failures that produce G1 cells with two centrosomes, and this may mask subsequent defects in centrosome duplication [159]. This may be a greater issue for actue inhibition of Mps1 versus gradual depletion of Mps1 by RNAi experiments. Third, the specific activity of centrosomal Mps1 is likely higher than cytoplasmic pool of Mps1 [200], and the dominant negative effect of the catalytically inactive Mps1 Kinase Dead (Mps1KD) suggests that Mps1 may have a non-catalytic function(s) [88]. Hence, Mps1 kinase activity may be dispensable, but inhibitor studies do not prove that the Mps1 protein itself is dispensable.

As the main objective of this study, we have attempted to address whether Mps1 is dispensable for centriole assembly. As the majority of BrdU positive cells complete centriole biogenesis, this clearly supports the suggestion that Mps1 is dispensable for centriole assembly (Figure 2.3). However, dispensable is not the equivalent of being unimportant or having no role, and both Cdk2 and Cetn2 were once thought to be essential for centrosome duplication, though it has now been shown that neither is
required. Thus, if Mps1 is dispensable, it is in abundant and good company, and the understanding of the mechanisms of centriole duplication requires an understanding of all centrosomal components that allow for accurate assembly, structure, and function of centrosomes, whether essential or dispensable to the process.

Accordingly, it would seem premature to conclude that Mps1 is dispensable based solely on data generated using Mps1 inhibitors. However, the data presented here are not likely to close the debate as to the dispensability of Mps1 for centriole assembly. It remains possible that since inhibitors act to inhibit the kinase activity of Mps1 but do not remove Mps1 from the centrosome, perhaps some of the functions of Mps1 require only its physical presence, or the presence of other factors that might bind to Mps1 such as another kinase, such as Plk4, that could phosphorylate and activate Mps1 or substrates of Mps1. To our knowledge, no inhibitors of Plk4 exist for examination under the same conditions.
Figure 2.1: Cell Cycle Profile of Mps1 protein and activity levels and its control by degradation.

The top panel shows the cell cycle profile of hMps1 protein levels (broken gray line), Mps1 protein kinase activity (solid black line), adapted from Hogg et al. 1994. After a sharp peak of Mps1 activity at the G1/S transition that is not accompanied by a rise in whole protein levels, both protein and activity peak in mitosis. After completing its function in the spindle checkpoint, Mps1 is targeted for degradation at mitotic exit through its D-Box. OAZ targets the centrosomal pool of Mps1 for degradation through the Mps1 Degradation Sequence (MDS). Phosphorylation of T468 within the MDS

Continued
Figure 2.1: Cell Cycle Profile of Mps1 protein and activity levels and its control by degradation (Continued).

transiently suppresses OAZ-mediated degradation, allowing accumulation of a highly active centrosomal pool coincident with centrosome duplication. The lower panel shows a schematic of the 853 amino acid hMps1 protein indicating the positions of the D-box (amino acids 256-263) and MDS (amino acids 420-507) in yellow as the binding sites for mitotic factors (blue) and OAZ (red), respectively, and the kinase domain in black. Figure Modified from Pike and Fisk, 2011 [198]; Reprinted with permission of Cell Division, BioMed Central.
Figure 2.2: The Mps1 Inhibitor, IN-1, is not equally as effective against all Mps1 substrates \textit{in vitro}.

In vitro kinase assays were performed as described in materials and methods, with GST-Mps1 (0.4mM) and either 6his-Cetn2 (Cetn2) or Myelin Basic Protein (MBP) as substrate (10mM). The IN-1 Mps1 inhibitor described by Kwiatkowski et al. (2010) was included at the indicated concentrations in μM. The top and bottom panels show autoradiographs of kinase assays with Cetn2 as substrate, the bottom inset panel cropped to show just Cetn2. The middle panel shows a similar kinase assay using MBP as substrate, cropped to show just MBP. Arrows to the left indicate the signals

Continued
Figure 2.2: The Mps1 Inhibitor, IN-1, is not equally as effective against all Mps1 substrates in vitro (Continued).

corresponding to Mps1 autophosphorylation (which is attenuated in the presence of Cetn2), Cetn2 phosphorylation, and MBP phosphorylation. While enzyme and substrate concentrations differ from those used by Kwiatkowski et al. (2010), Cetn2 phosphorylation and Mps1 autophosphorylation were observed at IN-1 concentrations that blocked MBP phosphorylation (5 and 10 µM IN-1), and residual Cetn2 phosphorylation was observed at even 100µM IN-1. Figure Derived from Pike and Fisk [198], 2011; Reprinted with permission of Cell Division, BioMed Central.
**Figure 2.3:** Mps1 Inhibitors, IN-1 and AZ3146, are not as effective against all Mps1 substrates in vitro.

*In vitro* kinase assays were performed as described in materials and methods, shown are Coomassie stained gels and one hour autoradiographic exposures of kinase assays combining 0.4mM GST-Mps1 (Mps1) with 10mM of the indicated substrate; 6his-Cetn2 (Cetn2) or Myelin Basic Protein (MBP), and the indicated concentrations of Mps1 inhibitors IN-1 or AZ3146.
Figure 2.4: Attenuation of Mps1 inhibits centriole biogenesis.

Schematic of experimental outline for assessing centriole biogenesis; RPE1 cells were transfected with siRNA against Mps1 (siMps1) or siControl (siCon) or treated with inhibitors (IN-1 or AZ3146) as indicated. To analyze cells in S phase, BrdU was added at 44 hours post transfection, and centrioles were analyzed in BrdU positive cells at 48 hours post transfection. (B) Bar graph shows the percentage of BrdU positive cells with \( \leq 2 \) Cetn2 foci (black) or \( \geq 4 \) Cetn2 foci (gray). Values represent the mean ±SD (Standard Continued
Figure 2.4: Attenuation of Mps1 inhibits centriole biogenesis (Continued).

Deviation) for triplicate samples with ≥100 cells per replicate. (C) Representative siRNA and Inhibitor images, with digitally magnified images of the centrosomes stained with; γ-tubulin (green), Cetn2 (red), BrdU (blue). Bar = 5µM.
Chapter 3: Cooperation Between Mps1 and Plk4

Derived from: Amanda N. Bliemeister*, Shubhra Majumder and Harold A. Fisk, et. al.

Mps1 and Plk4 Cooperate to Regulate Centriole Biogenesis. Under Review

*Subsequent name change from Pike to Bliemeister

3.1 Abstract

In human cells, the centriole biogenesis pathway is complex and despite recent advances in understanding the remarkable structural and functional conservation of centrioles, the exact molecular mechanisms of the centriole biogenesis pathway in human cells remains unclear. Plk4, the presumptive orthologue of C. elegans ZYG-1, is thought to be the major regulator of centriole assembly [80][201]. However, unlike C. Elegans Zyg-1, Plk4 has not been shown to phosphorylate hSas-6, nor is it required for recruitment of hSas-6 to the procentriole, and centriole amplification caused by overexpression of hSas-6 in human cells does not require Plk4 [202]. This suggests that Plk4 does not assume all of the functions ascribed to ZYG-1, and that other players must be involved.

While the role of Mps1 in centrosome duplication remains controversial, it is a centrosomal kinase whose mis-regulation causes centrosome re-duplication [88][107][175][177]. Thus, we set out to test the possibility that cooperation between the
two centrosomal kinases Mps1 and Plk4 might account for the discrepancy between studies in worms and humans. Either physiological levels of non-degradable forms of Mps1, or overexpression of Plk4 generates extra centrioles, and in each case this is dependent on the presence of the other kinase. Additionally, overexpression of Mps1 can compensate for depletion of Plk4 and vice versa, suggesting that Mps1 and Plk4 cooperate to promote centriole assembly. Furthermore, depletion of Plk4 leads to decreased centrosomal Mps1 levels, suggesting that Plk4 positively regulates centrosomal Mps1. Finally, dual knockdown of Mps1 and Plk4 synergistically inhibits proper recruitment of Sas-6 to the centriole and centriole assembly. Combined, our data suggest that Mps1 and Plk4 cooperate to regulate the centriole biogenesis pathway.

3.2 Introduction

Successful mitosis, defined as faithful segregation of genetic material, is dependent upon two precise duplication events, that of the genome and the centrosome. The centrosome is the main microtubule-organizing center (MTOC) of the cell, consisting of a pair of centrioles surrounded by the pericentriolar matrix (PCM). Similar to the genome, cells inherit a single centrosome through mitosis, which is precisely duplicated in the subsequent cell cycle in order to form the poles of the mitotic spindle that mediates chromosome segregation [203]. Failure to properly regulate centrosome duplication can lead to the generation of extra centrosomes or centrioles, generating aberrant mitotic spindles and leading to errors in chromosome segregation that produce
aneuploid cells. Excess centrosomes and aneuploid cells are an early hallmark of many human tumors [203][204].

The canonical centriole assembly pathway is a complex process [205] and recent estimations predict centrosome assembly, duplication, and regulation involves at least 464 proteins [206][205][207][208]. Due to the complex nature of this pathway, centriole biogenesis remains poorly understood. While variations exist between systems, the core features of the canonical centriole assembly pathway are evolutionarily conserved.

Studies in *C. elegans* have identified five core proteins involved in centriole biogenesis. Centriole biogenesis is initiated by SPD-2, which leads to the recruitment of ZYG-1, which recruits the SAS-5/SAS-6 complex, followed by SAS-4 [209]. Centrioles are highly conserved structures, and each of these centriole components in *C. elegans* has a direct human orthologue, except perhaps for Zyg-1 [210][211][209]. It has been argued that Plk4 is the orthologue of Zyg-1, but sequence analysis of the catalytic domains suggests that Zyg-1 cannot be placed within the Plk4 clan, indicating either rapid divergence of Zyg-1 or that an entirely different kinase has replaced Zyg-1 [212]. Additionally, one study has suggested convergent evolution of Zyg-1 and Plk4, and that Zyg-1 is more closely related to the NIMA and Mps1 families of centrosomal kinases [213]. Additionally, Plk4 and ZYG-1 are not interchangeable [213]. Functionally, ZYG-1 phosphorylates Sas-6 at Serine 123 and phosphorylation of this residue is required for recruitment of Sas-6 to the procentriole [214], and this site is not conserved from *C. elegans* to humans [213]. Moreover, Plk4 has not been shown to phosphorylate hSas-6,
and is not required for recruitment of hSas-6 to centrioles, although it does stabilize hSas-6 at centriole [215].

HsSAK/Plk4 causes centriole amplification in U2OS cells, while ZYG-1 is capable of localization to centrioles but fails to promote amplification [213]. Similarly, Drosophila SAK/Plk4 (DmSAK/Plk4) localizes to the centrioles in U2OS cells, but fails to promote amplification. This suggests that the sequence divergence has functional implications, leading to changes in protein regulation and/or function, and that Plk4 and Zyg-1 do not assume all the functions of the other kinase. Combined, these observations suggest either that recruitment of hSas-6 to the centriole does not require Plk4 kinase activity, or that another protein fulfills that role.

Evidence supporting the role of Mps1 at the centrosome continues to accumulate [88][177]. Mps1 is conserved from yeast to mammals [216] and is clearly a centrosomal protein [173][88][177][107][175] as most recently confirmed by mass-spec of isolated centrosomes utilizing PCP-SILAC [23]. Consistent with its centrosomal localization, Mps1 has several centrosomal substrates, including Mortalin [217] and Cetn2 [177], and was most recently shown to phosphorylate a peptide derived from Cep215 [217]. Cetn2 is a particularly robust substrate of Mps1, and causes Mps1-dependent centriole overproduction in a cell type specific manner [177]. Mps1 is required for Cdc25-dependent centrosome defects [218].

Consistent with a kinase important in centrosome duplication, centrosomal levels of Mps1 are precisely controlled. Cyclin A associated Cdk2 (Cdk2/A) phosphorylates Mps1 at T468 within the Mps1 centrosomal degradation signal, transiently suppressing
antizyme (OAZ) mediated degradation of Mps1 at centrosomes thus allowing accumulation of a centrosomal pool of Mps1 [88][107]. Due to this tight regulation, overexpression of wild type Mps1 is not capable of causing centriole re-duplication in human cells [173], but it does accelerate the onset of re-duplication in U2OS cells. However, preventing the degradation of Mps1 at centrosomes causes centriole re-duplication in all cell lines tested, and versions of Mps1 that cannot be degraded at centrosomes, such as Mps1Δ12/13 that lacks the centrosomal degradation signal or Mps1T468D that mimics Cdk2 phosphorylation cause centriole re-duplication even when expressed at levels that are just 20-50% that of the endogenous protein [219]. This appears to reflect at least two distinct functions of Mps1, a Cetn2-dependent function in centriole maturation, and an early Cetn2-independent function that leads to the production of excess hSas-6-containing structures even in the absence of Cetn2 [87].

All of this makes for an intriguing puzzle to be solved. Plk4 is not required for hSas-6 recruitment to the centriole, Mps1Δ12/13 produces excess hSas-6-containing structures in the absence of Cetn2, and failure to regulate Mps1 accurately leads to a hSas-6 recruitment defect. Captivated by these centrosomal mysteries, we decided to focus our efforts on understanding the roles of Mps1 and Plk4 in the early events of centriole assembly.
3.3 Materials and Methods

Cells and Cell Culture

U2OS cells were grown in DMEM (Hyclone, Logan UT) and RPE1 cells were grown in a 1:1 mixture of DMEM and Ham’s F12 (Hyclone). All media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta GA), 100U/mL penicillin (Hyclone), and 100μg/mL streptomycin (Hyclone). Cells were cultured at 37°C in a humidified chamber in the presence of 5% CO₂.

Plasmids

The following plasmids were used for this study. Previously described plasmids include, pHF65 GST-Mps1 in the pGEX-6p vector, pHF140 GFP-Mps1 T468D in the pECE-GFP vector, pHF286 pECE β-globin GFP, pHF287 pECE β-globin GFP-Mps1, pHF64 GST-Mps1, pHF65 pHF GST-Mps1KD. New plasmid constructs include, pHF287 pECE β-globin GFP-Mps1Δ12/13, pHF297 pECE-β-globin GFP-Mps1 T468D, pHF298 pECE-β-globin GFP-Mps1KD, and pHF313 pECE β-globin GFP-Plk4. GFP-tagged β-globin constructs were created by a two-step process, inserting the gene of interest into pECE-GFP, followed by insertion of the β-globin intron between the SV40 promoter and the gene of interest, both by Infusion cloning.

Transfections and siRNA

In RPE1 or U2OS cells, GFP and GFP-tagged constructs were expressed by transient transfection using Fugene 6 (Promega, Madison WI), and the following siRNAs
were transfected at 0.2μM using RNAiMAX (Invitrogen, Carlsbad CA); siMps1 (nucleotides 1360-1384), siPlk4 (3’ UTR 5’-ACTCCTTTCAGACATATAAG-3’ nucleotides) [80], and siGLO LaminA/C (siCon; Dharmaco, Lafayette CO). For experiments involving siRNA and expression constructs, cells were transfected first with siRNA, and transfected 24 h later with various plasmids as indicated, the further manipulated 20 h (in the case of BrdU incorporation) or 24 h (in the case of S-phase arrest) after plasmid transfection. Efficiency of siRNA depletion was determined by quantitative dual color immunoblot using the Odyssey imaging system (LICOR, Lincoln, NE) as previously described [159] using mouse anti-alpha-tubulin (Sigma, St. Louis MO) as a loading control, while efficiency of siRNA depletion was determined by by IIF for Plk4.

**Antibodies**

Primary antibodies for indirect immunofluorescence (IIF) were as follows: GTU-88 mouse anti-gamma-tubulin, 1:200 (Sigma); Rabbit anti-gamma-tubulin, 1:200 (T5192, Sigma); Rabbit anti-Cetn2 1:4000 [87]; Mouse anti-Sas-6, 1:100 (sc-81431 Santa Cruz Biotechnology); Rabbit anti-CP110, 1:50 was created for this study, by injection of the GST-tagged antigen described by [110] into rabbits, and affinity purification of rabbit serum against a similar 6his-tagged antigen; Goat anti-gamma-tubulin, 1:50 (Santa Cruz Biotechnology); Rabbit anti-Cep135, 1:1000 (Abcam); Rat anti-BrdU, 1:250 (Abcam). Secondary antibodies for IIF were Alexa-350-conjugated goat anti-rat, 488-, 594-, and 750-conjugated donkey anti-rabbit and donkey anti-mouse (Invitrogen). Excluding
experiments where BrdU was the only nuclear stain, DNA was stained with Hoechst 33342 (Sigma). Primary antibodies for immunoblot were DM1A mouse anti-alpha-tubulin, 1:10,000 (Sigma); mouse anti-Sas-6, 1:4000 (Santa Cruz Biotechnology); N1 mouse anti-hMps1, 1:1000 (Invitrogen); Rabbit anti-Plk4 (Cell Signaling). Secondary antibodies for immunoblot were Alexa 680 conjugated donkey anti-mouse and rabbit (Invitrogen), IRDye800-conjugated anti-mouse and rabbit (Rockland) and horseradish peroxidase (HRP)-conjugated donkey anti rabbit and mouse IgG, 1:5000 (GE Healthcare).

**Indirect Immunofluorescence**

For localization of GFP-Mps1, GFP-Plk4, cells were fixed in PBS containing 4% formaldehyde (Ted Pella, Redding CA) and 0.5mM MgCl₂, for 10 minutes at room temperature and processed for IIF as previously described [108][159]. However, experiments requiring visualization of GFP constructs and the use of the Sas-6 antibody, cells were fixed with MeOH and a GFP antibody utilized. For siRNA experiments, cells were fixed in ice cold -20°C methanol for 10 minutes at -20°C, washed four times with PBS, and processed for IIF as previously described [87]. Cells were then washed four times with PBST (0.05% Triton X-100 in PBS), and incubated with primary antibodies overnight at 4°C. Cells were then washed 3 times, and incubated with secondary antibodies for one hour at room temperature before mounting with Citiflour. Efficiency of siRNA depletion for Plk4 was determined by IIF using the described Plk4 antibody. All images were acquired at ambient temperature using an Olympus IX-81 microscope,
with a 63X or 100X Plan Apo oil immersion objective (1.4 numerical aperture), and a QCAM Retiga Exi FAST 1394 camera, and analyzed using the Slidebook software package (Intelligent Imaging Innovations, Denver CO).

**Cell Synchronization and BrdU Incorporation Assay**

For S-phase synchronization, arresting cells in S phase, RPE1 or U2OS cells were treated with 4mM hydroxyurea (HU), for 24 hours after the final transfection. The beginning of S phase arrest (t=0) was considered to be 24 hours after the addition of HU, and centriole number was assessed 24 hours after the addition of HU (a total of 24 hour S phase arrest). RPE1 cells do not normally undergo centrosome reduplication during any length S phase arrest. U2OS cells demonstrate spontaneous reduplication between t=48 and t=72 hours of S phase arrest; GFP-Mps1 does not increase the extent of reduplication in U2OS cells, but accelerates its onset so that it is apparent by t=24 hours of S phase arrest [159][89][107][88]. Plk4 accelerates its onset, increases its extent and produces characteristic centriolar rosettes [81]. For experiments in U2OS cells, centrosomal Plk4 was analyzed at t=0 of S phase arrest, while centriole number was assessed at t=24 hours of S phase arrest (e.g. 48 hours of total HU treatment). For BrdU pulse chase analysis in RPE1 cells transfected with siRNAs only, cells were transfected with siRNAs, BrdU (40 µM) was added 44 h post transfection, BrdU was removed and cells were incubated in medium lacking BrdU, and both BrdU incorporation and centriole number were assessed by IIF at 48 h (PULSE) or 52 h post transfection (CHASE). BrdU pulse
chase analysis was similar for RPE1 cells sequentially transfected with siRNAs and expression constructs, but BrdU was added 68 h post siRNA transfection, and BrdU incorporation and centriole number were assessed at 72 h (PULSE) and 76 h (CHASE) post siRNA transfection.

3.4 Results

3.4.1 Simultaneous knockdown of Mps1 and Plk4 Inhibits Recruitment of Sas-6 to the Centriole.

We examined the distribution of Sas-6 in BrdU positive cells depleted of Mps1, Plk4 or both in a pulse-chase assay (Figure 3.1-A). BrdU is a synthetic thymidine analog incorporated into nuclear DNA during replication, specifically labeling cells in S-phase. The pulse-chase aspect of this experiment allows for the following of cells through S-phase, which allows us to determine if loss of either Mps1 or Plk4 or both blocks centriole duplication or simply delays it (Figure 3.1-A). Depletion of Mps1 was measured by immunoblot (Figure 3.1-E) and Plk4 depletion was measured by IIF (Figure 3.1-F). In both the BrdU pulse and chase, the majority (95%) of BrdU-positive cells transfected with siCon alone showed the expected pattern for duplicated centrosomes of two hSas-6 foci and four Cetn2 foci (Figure 3.2-B), wherein two of the Cetn2 foci represent the two G1 centrioles, while together with the two hSas-6 foci the remaining two Cetn2 foci represent the two procentrioles formed upon entry into S-phase.
In the BrdU pulse, the percentage of cells that had completed centriole assembly was similar in BrdU-positive siMps1 and siPlk4 cells at roughly 60%. The remaining roughly 40% of BrdU-positive siMps1 or siPlk4 cells showed two different centriole assembly defects, with a slightly more severe effect seen in siPlk4 cells (Figure 3.1-B). This was not surprising, as slight defects in the recruitment of Sas-6 mediated by loss of Plk4 had been previously published [82]. Roughly 40% of BrdU-positive siMps1 cells had initiated, but failed to complete centriole assembly, having two hSas-6 foci and just two Cetn2 foci, which we presume represents the initiation of an hSas-6-containing procentriole that was not completely assembled as judged by addition of Cetn2. While just 25% of BrdU-positive siPlk4 cells showed this pattern, the remaining 15% showed a defect in the initiation of centriole assembly wherein there was just a single focus of hSas-6, a phenotype that was apparent in only a very small fraction of BrdU-positive siMps1 cells (Figure 3.1-B).

However, when we co-depleted Mps1 and Plk4 we made two striking observations. First, while roughly 60% of BrdU-positive cells were competent to complete centriole assembly in cells depleted singly of Mps1 or Plk4, only roughly 15% of BrdU-positive cells co-depleted of both Mps1 and Plk4 were able to complete centriole assembly. Second, co-depletion of Mps1 and Plk4 synergistically enhanced the initiation defect, and 25% of dual depleted cells had a single hSas-6 focus. This single focus of hSas-6 was typically localized closer to one of the two centrioles (81% of cells), but in 19% of cells was found between the two centrioles, as marked by either Cetn2 or CP110 staining. These observations suggest that Plk4 and Mps1 cooperate in the proper
assembly of hSas-6 into procentrioles. Similarly, siMps1 was not capable of inhibiting recruitment of Sas-6 to the centriole, but as previously published [107] and shown in Figure 3.1, clearly inhibited recruitment of Cetn2 in nearly 40% of cells. In contrast, approximately 9% of siPlk4 cells demonstrated a single focus of Sas-6, confirming that Plk4 does slightly inhibit recruitment of Sas-6 to the centriole. However, we were surprised to find that simultaneous knockdown of Plk4 and Mps1 generated a single focus of Sas-6 in approximately 30% of cells. Representative images of the pulse-chase cells can be found in Figure 3.1-C. As shown in Figure 3.1C, Sas-6 typically displays as two distinct foci of roughly equal size and intensity. However, in the pulse, we noticed that of the 2Sas-6:2Cetn2 phenotype, approximately 50% of these cells showed the phenotype of having one primary Sas-6 focus and a much less distinct second focus (Figure-3.1-D), and this phenotype was no longer apparent in the chase, further suggesting that while Mps1 and Plk4 dual depletion do not block recruitment, they indeed delay recruitment of Sas-6 and may be required for its stability at the centrosome.

3.4.2 Mps1 and Plk4 may be Dispensable for Centriole Assembly

Plk4 has emerged as the master regulator of centriole biogenesis, while the role of Mps1 has remained controversial. To address whether Mps1 or Plk4 is indeed required for centriole biogenesis, we utilized the BrdU pulse-chase assay in an identical manner to the BrdU pulse assay described in 3.4.1, except that centrioles are also analyzed in BrdU-positive cells four hours after removal of BrdU; these cells were labeled during the initial pulse, but given an additional four hours to complete centriole assembly. The dual
depletion of Mps1 and Plk4 experiment described in 3.4.1 used a short pulse of BrdU to show that depletion of Mps1 or Plk4 leads to centriole assembly defects that are synergistically enhanced by co-depletion.

As we recently used the BrdU pulse-chase approach to show that while centriole assembly defects are apparent in Cetn2-depleted cells after an initial BrdU pulse, centrioles can be assembled in the absence of Cetn2 if given more time [87], we reasoned this same approach would be useful to determine whether Mps1 or Plk4 is dispensable for centriole assembly. The observation that centriole assembly is only delayed demonstrates that Cetn2 is dispensable for centriole assembly, as Cetn2-depleted cells demonstrate a delay in centriole biogenesis when comparing the pulse and chase to that in control cells [87]. While Cetn2-depleted cells ultimately complete centriole assembly, this assay also clearly demonstrates that Cetn2 does have a centrosomal role, despite being dispensable for centriole assembly. Hence, the chase allows us to distinguish an outright block rather than a delay.

Consistent with the suggestion that Mps1 is dispensable for centriole assembly, we found that after a four hour chase period, less than 20% of BrdU-positive siMps1 cells had failed to complete centriole assembly, compared to 40% after the initial pulse. Surprisingly, and in contrast to expectations for a kinase that is essential for centriole assembly, we also found that less than 25% of BrdU-positive siPlk4 cells had failed to complete centriole assembly after the chase period, compared to 40% after the initial pulse. Even more surprisingly, the chase period allowed 55% of BrdU-positive cells that were co-depleted of both Mps1 and Plk4 to complete centriole assembly, compared to
just 15% that had completed centriole assembly after the initial pulse. The cell biology and centrosome fields are likely not ready to conclude that Plk4 is dispensable for centriole assembly. Yet it is remarkable that under similar experimental conditions Mps1 and Plk4 both appear to be equally dispensable for centriole assembly. Accordingly, we are hesitant to conclude from this data that Mps1 is dispensable, and this is discussed further below.

3.4.3 Mps1 and Plk4 are mutually dependent for Centriole Re-duplication in U2OS cells

Armed with the information that dual depletion of Mps1 and Plk4 leads to a delay in the recruitment of Sas-6 to the centriole, we next aimed to determine if Mps1 and Plk4 demonstrate any interdependency, specifically in their ability to cause centrosome amplification. Overexpression of Plk4 causes a striking “rosette” pattern of centriole overduplication in U2OS cells, and overexpression of Mps1 accelerates the onset of centrosome re-duplication in these cells. Accordingly, U2OS cells provide an ideal system in which to explore the dependency of centrosome duplication of Mps1 and Plk4. Hence, we examined centrosome re-duplication in S-phase arrested U2OS cells overexpressing Mps1 or Plk4 after depletion of the other kinase with either siMps1 or a Plk4-specific siRNA (siPlk4). Efficient depletion of Plk4 was determined by IIF (Figure 3.2-C), while efficient Mps1 depletion was determined by immunoblotting (Figure 3.2-D). In U2OS cells arrested in S phase for 48 hours, 95%, 38%, and 50% of cells expressing GFP-Plk4, GFP-Mps1, or the non-degradable GFP-Mps1T468D, respectively,
had undergone centriole over production (Figure 3.2-A). However, in each case this was dependent on the presence of the other kinase. Only 13% of siPlk4 cells expressing GFP-Mps1, had undergone re-duplication, representing a 3-fold reduction. While GFP-Mps1T468D had a significantly greater effect than did wild type Mps1, siPlk4 greatly reduced this re-duplication by nearly 4-fold to 13% (Figure 3.2-A). Hence, Plk4 is required for Mps1 driven centriole reduplication, even in the case of the non-degradable GFP-Mps1 T468D. Similarly, just 38% of GFP-Plk4 expressing siMps1 cells had extra centrioles, representing a 2.5-fold reduction compared to expression of GFP-Plk4 on its own. The effect of siMps1 was much more pronounced when we considered the ability of Plk4 to promote rosette formation, as judged with the centriole markers CP110 and Cetn2 [220]. In roughly 90% of GFP-Plk4 expressing U2OS cells extra centrioles were found in a rosette formation. However, only 26% of siMps1 cells expressing GFP-Plk4 show rosettes, a 3.5-fold reduction. Combined with the overall 2.5-fold reduction in reduplication of GFP-Plk4 in an siMps1 background, this further emphasizes a requirement for Mps1 in Plk4-driven centriole over duplication.

3.4.4 The roles of Mps1 and Plk4 in Centriole Duplication are mutually dependent in RPE1 cells

As U2OS cells normally undergo centrosome reduplication upon S-phase arrest in the absence of ectopic Mps1 or Plk4, we next examined the whether there was similar dependency between Mps1 and Plk4 for centrosome duplication in RPE1 cells that do not undergo centrosome reduplication. First, we assessed the effect of overexpression of Plk4 or Mps1Δ12/13 in RPE1 cultures that had been enriched in S-phase with a 24 hr
hydroxyurea (HU) treatment (Figure 3.3). Both GFP-Mps1Δ12/13 and GFP-Plk4 lead to
centriole amplification, as 31% and 42% of HU-arrested cells expressing GFP-
Mps1Δ12/13 or GFP-Plk4, respectively, had more than four centrioles as judged by the
number of Cetn2 foci (compared to just 4% for cells expressing GFP alone). Moreover,
as in U2OS cells the ability of each kinase to promote centriole over production was
dependent on the presence of the other kinase; GFP-Mps1Δ12/13 failed to produce extra
centrioles in siPlk4 cells, and GFP-Plk4 likewise failed to produce extra centrioles in
siMps1 cells (Figure 3.3). This was confirmed with two centriolar markers, Cetn2
(Figures 3.4, 3.5) and CP110 (Figure 3.3).

While our initial experiments in RPE1 cells used cells enriched in S-phase by
addition of HU for 24 hours (Figure 3.3), we wanted to determine if these results were
also true in an asynchronous cell population. We found a similar dependency between
Mps1 and Plk4 during an unperturbed cell cycle using the BrdU pulse approach described
above (Figure 3.1). While 65% of BrdU-positive siCon RPE1 cells expressing GFP-Plk4
had more than four centrioles, this was reduced by two-fold to only 31% by siMps1.
Likewise, 39% of BrdU-positive siCon RPE1 cells expressing GFP-Mps1Δ12/13 had
more than four centrioles, and this was reduced nearly 3.5-fold to just 12% by siPlk4
(Figures 3.4, 3.5). The BrdU pulse approach also yielded two additional important
observations. First, both Mps1Δ12/13 and Plk4 are capable of promoting centriole over
production in a single unperturbed S-phase. Second, Mps1Δ12/13 and Plk4 are mutually
dependent for centrosome re-duplication.
3.4.5 Cycling Cells are not Entering S Phase with Extra Centrioles

To determine if the presence of BrdU-positive cells with more than four centrioles might reflect cells that entered S-phase with extra centrioles that then duplicated normally, we used the PCNA/Cyclin B double labeling method described by Leidel et al [13] to examine centriole number in G1 cells. As PCNA labels nuclei of S phase cells while Cyclin B1 labels the cytoplasm of cells in G2 and mitosis, cells negative for both PCNA and Cyclin B1 with decondensed DNA represent G1 cells (Figure 3.6). We found that the vast majority of G1 cells expressing GFP-Mps1Δ12/13 had the normal two Cetn2 foci (Figure 3.7). While a statistically significant number (17%) of G1 cells expressing GFP-Plk4 were found to have four or more centrioles, this cannot account for the 30% BrdU-positive GFP-Plk4 expressing cells with more than four centrioles. Therefore, it appears that the extra centrioles in BrdU-positive cells expressing GFP-Mps1Δ12/13 or GFP-Plk4 largely arose after entry into S-phase. Second, while depletion of either Mps1 or Plk4 lead to the appearance of BrdU-positive cells that have not completed centriole assembly, overexpression of Mps1 or Plk4 can compensate for depletion of the other kinase. While 32% and 22% of BrdU-positive siPlk4 and siMps1 cells expressing GFP alone have just two centrioles, overexpression of GFP-Mps1 or GFP-Plk4 reduced this percentage to 17% and 4 %, respectively (Figure 3.7). While at least in principle an apparently normal centriole number could arise through an aberrant assembly process beginning with two unreplicated centrioles, this possibility can be ruled out in the case of siPlk4 cells overexpressing GFP-Mps1, because overexpression of wild type GFP-Mps1
does not cause any aberrant centrosome phenotype in RPE1 cells. Accordingly, our data suggest that overexpression of Mps1 can compensate for depletion of Plk4, and perhaps vice versa.

3.4.6 Centrosomal Levels of Mps1 and Plk4 are Interdependent

The centrosomal levels of Mps1 and Plk4 are both tightly controlled through phosphorylation-regulated degradation [88][221]. Accordingly, given that the two kinases phosphorylate each other in vitro, we set out to test whether Mps1 and Plk4 might regulate the centrosomal levels of the other kinase. We found that in siPlk4 cells, staining of Mps1 at the centrosome decreased by 35% (Figure 3.8). This suggests that Plk4 has a role in the maintenance of Mps1 at the centrosome, and further supports the hypothesis that Mps1 and Plk4 cooperate to regulate centriole biogenesis.

This is of particular interest, because it suggests that depletion of Plk4 also causes a modest depletion of Mps1 from the centrosome, and suggests that previous assessments of Plk4 depletion are actually an assessment of simultaneous depletion of Mps1 and Plk4. It might also explain the more severe phenotype we observe here in siPlk4 cells, such as the 9% of siPlk4 cells that show a single Sas-6 focus. This may also contribute to an assessment by the field of Plk4 as the essential kinase for centriole duplication, and of Mps1 as dispensable. Plk4 has been shown to auto-regulate its stability [222]. However, the possibility exists that additional proteins may affect the activity of Plk4, and therefore has an effect on its stability at the centrosome. To test the hypothesis that Mps1 may have a role in the regulation of stability of Plk4 at the centrosome, we again utilized siRNAs
specific to Mps1, and measured the pixel intensity of Plk4 at the centrosome. In the absence of Mps1, staining of Plk4 at the centrosome was not affected.

In contrast, GFP-Mps1 reduced centrosomal Plk4 nearly 2 fold in S phase arrested U2OS cells, and GFP-Mps1Δ12/13 had a similar effect (Figure 3.9). These observations suggest that Plk4 positively regulates centrosomal Mps1. Interestingly, a small number of cells had very strong centrosomal GFP-Mps1Δ12/13; those cells also had high centrosomal Plk4 levels (Figure 3.9), and those cells had excess centrosomes.

3.5 Discussion

Based on evidence that Plk4 does not assume all the functions ascribed to the C. elegans Zyg-1[215][213][223], and studies suggesting that Zyg-1 does not cluster with the polo-like kinase clan and may be more closely related to Mps1[213], we set out to examine the relationships between Plk4 and another important centrosomal kinase, Mps1. Data presented here suggests that Mps1 and Plk4 cooperate to promote proper centriole biogenesis. Interestingly, we found that overexpression of Plk4 or Mps1Δ12/13 can cause centriole over duplication in a single, unperturbed cell cycle. This did not reflect entry into S-phase with excess centrioles that were then replicated normally, because G1 cells overexpressing Mps1Δ12/13 had the expected number of two centrioles, and the percentage of G1 cells overexpressing Plk4 with more than two centrioles was far too low to explain the percentage of BrdU positive cells with more than four centrioles.

More importantly, neither kinase can promote centriole over duplication in the absence of the other, whether during a prolonged S-phase arrest in U2OS cells, after S-
phase enrichment of RPE1 cells, or during a single unperturbed S-phase in RPE1 cells. Moreover, overexpression of one kinase can restore normal centrosome duplication in cells depleted of the other kinase. Because each kinase can also promote centriole over duplication, we considered the possibility that this apparent compensation reflected not restoration of normal duplication but rather entry into S-phase with too few centrioles, followed by an aberrant “re-duplication” event from centrioles that were not first replicated through the normal duplication process. However, because wild type Mps1 does not cause centriole re-duplication in RPE1 cells, its ability to restore normal centriole numbers in cells depleted of Plk4 cannot be the result of abnormal re-duplication of un-replicated centrioles. Moreover, catalytically inactive Mps1 KD does not restore normal centriole numbers in siPlk4 cells to the same degree as wild type Mps1 or Mps1Δ12/13, suggesting that it is not just the presence of Mps1, but its kinase activity that compensates for knockdown of Plk4.

In further support of the suggestion that Mps1 and Plk4 cooperate to promote centriole biogenesis, we also found synergistic centriole assembly defects upon simultaneous knockdown of Mps1 and Plk4, wherein only 15% of BrdU-positive cells have completed centriole assembly and the recruitment of hSas-6 to the procentriole is defective. Moreover, depletion of Plk4 leads to reduced centrosomal levels of Mps1, suggesting that they may cross-regulate each other. This suggests that some of the Plk4 phenotype may be due to a concomitant reduction of Mps1, which may explain the observation that knockdown of Plk4 alone shows a hSas-6 recruitment defect in approximately 9% of RPE1 cells, while knockdown of Mps1 alone does not. However,
we were unable to identify any changes in Plk4 levels upon knockdown of Mps1 in RPE1 cells. In contrast, cells expressing GFP-Mps1 demonstrate a 2 fold reduction of centrosomal Plk4, suggesting that Plk4 can positively regulate Mps1. Combined with the result that cells with a high level of GFP-Mps1Δ12/13 also had high levels of centrosomal Plk4, and those cells had excess centrosomes. It is therefore tempting to hypothesize the possibility that Mps1 might regulate Plk4 in turn. This is consistent with the hypothesis that both Mps1 and Plk4 are required for centrosome reduplication, while Mps1 or Plk4 alone are not, and suggests cells must escape the negative regulation of Plk4 to undergo reduplication. It also suggests the existence of a negative feedback loop, whereby centrosomal Mps1 decreases centrosomal Plk4, the loss of which then reduces centrosomal Mps1.
3.6: Chapter 3 Figures

Figure 3.1: Simultaneous knockdown of Mps1 and Plk4 Inhibits Recruitment of Sas-6 to the Centriole.
Figure 3.1: Simultaneous knockdown of Mps1 and Plk4 Inhibits Recruitment of Sas-6 to the Centriole (Continued).

Schematic of experimental outline for assessing centriole biogenesis; RPE1 cells were transfected with control (SiCon), Mps1-specific (siMps1), Plk4-specific (siPlk4) or a combination of the siRNAs for 48 hr in cycling cells. Cells were labeled in S phase by the addition of BrdU for 4hr at 44 hr post siRNA transfection, and cells were collected at 48 hours (pulse) and 52 hours (chase), to be analyzed by IIF. (B) Bar graph showing the percentage of BrdU positive cells with 1Sas-6 – 2 Cetn2 foci (Black), 2 Sas-6 – 4 Cetn2 foci (red), or 2 Sas-6 – 2 Cetn2 foci (gray). Values represent the mean of triplicate independent samples, with at least 100 cells per replicate. (C and D) Digitally magnified images of centrioles from representative cells expressing the indicated siRNA constructs; green, Cetn2; red, Sas-6; blue, BrdU. © Whole cell Mps1 levels were analyzed by immunoblot. Mps1 levels were reduced by approximately 87% and the transfection efficiency of the siRNA was roughly 90%. (F) Representative images of centrosomal levels of Plk4 were assessed by IIF as described in methods. Plk4 levels were reduced by approximately 85%.
Figure 3.2: Mps1 and Plk4 are mutually dependent for Centriole re-duplication in U2OS cells.

Continued
Figure 3.2: Mps1 and Plk4 are mutually dependent for Centriole re-duplication in U2OS cells (Continued).

U2OS cells were plated for 24 hours; Day 1. Cells were transfected with indicated siRNA (siCON, siMps1, siPlk4); Day 2, followed by transfection with the indicated plasmid (GFP-Mps1, GFP-Mps1T468D, GFP, or GFP-Plk4); Day 3. Hydroxyurea (HU) was added on day 4 and day 5 for a total of 48 hour HU arrest. Bar graph shows the percentage of GFP positive cells with 2 centrioles (black), 3-4 centrioles (green), more than 4 centrioles – no rosette (Orange), and more than 4 centrioles – forming a rosette (gray). The graph also indicates the percentage of GFP positive cells with centrosomal GFP. (B) Representative images for the experiment described in A. (C) Centrosomal levels of Plk4 were assessed by indirect immunofluorescence (IIF) using the Plk4 antibody described in methods. Plk4 levels were reduced by approximately 85%. (D) Protein levels were analyzed by immunoblot. Mps1 levels were reduced by approximately 90% and the transfection efficiency of the siRNA was roughly 90%.

Figure derived from Amanda N. Bliemeister, Shubhra Majumder and Harold A. Fisk, Mps1 and Plk4 Cooperate to Regulate Centriole Biogenesis. Under Review. The data in this figure was generated by Dr. Shubhra Majumder.
Figure 3.3: Mps1 and Plk4 are mutually dependent for reduplication in RPE1 Cells.

Experimental outline; RPE1 cells were sequentially transfected with control (siCon), Mps1-specific (siMps1), or Plk4 specific (siPlk4) siRNAs and the indicated GFP expression constructs. Cells were arrested in S phase by the addition of HU for 24h, and centrioles were analyzed at IIF 72 hours post siRNA transfection. (B) Bar graph showing the percentage of GFP positive S-phase arrested cells expressing the indicated constructs with >4 CP110 foci (gray), 4 CP110 foci (red), or ≤2 CP110 foci (black). Values represent the mean±SD of 3 independent samples, with at least 100 cells per replicate.
Figure 3.4: Plk4 Dependent Reduplication Requires Mps1.

Schematic of experimental outline for assessing centriole biogenesis; RPE1 cells were sequentially transfected with control (SiCon) or Mps1-specific (siMps1) siRNAs and the indicated GFP expression constructs in cycling cells. Cells were labeled in S phase by the addition of BrdU for a 4hr pulse at 68 hr post siRNA transfection, to be analyzed by IIF after 72 hr. (B) Bar graph showing the percentage of BrdU positive cells expressing the indicated constructs with more than 4 Cetn2 foci (Gray), 4 Cetn2 foci (Red), or less than or equal to 2 Cetn2 foci (Black). Values represent the mean of 5 independent samples, with at least 25 cells per replicate. (C) Digitally magnified images of centrioles from representative cells expressing the indicated constructs; green, γ Tubulin; red, Cetn2.
Figure 3.5: Mps1 Δ12/13 Dependent Reduplication Requires Plk4.

Schematic of experimental outline for assessing centriole biogenesis; RPE1 cells were sequentially transfected with control (SiCon) or Plk4-specific (siPlk4) siRNAs and the

Continued
Figure 3.5: Mps1 Δ12/13 Dependent Reduplication Requires Plk4 (Continued).

indicated GFP expression constructs, in cycling cells, labeled in S phase by the addition of BrDU for 4 hours at 68 hr post siRNA transfection, to be analyzed by IIF after 72 hr. (B) Bar graph showing the percentage of BrdU positive cells expressing the indicated constructs with more than 4 Cetn2 foci (Gray), 4 Cetn2 foci (Red), or less than or equal to 2 Cetn2 foci (Black). Values represent the mean of 5 independent samples, with at least 25 cells per replicate. (C) Digitally magnified images of centrioles from representative cells expressing the indicated constructs; green, γ Tubulin; red, Cetn2.
Figure 3.6: Analysis of centrioles in G1 cells.

CyclinB/PCNA/Cetn2 labeling through the cell cycle. Asynchronously growing RPE1 cells were stained with antibodies against PCNA (stains S-phase cells), Cyclin B (stains G2/M cells), and Cetn2 (stains centrioles throughout the cell cycle) as described by Strnad et al., 2009 [82]. G1 cells are negative for both PCNA and Cyclin B and have two centrioles; S-phase cells have nuclear PCNA and are Cyclin B negative, and generally have four centrioles. G2 cells are negative for PCNA and show either cytoplasmic Cyclin B with no nuclear staining, or, as shown, nuclear and cytoplasmic Cyclin B, and have 4 centrioles. Mitotic (M) phase cells have strong cytoplasmic staining, no nuclear membrane, condensed DNA, and two well separated centrosomes. Despite background staining in G1 cells, nuclear intensity clearly distinguishes G1 cells from S-phase cells, and cytoplasmic intensity clearly distinguishes G2 cells from S-phase cells.
Figure 3.7: Confirmation that Cycling Cells are Not Entering S Phase with Extra Centrioles.

(A) Asynchronously growing RPE1 cells were sequentially transfected with control...
Figure 3.7: Confirmation that Cycling Cells are Not Entering S Phase with Extra Centrioles (Continued).

(SiCon), Mps1-specific (siMps1) or Plk4 specific (siPlk4) siRNAs and the cells negative for both PCNA and Cyclin B (indicative of G1 cells) expressing indicated GFP constructs were counted. Bar graph showing the percentage of cells expressing the indicated constructs with more than 4 Cetn2 foci (Black), 4 Cetn2 foci (gray), or less than or equal to 2 Cetn2 foci (red). Values represent the mean of 3 independent samples, with 100 cells per replicate. (B) Representative images of G1 and S phase cells labeled with PCNA, Cyclin B, and Cetn2.
Figure 3.8: Knockdown of Plk4 affects Mps1 Centrosomal Levels.

RPE1 cells were transfected with control (SiCon), or Plk4 specific (siPlk4) siRNAs and the cells labeled with BrdU for 4 hours. Representative images of cells are shown, using an Mps1 specific antibody, Mps1 MO2 (Red) and γ Tubulin (Green). (B) Indicates the relative measured value of Mps1 at the centrosome. Box and whisker plots show the ratio of centrosomal $F_{Plk4}/F_{\gamma-Tub}$ in siPlk4 cells to that paired in siCon transfected cells. Values represent independent triplicate counts of at least 8 cells.
Figure 3.9: Overexpression of Mps1 reduces centrosomal Plk4 levels.

(A-D) U2OS cells were transfected with the indicated constructs, and centrosomal Plk4 was analyzed after a 24 hour HU treatment. Representative cell pairs; green = GFP, red = γ-Tub, blue = Plk4, bar = 5µM, * = high centrosomal GFP-Mps1Δ12/13. © Box and whisker plots show the ratio of centrosomal F_{Plk4}/F_{γ-Tub} in GFP positive cells to that in paired untransfected cells for 20 cell pairs; GFP-Mps1Δ12/13* includes only 5 cells, and no statistical analysis was applied. Figure derived from Amanda N. Bliemeister, Shubhra Majumder and Harold A. Fisk, Mps1 and Plk4 Cooperate to Regulate Centriole Biogenesis. Under Review. The data in this figure was generated by Dr. Shubhra Majumder.
Chapter 4: Mps1 and Plk4 Cross Phosphorylate and May Share Similar Substrates

Derived from: Amanda N. Bliemeister, Shubhra Majumder and Harold A. Fisk, Mps1 and Plk4 Cooperate to Regulate Centriole Biogenesis. Under Review


*Subsequent name change from Pike to Bliemeister

4.1 Abstract

Phosphorylation is a complex and prominent posttranslational modification in centrosome biology. It regulates cell cycle events including mitotic spindle formation and centrosome duplication. Therefore, identification of substrates for the centrosomal kinases Mps1 and Plk4 could identify critical phosphorylated residues essential for localization of centrosomal components or the regulation of centriole biogenesis. We have purified recombinant SUMO-His-Plk4, SUMO-His-Plk4KD and a control SUMO-His-MBP for examination by in vitro kinase assays with recombinant GST-Mps1, GST-Mps1KD, and GST alone. As Mps1 and Plk4 are both known to autophosphorylate, it was necessary to produce the kinase dead versions of both kinases. We have found that Mps1 phosphorylates Plk4, and Plk4 phosphorylates Mps1, in vitro. Additionally, it
appears that the Plk4 phosphorylation of Mps1 is more robust in comparison to the phosphorylation of Plk4 by Mps1. For the first time in our lab, we have also isolated centrosomes from HeLa cell lysates by sucrose gradient and ultracentrifugation. These centrosomal preparations were then used in an in vitro kinase assays and have shown that Mps1 and Plk4 may share similar unidentified substrates.

4.2 Introduction

The MTOC activity of the centrosome includes the nucleation and organization of microtubules, and therefore has a profound influence on all microtubule-dependent processes. Upon entry to mitosis, the daughter centrosomes nucleate the astral arrays that contribute the majority of microtubules to the formation of the spindle. These microtubule asters allow centrosomes to determine spindle polarity, orientation of the spindle, and the plane of cleavage. Hence, the centrosome must be duplicated only once, so the cell will have two centrosomes upon entry into mitosis, as cells with more than two centrosomes are prone to multipolar mitosis. Multipolar mitosis can be detrimental to the cell and the whole organism, leading to multipolar divisions or an increased incidence of lagging chromosomes [119] as the resulting aneuploidy can lead to the elimination of tumor suppressor genes or normal alleles that can promote unregulated growth characteristics and/or a diminished apoptotic response to cellular damage [224][143]. Common hallmarks of human cancers include aneuploidy, genomic instability, and a high incidence of centrosome amplification [128][225]. Hence, regulation of centriole
biogenesis and centrosome duplication is critical. Part of this regulation is due to posttranslational modifications, such as phosphorylation.

It is well known that protein phosphorylation can modulate centrosome function [24][226]. The cell cycle phosphoproteome of the yeast centrosome has been delineated, demonstrating conserved phosphorylation residues, clustering of phosphorylation sites and different modes of phosphoregulation based on the specific protein kinase [24]. The extent and complexity of phosphoregulation of the centriole biogenesis pathway and centrosome duplication is extensive, and phosphorylation of substrates by their kinases is a prominent posttranslational modification. The centrosome is comprised of many kinases, including but not limited to: Nek2 [227], Plk4 [80], Mps1[159], Plk1 [148], Aurora A [144], Aurora B [145], and Cdk2/CyclinA [228]. Of these kinases, we have limited our focus to Plk4, the presumptive master regulator of centriole biogenesis, and Mps1, the controversial centrosomal kinase.

Conservation of Plk4 can be found in most organisms that possess a single posterior flagellum (opisthokonts) [229]. Plk4 is a centrosomal kinase [80], and has several centrosomal proteins it interacts with and centrosomal substrates including GCP6 [230], Cep152 [29], and Cep192 [78]. Plk4 overexpression causes centriole reduplication, and this kinase is capable of autoregulating its own degradation, and preventing this autophosphorylation causes centrosome amplification [231]. As the centrosomal kinase Plk4 has such a striking effect on centriole biogenesis, it is likely that it has many centrosomal substrates and interacting partners beyond those already determined.
Conservation of Mps1 can be found from yeast to mammals [216] and is clearly a centrosomal protein [173][88][177][107][175] and was most recently confirmed as a centrosomal kinase by mass-spectrometry of isolated centrosomes utilizing PCP-SILAC [23]. Consistent with its centrosomal localization, as a protein kinase, Mps1 has several centrosomal substrates, including Mortalin [217] and Cetn2 [177], and was most recently shown to phosphorylate a peptide derived from Cep215 [217]. One of the most well characterized substrates of Mps1 is Cetn2, and is a particularly robust substrate of Mps1, causing Mps1-dependent centriole overproduction in a cell type specific manner [177].

Thus, as Mps1 and Plk4 are two centrosomal kinases recruited to the site of procentriole assembly early in the process, and we have shown that there is cooperation between Mps1 and Plk4 at the centrosome. Specifically, that Mps1 and Plk4 are mutually dependent for centrosome re-duplication, dual depletion leads to a recruitment defect of Sas-6, and Mps1 and Plk4 centrosomal levels are interdependent. With this data in mind, we set out to determine if this could be explained by these centrosomal kinases phosphorylating one another and to determine if these kinases share similar substrates in centrosomal preparations.

4.3 Materials and Methods

Construction of SUMO-His expression vectors

Plk4 and MBP (Myelin Basic Protein) were amplified via PCR using InFusion Primers containing at least 15bp of homology from the Gene of Interest (GOI) at the 3’
end and at least 15bp of homology from the vector on the 5’ end [232]. Forward primer utilized the restriction enzyme BsaI and and reverse primer with XbaI. The identity of all constructs was verified by sequence analysis.

**Expression of Recombinant Proteins**

The verified Sumo-His vector containing the correct GOI was transformed into E. Coli strain BL21 (LysS) (Invitrogen) and spread on plates containing ampicillin and Chloramphenicol antibiotics. This particular strain expresses tRNA for codons rarely used in bacteria and constitutively expresses the T7 lysozyme, an inhibitor of T7 RNA polymerase, thus reducing RNA polymerase activity in uninduced cells. The next day, all colonies were removed from the plates using LB, and used to make a starter culture of LB medium containing 100µg/mL ampicillin and 100µg/mL chloramphenicol, which was used to inoculated the one liter for large-scale growth. Cells were grown at 37°C for approximately 2.5 hours, until reaching log phase growth, determined by an OD600 between 0.5 and 0.7, when induced by inoculation with IPTG (isopropyl-1-thio-8-D-galactopyranose) to a final concentration of 1mM. Induction samples were collected at every hour for a four hour time period, at which the total culture was harvested by centrifugation. Pellets were stored at -20°C for confirmation of induction of the GOI and for further purification.
Recombinant Protein Preparation and Induction Confirmation

The induction samples were thawed on ice and resuspended in lysis buffer (500mM NaCl₂, 50mM Na₂H₂PO₄ pH 8.0) containing 1mg/mL lysozyme. Following a 30 minute incubation on ice, this lysate was sonicated at high intensity in bursts of 30 seconds at least four times, with a minimum 30 second cooling period between each burst. The lysates were separated by centrifugation for 10 minutes at 4°C at 13,000 rpm. This separates the soluble (supernatant) from the insoluble (pellet) fractions. The pellet was resuspended with lysis buffer and both pellet and supernatant were prepared for SDS-PAGE by addition of sample buffer and boiling for 5 minutes. Analysis of induced recombinant proteins was accomplished SDS-PAGE and coomassie staining.

Recombinant protein Purification

The large scale 1L harvested bacteria, after confirmation of induction, was thawed on ice. Cells were resuspended in lysis buffer (500mM NaCl₂, 50mM Na₂H₂PO₄ pH 8.0) containing 1mg/mL lysozyme, and incubated on ice for thirty minutes. The cells were sonicated at high intensity for thirty seconds at least four times, with a 30 second cooling period between each burst, until the lysate was homogenous. The lysates were cleared by centrifugation at least two times at 12,000 rpm for 30 minutes at 4°C. Simultaneously, Ni-NTA resin (Invitrogen), for the purification of 6-His recombinant proteins, was prepared by equilibration in lysis buffer. The supernatant, which contains the soluble recombinant proteins, were incubated with the equilibrated resin for 2 hours with gentle mixing at 4°C. Proteins bound to the resin were washed extensively with lysis buffer, and
the recombinant 6-His proteins bound to the column were eluted by use of lysis buffer containing 250mM imidazole.

**Plasmids**

pHF319 SUMO-Pro3 (Lifesensors Inc, Malvern PA), pHF321 SUMO-MBP, pHF321 SUMO-Plk4, pHF322 SUMO-Plk4KD. GFP-tagged β-globin mammalian constructs were created by a two step process, inserting the gene of interest into pECE-GFP by Infusion, followed by insertion of the β-globin intron downstream of the SV40 promoter. SUMO constructs (pHF321, pHF322, pHF323) were also created by InFusion cloning using Bsal and XbaI restriction sites. The identity of all constructs was verified by sequence analysis.

**Centrosome Isolation from Cultured Cells**

Centrosome isolation was primarily based on previously described methods [233]. HeLa cells were cultured in DMEM supplemented with 10% FBS (Atlanta Biologicals, Atlanta GA), 100U/mL penicillin (Hyclone), and 100µg/mL streptomycin (Hyclone). Cells were cultured at 37°C in a humidified chamber in the presence of 5% CO₂. Cells were incubated with 1µM nocodazole and 1µg/mL cytochalasin D for one hour at 37°C to depolymerize actin and microtubule filaments. Cells were trypsinized, collected, and washed with PBS. Cells were resuspended and incubated with agitation at 4°C in lysis buffer, which is comprised of: 1mM TrisHCl pH 7.4, 0.5mM MgCl₂, 0.4mM DTT, 1X protease inhibitor (Complete tabs Roche), 1X phosphatase inhibitor (Roche-PhosSTOP),
and 0.5% NP-40. Confirmation of lysis can be visualized after approximately 5 minutes. Cell lysates were then centrifuged at 2000g to remove cell debris and nuclei. The supernatant was adjusted with PIPES buffer (50X stock: 500mM PIPES pH 7.2). Dnase was then added to a final concentration of 2U per mL and incubated on ice for 30 minutes. Lysate was then underlayed with 6mL of a 60% sucrose solution (60% sucrose in 10mM PIPES pH 7.2, 0.1% TritonX100, and 0.4mM DTT). The centrosomes were then sedimented into the sucrose cushion by centrifugation at 10,000g for 30 minutes at 4°C. The supernatant was carefully removed, specifically, avoid disturbing the aqueous layer between the 60% sucrose and the supernatant. Gently mix the aqueous layer and the sucrose. This is the crude centrosome preparation. This was then layered on a discontinuous sucrose gradient, consisting of 2.25mL 70% sucrose, 1.35mL 50% sucrose, and 1.35mL 40% sucrose (10mM PIPES pH7.2, 0.1% TritonX100, and 0.4mM DTT) and centrifuged at 120,000g for one hour at 4°C using a Beckman Optima Max ultracentrifuge and a Beckman MLS-50 swinging bucket rotor. Note: It is absolutely critical that your ultracentrifugation tube is at least 90% full to prevent tube collapse in the rotor. After ultracentrifugation, fractions were collected and each fraction checked for the presence of centrosomal components by immunoblotting for verification. Fractions were then stored at -80°C.

**In Vitro Kinase Assays**

GST, GST fusion proteins, and SUMO-His fusion proteins were purified as previously described [107]. Kinase assays were performed as previously described.
using 1µg of recombinant protein, 0.5µg of recombinant kinase. Kinase assay reactions were analyzed by SDS-PAGE, followed by autoradiography of dried gels. Isolated centrosomes (20µg per reaction) used in kinase assays were isolated as previously described [60].

4.4 Results

4.4.1 Plk4 Phosphorylates Mps1 in Vitro

As both Mps1 and Plk4 are centrosomal kinases whose roles in centriole assembly are co-dependent, we sought to determine if they might be substrates of each other. We started by the production of in vitro recombinant Plk4. To this end, as it had been previously established that SUMO-His-Plk4 is active in both auto- and substrate phosphorylation [177][234], we expressed and purified recombinant SUMO-His-Plk4 (Figure 4.1). This induction, purification, and verification by immunoblot utilizing a Plk4 antibody are shown in Figure 4.1. Due to autophosphorylation of the Mps1 kinase, which robustly autophosphorylates (Figure 4.4), it was necessary to produce Mps1KD, and the production of both Mps1 and Mps1KD is a common protocol in the lab [159][87]. Mps1KD is a version of Msp1 with a critical aspartate residue mutated to an alanine, specifically D664A. Recombinant GST-Mps1KD does not show kinase activity (Figure 4.3). We have shown that SUMO-His-Plk4 does not phosphorylate GST (Figure 4.3). However, we found that SUMO-His-Plk4 phosphorylates GST-Mps1 KD in vitro.
4.4.2 Mps1 Phosphorylates Plk4 in Vitro

As Mps1 is an in vitro substrate of Plk4, we sought to determine if the opposite was also true, that Plk4 may be a substrate of Mps1 in vitro. It has been previously established that recombinant GST-Mps1 is active in both auto- and substrate phosphorylation [177][234] (Figure 4.4). Because Plk4 kinase is capable of autophosphorylation (Figure 4.3), it was necessary to use the kinase dead (KD) version Plk4 wherein a critical aspartate residue is mutated to alanine, Plk4 D154A, as a test substrates. Recombinant SUMO-His-Plk4KD was expressed and purified in the same manner as active SUMO-His-Plk4 (Figure 4.1). Recombinant SUMO-Plk4 KD showed no kinase activity (Figure 4.4), though recombinant GST-Mps1 demonstrates robust auto-phosphorylation in vitro (Figure 4.4). As a necessary control, we generated recombinant SUMO-His-MBP in vitro (Figure 4.2).GST-Mps1 does not phosphorylate a SUMO-His-MBP (Maltose Binding Protein) fusion protein. However, we found that GST-Mps1 phosphorylates SUMO-Plk4 KD in vitro (Figure 4.4).

4.4.3 The Many Potential Substrates of Mps1 and Plk4

As we are interested in determining further substrates of Mps1, we completed an in vitro kinase assay utilizing centrosomal preparations of HeLa cells with GST-Mps1 or SUMO-Plk4, to quickly assess the number of potential centrosomal substrates for either kinase. Centrosome preparations were accomplished using HeLa cells by combination of classical protocols based on the enrichment of cellular organelles by density gradient ultracentrifugation [233] (Figure 4.5).
The recombinant proteins Sumo-His-Plk4, GST-Mps1, GST-Mps1Δ12/13, and the centrosomal preparation alone all show autophosphorylation in vitro (Figure 4.6). GST-Mps1KD (Figure 4.3) and SUMO-His-Plk4KD (Figure 4.4) do not show autophosphorylation. Combination of the centrosomal preparation with SUMO-His-Plk4KD or active SUMO-His-Plk4 demonstrates potentially shared substrates with GSTMps1KD, GST-Mps1Δ12/13, and GST-Mps1, at approximately 20kD and 25kD in size, as marked by the asterisks (Figure 4.6). GST-Mps1 and GST-Mps1Δ12/13 also has an unidentified substrate in the centrosomal preparation unique from Plk4 at approximately 30kD in size, as marked by arrows. This potential substrate is absent from GST-Mps1KD. This also appears to be true for SUMO-His-Plk4, as when combined with the centrosomal preparation, there appears to be a unique and unidentified substrate of approximately 32kD in size, as marked by the carat (Figure 4.6). Hence, both Mps1 and Plk4 have unique and unidentified potential substrates, and also appear to share two potential substrates in the centrosomal preparation.

4.5 Discussion

The posttranslational modification, phosphorylation, is complex and prominent in the field of centrosome biology. It is common for phosphorylation events to be responsible for the regulation of cell cycle events, including centrosome duplication and mitotic spindle formation [24][235]. We have shown that the centrosomal kinases Mps1 and Plk4 are in vitro substrates of one another. The fact that Mps1 and Plk4 are mutual substrates may explain the previous result that centrosome reduplication due to Mps1 or
Plk4 overexpression is also mutually dependent on the presence of both kinases. This in vitro kinase assay has shown that the Plk4 phosphorylation of Mps1 is more robust in comparison to the phosphorylation of Plk4 by Mps1. Additionally we have utilized centrosomal preparations from HeLa cells in an in vitro kinase assay with Mps1 and Plk4, identifying several potential substrates of Mps1 and Plk4, some of which may be shared.

It is interesting, but not surprising that Mps1 and Plk4 have potentially shared substrates, as they both localize to the site of procentriole formation early in the process of centriole biogenesis. The observation that both the kinase dead versions of Mps1 and Plk4 can enhance or inhibit phosphorylation of multiple substrates supports that these kinases may have a centrosomal role even without their catalytic activity. Both Mps1 and Plk4 are large proteins, comprised of many domains, including domains for kinase activity, degradation, localization, and of particular interest, for binding to other proteins. Hence, it is possible that Mps1 or Plk4 interact directly or indirectly with centrosomal component(s), inducing conformational changes that allow or inhibit phosphorylation by another centrosomal kinase. It is also possible that this binding occurs with another centrosomal kinase, again inducing a conformational change that allows for auto-activation or inhibits auto-activation of that kinase. Combined, the fact that kinase dead versions do show effects on phosphorylation levels in vitro, strongly supports that other centrosomal factors may be interact with Mps1 or Plk4.

Mps1 is an intriguing multifunctional enzyme with multiple roles in both the centriole and nuclear cell cycles. Here, we have provided evidence that further places
Mps1 in the puzzle that is centriole assembly. Based on the *in vitro* kinase data utilizing purified Mps1, Plk4, and purified centrosomes from HeLa cell lysates, centrosomal substrates of both Plk4 and Mps1 remain to be identified. We are genuinely intrigued at the number of potential substrates and plan to utilize a more systematic approach to identify the centrosomal substrates of Mps1, including the potentially shared Plk4 substrates. This will involve isolation of the *in vitro* phosphorylated bands for identification by mass spectrometry. Additionally, the lab has produced a Biotin-Mps1 construct, which can be immunoprecipitated (IP) from cells to identify potential substrates utilizing antibodies specific to centrosomal proteins, or again could be analyzed by mass spectrometry for identification. Biotin tagged Plk4 and Sas-6 could also be used for the same purpose. This will further characterize the role of Mps1 phosphorylation of these substrates and and help to determine its role and contributions to centriole assembly. In the future, the lab looks forward to determining the exact phosphorylation sites of Mps1 and Plk4 and determining the *in vivo* relevance of these phosphorylation events.
Figure 4.1: Expression and Purification of Plk4.

SUMO-His-Plk4 was induced at 30°C for 4 hours. Samples were collected every hour.
Figure 4.1: Expression and Purification of Plk4 (Continued).

from 0-4 hour time period and analyzed by SDS-PAGE. (B) After the 4 hour induction

the culture was resuspended in lysis buffer and was further subjected to sonication,
centrifugation, and purification, where: Molecular Mass Ladder (M), Flow through (FT),
Unconcentrated (Unconc.), Concentrated (Conc.). (C) Purified Sumo-His-Plk4 and
Sumo-His-Plk4KD were analyzed by immunoblot using an antibody against Plk4.
Figure 4.2: Expression and Purification of SUMO-His-MBP.

SUMO-His-Plk4 was induced at 30°C for 4 hours. Samples were collected every hour from 0-4 hour time period and analyzed by SDS-PAGE. After the 4 hour induction the culture was resuspended in lysis buffer and was further subjected to sonication, centrifugation, and purification, where: Molecular Mass Ladder (M), Flow through (FT), Unconcentrated (Unconc.), Concentrated (Conc.).
Figure 4.3: In Vitro Phosphorylation: Plk4 Phosphorylates Mps1KD.

Kinase assays consisting of purified recombinant GST-Mps1 Kinase Dead (GST-Mps1 KD) and SUMO-6His-Plk4 (S-His-Plk4), or (B) GST and SUMO-6His-Plk4 (S-His-Plk4), were analyzed by SDS-PAGE. Shown are coomassie stained gels and corresponding 1 hour autoradiographic exposures.
Figure 4.4: *In Vitro* Phosphorylation: Mps1 Phosphorylates Plk4KD

Kinase assay consisting of purified recombinant GST-Mps1, and SUMO-6His-Plk4
Kinase Dead (S-His-Plk4 KD) and (B) GST-Mps1 and SUMO-6His-MBP (Myelin Basic
Protein), were analyzed by SDS-PAGE. Shown are coomassie stained gels and
corresponding 1 hr autoradiographic exposures.
Figure 4.5: Centrosomal Preparation

Sucrose gradient fractionation (from 70% to 40%) of nuclei depleted extracts from HeLa cells, where the graph represents the total protein concentrations of each fraction and the immunoblots show the distribution of the indicated centrosomal protein in these fractions (Mps1 and Gamma Tubulin).
Figure 4.6: Mps1 and Plk4 may share potential substrates.

Phosphorylation by in vitro kinase assay, where abbreviations correspond to: CP (Centrosomal Preparation), S-His-Plk4 (SUMO-His-Plk4), and KD (Kinase Dead). Mps1 and Plk4 have two potential shared substrates in the purified centrosomal preparations, marked by asterices. Mps1 has a single unshared substrate as marked by the arrows, and SUMO-His-Plk4 also has an unshared substrate, as marked by the carat.
Chapter 5: The Interplay of Mps1 and Sas-6

5.1 Abstract

The mature centriole is comprised of a cylinder of nine radially arranged triplet microtubules. One of the first steps of the generation of a centriole is the recruitment of the cartwheel that acts as a structural base and determines the 9 fold symmetry of the centriole. The hub of the cartwheel is a single protein, hsSas-6. It is known that Sas-6 can self-arrange into oligomers and is essential for centrosome duplication in human cells. Additionally, Sas-6 overexpression leads to centrosome amplification. By generation of recombinant proteins and \textit{in vitro} kinase assays, we have identified Sas-6 as robust substrate of Mps1. Mass spectrometry did not identify the phosphorylation sites, but did narrow down the potential sites. Generation of Sas-6 truncations has shown that Mps1 phosphorylates both the N and C terminus of Sas-6, but the most robust phosphorylation is in the C terminus. Furthermore, we have generated non-phosphorylatable Sas-6 mutants, and have shown that Mps1 likely has multiple phosphorylation sites within Sas-6. Plk4 also phosphorylates Sas-6, but not to the same degree as Mps1.
5.2 Introduction

The centrosome is a single-copy organelle that serves as the MTOC in mammalian cells, coordinating the assembly of a mitotic spindle that accurately separates the duplicated genome, maintaining genomic integrity. Loss of this genomic integrity can cause aneuploidy, an abnormal number of chromosomes, which is a hallmark of many human cancers and tumors. The centrosome is the main microtubule organizing center of the cell (MTOC). It directs bipolar spindle assembly and contributes to the correct segregation of genetic material.

The centrosome is located within the PCM, where it is comprised of two cylindrical microtubule-based centrioles. The most defining characteristic of centrioles is their specific arrangement, ninefold radial symmetric microtubules. At the G1/S transition in proliferating cells, a new procentriole assembles at the proximal end of each existing centriole. These procentrioles form around a cartwheel structure, that consists of a central hub and nine spokes that emanate from it toward the microtubules. Sas-6 is at the heart of this cartwheel structure, and yields the defining 9 fold arrangement of the entire centriole [185]. Sas-6 is required for cartwheel assembly and is necessary for efficient centriole formation [185].

Ssa6 is comprised of several domains, including a globular N-terminal head, followed by a coiled coil domain, and a C-terminal tail that appears to be unstructured [16][188]. All three of these domains, e.g. the entire Sas-6 protein, is required for procentriole formation in cells, though the coiled coil domain is sufficient for
centrosomal localization [104]. In the process of centriole biogenesis, Sas-6 is one of the earliest proteins recruited and remains at the proximal end of the existing centriole where the cartwheel resides until metaphase, when Sas-6 is degraded in an SCF (Skp1-Cullin-F-box) and APC (anaphase-promoting complex) dependent manner [236][82]. Regulation of Sas-6 degradation is mediated by the KEN box, which is located in the C-terminus. Depletion of Sas-6 in human cells leads to failure of centriole formation, while overexpression leads to centriole amplification [13][82].

The current model for Sas-6 localization at the centrosome is as follows: Sas-6 initially resides in the cytoplasm and exists a mixture of monomers and predominantly homodimers. During G1/S, Sas-6 is recruited to the proximal region of the mother centriole which marks the beginning of cartwheel assembly. This localization may enable concentrations of Sas-6 that are capable of favoring oligomerization, leading to incorporation and assembly of the nine fold symmetrical cartwheel [104]. This model fits with current data, but doesn’t explain how Sas-6 is initially recruited to the centriole or how it is maintained. Based on data in C. elegans, and that dual knockdown of Mps1 and Plk4 leads to a recruitment defect in Sas-6 to the centriole, in conjunction with the peak of Mps1 kinase activity during S phase, we hypothesized that Mps1 and Plk4 may have roles in the organization of Sas-6 at the procentriole, or may aid in favoring oligomerization and maintenance of Sas-6 at the procentriole.
5.3 Materials and Methods

Cells and Cell Culture

RPE1 cells were grown in a 1:1 mixture of DMEM and Ham’s F12 (Hyclone). All media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta GA), 100U/mL penicillin (Hyclone), and 100μg/mL streptomycin (Hyclone). Cells were cultured at 37°C in a humidified chamber in the presence of 5% CO₂.

Plasmids

The following plasmids were used for this study. Previously described plasmids include, pHF286 pECE β-globin GFP and pHF287 pECE β-globin GFP-Mps1, pECE-MBP-Sas-6. Mutations of Sas-6 were generated using QuikChange XL Site directed Mutagenesis System (Agilent Technologies) and included: pECE-MBP-Sas-6-S25A, pECE-MBP-Sas-6-460A, pECE-MBP-Sas-6-T550A S551A, pECE-MBP-Sas-6-S55A T557A, pECE-MBP-Sas-6-T566A S557A, pECE-MBP-Sas-6-T566A S571A, pECE-GFP-Sas-6-S577A T580A S582A, and pECE-MBP-Sas-6-S586A T587A. New plasmid constructs include, pECE β-globin GFP-Sas-6, pHF287 pECE β-globin GFP-Mps1Δ12/13, pHF298 pECE-β-globin GFP-Mps1KD, and pHF313 pECE β-globin GFP-Plk4. GFP-tagged β-globin constructs were created by a two step process, inserting the gene of interest into pECE-GFP, followed by insertion of the β-globin intron between the SV40 promoter and the gene of interest, both by Infusion cloning.
Transfections and siRNA

In RPE1 cells, GFP and GFP-tagged constructs were expressed by transient transfection using Fugene 6 (Promega, Madison WI), and the following siRNAs were transfected at 0.2μM using RNAiMAX (Invitrogen, Carlsbad CA); siMps1 (nucleotides 1360-1384), siPlk4 (3’ UTR 5’-ACTCCTTTCAGACATATAAG-3’ nucleotides) [80], and siGLO LaminA/C (siCon; Dharmaco, Lafayette CO). For experiments involving siRNA and expression constructs, cells were transfected first with siRNA, and transfected 24 h later with various plasmids as indicated, followed by a 24 hour HU treatment (for S-phase arrest) after plasmid transfection. Efficiency of siRNA depletion was determined by quantitative dual color immunoblot using the Odyssey imaging system (LICOR, Lincoln, NE) as previously described [159] using mouse anti-alpha-tubulin (Sigma, St. Louis MO) as a loading control, while efficiency of siRNA depletion was determined by by IIF for Plk4.

Antibodies

Primary antibodies for indirect immunofluorescence (IIF) were as follows: Goat anti-gamma-tubulin, 1:50 (Santa Cruz Biotechnology); Rabbit anti-Cetn2 1:4000 [87]; . Secondary antibodies for IIF were Alexa-488-, 594-, and 750-conjugated donkey anti-rabbit and donkey anti-mouse and donkey anti goat (Invitrogen). DNA was stained with Hoechst 33342 (Sigma).
Indirect Immunofluorescence

For localization of GFP and GFP-Sas-6 constructs, cells were fixed in ice cold -20°C methanol for 10 minutes at -20°C, washed four times with PBS, and processed for IIF as previously described [87] and a GFP antibody utilized. Cells were then washed four times with PBST (0.05% Trition X-100 in PBS), and incubated with primary antibodies overnight at 4°C. Cells were then washed 3 times, and incubated with secondary antibodies for one hour at room temperature before mounting with Citiflour.

Cell Synchronization and BrdU Incorporation Assay

For S-phase synchronization, arresting cells in S phase, RPE1 or U2OS cells were treated with 4mM hydroxyurea (HU), for 24 hours after the final transfection. The beginning of S phase arrest (t=0) was considered to be 24 hours after the addition of HU, and centriole number was assessed 24 hours after the addition of HU (a total of 24 hour S phase arrest).

5.4 Results

5.4.1 Sas-6 is a Robust Substrate of Mps1

Recombinant Maltose binding protein (MBP)-Sas-6 was expressed and purified from bacteria (Figure 5.1). This MBP-Sas-6 was subjected to an in vitro kinase assay with GST-Mps1 (Figure 5.2). GST-Mps1 shows robust autophosphorylation, while hsSas-6 alone shows no phosphorylation. MBP-Sas-6 is a robust in vitro substrate of
GST-Mps1. Recombinant full length MBP-Sas-6 was subjected to mass spectrometry alone and in the presence of GST-Mps1, e.g. Mps1 phosphorylated MBP-Sas-6, to identify phosphorylation sites. Mass spectrometry did not identify the phosphorylation sites, as it covered only 65% of the protein, leaving a total of 22 potential phosphorylation sites including two in the N-terminus (S2 and S25), nine in the coiled-coil domain (T184, S211, T215, S255, T265, Y269, T369, S377, and S460), and 11 in the C-terminus (T550, S551, S555, T557, T566, S571, S577, T580, S582, S586, and S587) as shown in Figure 5.3.

5.4.2 The C-terminus of Sas-6 is Phosphorylated by Mps1 in vitro

Endogenous Sas-6 is a 667 amino acid (AA) protein. In an attempt to limit the number of phosphorylation sites, truncations of Sas-6 were generated using the domains specific to Sas-6. The N-terminus contains two conserved regions, CR1, which is also known as PISA (Present In Sas-6), and CR2. The middle of Sas-6 is comprised of a coiled-coil domain, and the C-terminus is currently thought to be unstructured and no specific domains have been identified in that region (Figure 5.4). Specifically, the N-terminus comprises AA 1-169, the middle AA comprises 167-480, and the C-terminus comprises 400-657 (Figure 5.4). These truncated versions of Sas-6 were subjected to in vitro kinase assay in the same manner as full length Sas-6. None of the individual truncated Sas-6 proteins alone show autophosphorylation, but Mps1 shows robust autophosphorylation (Figure 5.5). Full length MBP-Sas-6 is robustly phosphorylated by GST-Mps1 (Figure 5.5). The N-terminus of Sas-6 does show a small amount of
phosphorylation, while the middle of Sas-6 shows no phosphorylation by GST-Mps1 \textit{in vitro} (Figure 5.5). However, the C-terminus of Sas-6 shows robust phosphorylation by GST-Mps1 \textit{in vitro} (Figure 5.5).

5.4.3 The search for Mps1 phosphorylation sites within the C-terminus of Sas-6

Seven non-phosphorylatable mutant Sas-6 constructs were created by site directed mutagenesis from serine/threonine to alanine covering a total of 13 potential phosphorylation sites including: S25A, S460A, T550A S551A, T555A T557A, T566A S571A, S577A T580A S582A, and S586A T587A. The expression and purification of these constructs is shown in Figure 5.6. Each of these MBP-Sas-6 Alanine Mutants was subjected to \textit{in vitro} kinase assay, and we did not observe a difference between any of these mutants (Figure 5.7). While the evidence is not conclusive, it appears that MBP-Sas-6 is phosphorylated \textit{in vitro} by GST-Mps1 at multiple sites within the C-terminus (Figure 5.7).

5.4.4 MBP-Sas-6 is an \textit{in vitro} Substrate of SUMO-His-Plk4

MBP-Sas-6 was subjected to an \textit{in vitro} kinase assay with SUMO-His Plk4 (Figure 5.8-A). SUMO-His-Plk4 shows robust autophosphorylation, while hsSas-6 alone shows no phosphorylation. MBP-Sas-6 is a weak \textit{in vitro} substrate of SUMO-His-Plk4 (Figure 5.8-B). The N-, Middle- and C-terminal truncations of MBP-Sas-6 were also subjected to \textit{in vitro} kinase assay with SUMO-His-Plk4. While the N-terminus and
middle truncations of MBP-Sas-6 showed no phosphorylation, the C-terminus of MBP-Sas-6 showed mild phosphorylation by SUMO-His-Plk4 (Figure 5.8-A).

5.4.5 Overexpression of Sas-6 does not compensate for Mps1 and Plk4 Dual Depletion

As Mps1 and Plk4 dual depletion shows an inhibition of Sas-6 recruitment to the centriole, and there is obvious cooperation between Mps1 and Plk4 from centrosomal levels, to their ability to cause centriole amplification, and mutual phosphorylation, we next set out to examine if Sas-6 overexpression can compensate for the loss of Mps1, Plk4, or both, and to determine if Mps1, Plk4, or both are required for Sas-6 reduplication. We again utilized a protocol to deplete Mps1, Plk4 or both, followed by overexpression of GFP-Sas-6 in cells arrested in S phase as indicated in Figure 5.9-A.

Control cells, treated with a control siRNA (siCon) and GFP show a normal distribution of centrosome duplication with the majority of cells displaying 4 Cetn2 foci, with a small amount of reduplication at approximately 8%. As previously shown, depletion of either Mps1 or Plk4 alone followed by GFP expression shows that Mps1 and Plk4 cause a defect in the recruitment of Cetn2, with nearly 50% of cells with only 2 Cetn2 foci. Dual depletion aggravates this phenotype, with just over 80% of cells showing defects in Cetn2 recruitment.

Overexpression of Sas-6 in S-phase of cycling RPE1 cells shows centriole reduplication in nearly 40% of cells, indicated by cells with more than 4 Cetn2 foci, while 60% demonstrate a normal centrosome duplication with 4 Cetn2. Depletion of
Mps1 or Plk4 by siRNA, followed by overexpression of Sas-6 shows a minor change in the amount of centriole reduplication, at 40% and 32% respectively. However, Overexpression of Sas-6 does not correct the Cetn2 phenotype of depletion of Mps1 or Plk4, as nearly 30% of cells still show a defect in the recruitment of Cetn2. Hence, overexpression of Sas-6 is unable to compensate for dual depletion of Mps1 and Plk4, as nearly 70% of cells show a defect in Cetn2 recruitment.

This experiment also demonstrated a very unexpected and intriguing observation, that Sas-6 can cause centrosome reduplication even in the absence of both Mps1 and Plk4 in 20% of cells, more than twice the control cells (SiCon, GFP). However, this reduplication is two-fold less than overexpression of Sas-6 in SiCon cells.

5.5 Discussion

This chapter provides further evidence linking Mps1 and Plk4 at the centrosome. Specifically, that dual depletion of Mps1 and Plk4 is not rescued by Sas-6, and that Sas-6 does not require Mps1 or Plk4 for reduplication. This could be indicative of yet another player involved in centriole biogenesis that can bypass the requirement of both Mps1 and Plk4, or that Sas-6, when expressed to a certain threshold may possess the internal ability to form a labile cartwheel structure for centriole assembly, doesn’t require Mps1 or Plk4.

Additionally, we have found that a protein essential to centrosome duplication in human cells as it serves as the base of the cartwheel, Sas-6, is a shared substrate of Mps1 and Plk4, and both phosphorylate Sas-6 within its C-terminus. We have also shown that
Mps1 robustly phosphorylates Sas-6 and is likely to phosphorylate Sas-6 at multiple sites within the C-terminus. It is not surprising given the previous data that Mps1 and Plk4 cross-phosphorylate, require each other for centrosome reduplication, that these kinases would share such an integral protein as Sas-6 as a substrate. This chapter lays the foundation for an entire thesis project, including the identification of phosphorylation sites within Sas-6 by Mps1 and Plk4, and determining the relevance of this phosphorylation in vivo.
Figure 5.1: Expression and Purification of HsSas-6.

MBP-HsSas-6 was induced at 30°C for 4 hours. Samples were collected every hour during the induction, 0-4 hour time period and analyzed by SDS-PAGE. (B) The 4 hour sample was suspended in lysis buffer and subjected to sonication, centrifugation, and purification (with specific details available in the materials and methods). M: Marker; FT: Flow through; Conc: Concentrated; Unconc: Unconcentrated.
Figure 5.2: Mps1 Phosphorylates Sas-6 in vitro.

In vitro kinase assays consisting of purified recombinant GST-Mps1 and MBP-HsSas-6 were analyzed by SDS-PAGE that were Coomassie stained. Red arrows indicate GST-Mps1 and green arrows indicate MBP-Sas-6.
Figure 5.3: Potential Mps1 Phosphorylation sites within Sas-6

Recombinant full length MBP-HsSas-6 was subjected to mass spectrometry alone and in the presence of recombinant GST-Mps1 to identify phosphorylation sites. Approximately 70% of the protein was covered by mass spectrometry (shown in gray) and no sites were identified. However, the regions in shown in white were not covered by mass spectrometry, and thus there are 22 potential phosphorylation sites, serines, threonines, and tyrosines (shown in red). 13 of these sites are within the N or C terminus of MBP-Sas-6. Utilizing site directed mutagenesis, all of these potential sites within the N- and C-terminus have been mutated to alanine for in-vitro kinase assays to determine relevant in-vitro phosphorylation sites.
Figure 5.4: MBP-HsSas-6 Truncations.

Endogenous HsSas-6 is a 657 AA protein. To limit the number of phosphorylation sites, truncations of Sas-6 were completed utilizing the domains specific to Sas-6. Conserved regions within Sas-6 include Conserved Region 1 (CR1) also known as PISA (Present in Sas-6), shown in red, and Conserved Region 2 (CR2), shown in green. Sas-6 also contains a large coiled coiled region, shown in gray. The MBP (Maltose Binding Protein) tag utilized to purify recombinant Sas-6 is shown in blue.
Figure 5.5: The N and C terminus of MBP-Sas-6 contain potential in-vitro phosphorylation sites by GST-Mps1.

Truncations of MBP-HsSas-6 were generated and utilized in a radioactive kinase assay using GST-Mps1 to determine the specific region of phosphorylation. Mps1 readily autophosphorylates. Full length MBP-HsSas-6 is phosphorylated by GST-Mps1. Additionally, the C-terminal fragment of HsSas-6 is heavily phosphorylated and the N-terminal fragment is moderately phosphorylated. The middle fragment remains unphosphorylated. Hence, efforts have been focused on identification of potential phosphorylation sites within the N and C terminus of MBP-HsSas-6.
Figure 5.6: Expression and Purification of Sas-6 Alanine mutations in the N- or C-terminus of Sas-6.
(A-G) MBP-HsSas-6 was induced at 30°C for 4 hours. Samples were collected every hour during the induction, 0-4 hour time period and analyzed by SDS-PAGE. The 4 hour sample was suspended in lysis buffer and subjected to sonication, centrifugation, and purification (with specific details available in the materials and methods). M: Marker; FT: Flow through; Conc: Concentrated; Unconc: Unconcentrated. (A) MBP-Sas-6 S25A (B) MBP-Sas-6 S460A (C) MBP-Sas-6 T550A S551A (D) MBP-Sas-6 T555A T557A © MBP-Sas-6 T566A S571A (F) MBP-Sas-6 S577A T580A S582A (G) MBP-Sas-6 S586A T587A.
Figure 5.7: GST-Mps1 potentially phosphorylates MBP-Sas-6 in vitro at Multiple Sites

The recombinant MBP-Sas-6 alanine mutants (S25A, S460A, T550A S551A, S555A T557A, T566A T571A, S577A T580A S582A, and S586A T587A) were subjected to in vitro kinase assay with recombinant GST-Mps1.
**Figure 5.8: The C terminus of MBP-Sas-6 contains potential *in-vitro* phosphorylation sites for SUMO-His-Plk4.**

Truncations of MBP-HsSas-6 were generated and utilized in a radioactive kinase assay using SUMO-His-Plk4 to determine the specific region of phosphorylation. SUMO-His-Plk4 readily autophosphorylates. Full length MBP-HsSas-6 is phosphorylated by SUMO-His-Plk4. Additionally, the C-terminal fragment of HsSas-6 is weakly phosphorylated by SUMO-His-Plk4. The middle and N-terminal fragments remain unphosphorylated.
Figure 5.9: Centriole Amplification caused by Overexpression of Sas-6 in RPE1 cells does not compensate for dual depletion of Mps1 and Plk4.

(A) RPE1 cells were sequentially transfected with control (siGLO) or Mps1-specific
Figure 5.9: Centriole Amplification caused by Overexpression of Sas-6 in RPE1 cells does not compensate for dual depletion of Mps1 and Plk4 (Continued).

(siMps1) or Plk4-specific (siPlk4) or a combination of Mps1 and Plk4 (siMps1/siPlk4) siRNAs; followed by GFP or GFP-Sas-6 expression constructs, in RPE1 cells arrested in S phase by the addition of HU. (B) Bar graph showing the percentage of GFP positive cells expressing the indicated constructs with more than 4 Cetn2 foci (gray), 4 Cetn2 foci (red), or less than or equal to 2 Cetn2 foci (black). Values represent the mean of 3 independent samples, with at least 100 cells per replicate. (C) Representative images of the experiment outlined in A; GFP (green), Cetn2 (red) and γ-tubulin (pink).
Chapter 6: Conclusions and Future Directions

This thesis provides evidence for the following:

- Mps1 has a role in Centriole biogenesis
- Mps1 Inhibitors, specifically IN-1 and AZ3146, are not equally effective against all Mps1 substrates
- Mps1 Inhibition or Depletion Attenuates Centriole Biogenesis
- Both Mps1 and Plk4 may be dispensable for Centriole Assembly
- Mps1 and Plk4 are mutually dependent for Centriole Reduplication
- The roles of Mps1 and Plk4 in centriole biogenesis are mutually dependent
- Simultaneous knockdown of Msp1 and Plk4 inhibits recruitment of Sas-6
- Mps1 and Plk4 Co-Regulate their centrosomal levels
- Mps1 and Plk4 are Mutual Substrates that May phosphorylate a similar subset of Centrosomal Proteins
- Mps1 and Plk4 phosphorylate Sas-6 within its C-terminus
- Dual depletion of Mps1 and Plk4 is not rescued by Sas-6
- Sas-6 driven re-duplication does not require Mps1 or Plk4

This thesis raises many important questions:

- Mps1 and Plk4 are mutual substrates. What are the phosphorylation sites and are they relevant in-vivo?
- Mps1 and Plk4 share the substrate Sas-6. What are the phosphorylation sites and are they relevant in-vivo?
- What are the shared substrates of Mps1 and Plk4 in the centrosomal preparation?
- How do Mps1 and Plk4 regulate each other?
• What are the non-catalytic roles of Mps1 and Plk4?
• What are the other shared substrates of Mps1 and Plk4?

Centrosomes are single copy organelles composed of a pair of centrioles, surrounded by a pericentriolar matrix. Proliferating cells are born with a single centrosome, which is duplicated once every cell cycle in a tightly controlled and semi-conservative manner. The contributions of the centrosome are essential for multiple cellular processes including microtubule nucleation, spindle assembly, and chromosome segregation. Faithful chromosome segregation is dependent upon proper centrosome duplication, and hence, the duplication process must be tightly controlled, as excess centrosomes generate aberrant spindles that cause chromosome segregation errors, leading to aneuploidy, a common characteristic of human tumors.

In human cells, centrosome duplication is initiated at the G1/S transition when a single procentriole forms orthogonally to the proximal end of each centriole. This process has been well defined in the model system C. elegans, SPD-2 and ZYG-1 are required for the initiation of pro-centriole formation. Sas-5 and Sas-6 are two coiled-coil proteins recruited next to the centrioles and are dependent on SPD-2 and ZYG-1 activity. Sas-5 and Sas-6 are required for the recruitment of Sas-4. Homologues to all of these proteins in humans have been identified including: SPD-2/Cep192, Sas-5/STIL, Sas-6/HsSas-6, Sas-4/CPAP, except for the protein kinase, Zyg-1. The presumptive ortholog is Plk4, but it does not serve all the same prescribed functions as the C. elegans Zyg-1 (Figure 6.1). This suggests that other centrosomal players may be involved. We suggest that one of these key players is Mps1.
Despite suggestions that Mps1 has no role in centriole biogenesis, this thesis not only provides evidence for a role of Mps1 at the centrosome, it suggests that there are actually at least two key regulators of centriole biogenesis and centrosome duplication, the protein kinases Mps1 and Plk4. Why has the centrosomal role of Mps1 been missed by other studies? It is possible that it was difficult to find, requiring a direct analysis of centriole biogenesis and simultaneous manipulation of Mps1 and Plk4. Moreover, the ability of Mps1KD to enhance radiolabeling in isolated centrosomes suggests that Mps1 may also have a non-catalytic role at centrosomes, which may not have been affected by inhibition. Regardless, two observations suggest that centriole biogenesis defect in siMps1 cells are profound. First, defects associated with Cetn2 depletion are completely reversed by the chase [87], but siMps1 cells recover only partially. Second, the ability of siMps1 cells to complete centriole biogenesis during the pulse and recover during the chase are similar to that in siPlk4 cells. Because even co-depleted cells recover significantly during the chase, perhaps human cells have as yet unidentified compensatory mechanisms.

The dependence of centriole biogenesis on both Mps1 and Plk4, the ability of overexpression of one kinase to compensate for depletion of the other, the synergistic effects of co-depletion, co-regulation and cross-phosphorylation, and similarities in substrate profile all strongly suggest the hypothesis that Mps1 and Plk4 cooperate to regulate centriole biogenesis. We find this to be a plausible possibility that may contribute to the perception that Mps1 is dispensable; while siPlk4 perturbs centriole biogenesis more severely than siMps1, it also reflects a
partial loss of Mps1, and while overexpression of Mps1 causes less severe reduplication than Plk4 it also reduces Plk4. While the molecular basis of the cooperation between Mps1 and Plk4 is not clear, the pattern of radiolabeling in isolated centrosomes is consistent with the suggestion that Mps1 and Plk4 regulate a common subset of centrosome proteins. This is further supported by the finding that Mps1 and Plk4 both phosphorylate Sas-6 in vitro.

This study raises many important questions. How do Mps1 and Plk4 regulate each other, what are their non-catalytic roles, and what are their shared substrates? Because both Mps1 [88] and Plk4 [237] are controlled by degradation, perhaps Plk4 prevents Mps1 degradation while Mps1 promotes Plk4 degradation. T436 phosphorylation both stabilizes and activates Mps1 [238], so Plk4 might also activate Mps1 (Figure 6.2). The ability of GFP-Mps1Δ12/13 to escape the negative feedback loop, and of Mps1 and Plk4KD to enhance phosphorylation of the same centrosomal species, are both consistent with this suggestion. Plk4 might also regulate the binding of Mps1 to VDAC3 at centrosomes [59]. Mps1 could control Plk4 through βTrCP, or by activating Plk4 to promote its own degradation [237]. Regardless of the precise mechanisms, our observations that Mps1 and Plk4 cross-regulate each other suggests that care must be taken when interpreting experiments on Mps1 or Plk4.

Mps1 is an intriguing multifunctional enzyme with roles in centriole biogenesis and the spindle checkpoint, but also in DNA damage response [239], the post mitotic checkpoint [240] and meiosis [241]. Fully understanding centriole
biogenesis will require knowledge of all pathway components, so regardless of whether cells can ultimately assemble centrioles without Mps1, we look forward to identifying the exact phosphorylation sites within Mps1 and Plk4, determining their in vivo relevance, and identifying both the Mps1-specific and Plk4-overlapping centrosomal substrates.
Many of the proteins in *C. elegans* have direct homologs in human cells, including SPD-2/Cep192, Sas-5/STIL, Sas-6/hSas-6, and Sas-4/CPAP. The *C. elegans* protein kinase Zyg-1 does not have a direct human homolog, but evidence supports the possibility that a combination of Mps1 and Plk4 may be the functional analogs.
Figure 6.2: Preliminary Model for Potential Feedback Loop between Mps1 and Plk4

Overexpression of Mps1 causes a decrease in Plk4 centrosomal levels. Knockdown of Plk4 causes a decrease in centrosomal Mps1 levels. This is suggestive of a potential feedback loop, if knockdown of Mps1 causes an increase in centrosomal Plk4 levels, and if overexpression of Plk4 causes an increase in centrosomal Mps1 levels, neither of which is known.
References


153


161


