REGULATION OF CENTRIOLE GROWTH BY THE DISTAL TIP COMPLEX

by

John Ryniawec

Copyright © John Ryniawec 2021

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF CELLULAR AND MOLECULAR MEDICINE

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2021
As members of the Dissertation Committee, we certify that we have read the dissertation prepared by: John Ryniawec
titled: Regulation of Centriole Growth by the Distal Tip Complex

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Ghassan Mouneimne
Date: Apr 26, 2021

GREGORY C ROGERS
Date: Apr 26, 2021

GREGORY C ROGERS
Date: Apr 26, 2021

Joyce Schroeder
Date: Apr 26, 2021

Joyce Schroeder
Date: Apr 26, 2021

Paul Krieg
Date: Apr 28, 2021

Final approval and acceptance of this dissertation is contingent upon the candidate’s submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Ghassan Mouneimne
Date: Apr 26, 2021

Ghassan Mouneimne
Date: Apr 26, 2021
ACKNOWLEDGEMENTS

I would like to thank my collaborators in the Rusan Lab at the National Institute of Heart, Lung, and Blood for their contributions to our collaborative research and Dr. Kevin Slep at University of North Carolina – Chapel Hill for his assistance in generating mutants that enabled work in Chapter 3 and 4.

I would like to thank everyone in the Rogers Lab, especially those who have contributed to the work in this dissertation. Thank you to Dr. Daniel Buster for listening to my rambling, incoherent ideas and helping mold them into actual thoughts. Thank you to my fellow graduate students, Dr. Tiffany McLamarrah, Dr. Mengdie Wang, and Dr. Cody Boese who trained and learned with me. Especially thank you to Melanie Zibrat, Spencer Dean, Sophia Aguirre, and Bethany Guice for working with me to advance this research forward. Finally, thank you Dr. Gregory Rogers for giving me the freedom to follow my curiosity and trusting me to pursue new ideas.
# TABLE OF CONTENTS

ABSTRACT..............................................................................................................................................page 8

CHAPTER 1 – The centrosome: a tiny organelle with big responsibilities.................................page 9
  Section 1.1 – A historical perspective of centrosomes.................................................................page 9
  Section 1.2 – Why do we need centrosomes?...........................................................................page 11
    Sub-Section 1.2.1 – Interphase centrosome functions.........................................................page 12
    Sub-Section 1.2.2 – Mitotic centrosome functions............................................................page 15
  Section 1.3 – When good centrosomes go bad.........................................................................page 21
    Sub-Section 1.3.1 – Cellular responses to centrosome defects............................................page 21
    Sub-Section 1.3.2 – Selecting against centrosome instability.............................................page 24
    Sub-Section 1.3.3 – Centrosome instability in disease.........................................................page 26

CHAPTER 2 – Centriole assembly........................................................................................................page 30
  Section 2.1 – Centriole structure...............................................................................................page 30
    Sub-Section 2.1.1 – Centriole ultrastructure .......................................................................page 30
    Sub-Section 2.1.2 – Molecular composition of the centriole.............................................page 33
  Section 2.2 – Centriole duplication.............................................................................................page 37
    Sub-Section 2.2.1 – Stabilizing the pre-procentriole..............................................................page 40
  Section 2.3 – Centriole assembly...............................................................................................page 42
    Sub-Section 2.3.1 – Cartwheel assembly .............................................................................page 43
    Sub-Section 2.3.2 – Distal centriole assembly ......................................................................page 45
  Section 2.4 – How do centrioles grow?.......................................................................................page 50

CHAPTER 3 – Cep104 is a member of the distal tip complex.........................................................page 52
  Section 3.1 – Identifying *Drosophila* Cep104.........................................................................page 53
  Section 3.2 – Cep104 is a bona fide member of the distal tip complex.....................................page 55
  Section 3.3 – Cep104 interacts with Cep97 to target distal tips..............................................page 60
  Section 3.4 – Does Cep104 bind microtubules to regulate centriole length?.........................page 67
Section 3.5 – Modifications and regulation of the distal tip complex..................page 67
Section 3.6 – Materials and methods.................................................................page 68

CHAPTER 4 – Plk4 homodimerization precedes relief of autoinhibition........................................page 72
Section 4.1 – Developing a Plk4 dimerization mutant.............................................page 74
Section 4.2 – Plk4 can activate independent of homodimerization............................page 75
Section 4.3 – Plk4 dimerization is required to bind Asterless....................................page 77
Section 4.4 – Is Plk4 homodimerization required to relieve autoinhibition but dispensable for catalytic activity?..........................................................page 80
Section 4.5 – A revised model of Plk4 activation.....................................................page 81
Section 4.6 – Materials and methods.......................................................................page 82

CHAPTER 5 – Towards a molecular understanding of centriole assembly....................page 87
Section 5.1 – A hypothetical model of centriole growth at the distal tip.......................page 87

APPENDICES.................................................................................................................page 92
Appendix A – Cep104 evolutionary alignment.........................................................page 92
Appendix B – Primer sequences...............................................................................page 94

BIBLIOGRAPHY.............................................................................................................page 96
LIST OF FIGURES

CHAPTER 1
Figure 1.1 – Centrosome functions........................................................................................................page 11

CHAPTER 2
Figure 2.1 – Centrosome architecture....................................................................................................page 31
Figure 2.2 – Molecular composition of the centriole............................................................................page 35
Figure 2.3 – Proximal centriole structure.............................................................................................page 36
Figure 2.4 – Centriole duplication cycle..............................................................................................page 39
Figure 2.5 – Pre-procentriole assembly................................................................................................page 41
Figure 2.6 – Distal tip complex proteins in *Drosophila* cells.................................................................page 49
Figure 2.7 – Model of distal tip complex function................................................................................page 50

CHAPTER 3
Figure 3.1 – Identifying *Drosophila* Cep104.......................................................................................page 54
Figure 3.2 – Developing tools to study the distal tip complex.................................................................page 56
Figure 3.3 – Cep104 localizes to the centriole distal tip..........................................................................page 57
Figure 3.4 – Cep104 is undetectable in S2 cells....................................................................................page 57
Figure 3.5 – Cep104 knockdown results in shorter centrioles...............................................................page 58
Figure 3.6 – Cep104 overexpression causes centriole elongation.........................................................page 59
Figure 3.7 – Understanding Cep104 targeting to the distal tip.............................................................page 61
Figure 3.8 – Cep104 interacts with Cep97 and Cep104.........................................................................page 62
Figure 3.9 – Mapping Cep104 homotypic interactions.......................................................................page 63
Figure 3.10 – Mapping Cep104 interactions with Cep97.................................................................page 65
Figure 3.11 – Distal tip complex interactome........................................................................................page 65
Figure 3.12 – Cep97 is required for proper localization of Cep104.......................................................page 66
Figure 3.13 – Developing microtubule binding TOG mutants............................................................page 66
Figure 3.14 – Klp10A is phosphorylated by Plk4 *in vitro*....................................................................page 68
CHAPTER 4

Figure 4.1 – Developing a Plk4 dimerization mutant....................................................page 73
Figure 4.2 – Plk4 dimerization mutants have reduced activity........................................page 75
Figure 4.3 – A new mechanism of Plk4 activation.........................................................page 78
Figure 4.4 – Asterless does not regulate Plk4 dimerization mutant...............................page 80
Figure 4.5 – Plk4 must dimerize to bind Asterless.........................................................page 81
Figure 4.6 – Developing a phospho-T loop antibody......................................................page 83

CHAPTER 5

Figure 5.1 – Model of Cep104’s role in the distal tip complex.......................................page 90
ABSTRACT

The centrosome is a tiny organelle that builds and coordinates complex microtubule machines in cells. Centrioles, the core components of each centrosome, are barrel-shaped structures with intricate architecture. Establishing and maintaining proper centriole structure is critical for proper centrosome function; however, deciphering mechanisms underlying centriole assembly remains a major hurdle in the field of centrosome biology. To address this gap, I sought to elucidate mechanisms by which the centriole grows. Herein, I focus on a key question in the field: How do the proteins that reside at the distal tip of the centriole coordinate processive growth during specific stages of centriole biogenesis? First, I identify a novel component of the conserved distal tip complex, Cep104, that promotes centriole growth. Second, I decipher a mechanism regulating activity of the key centriolar kinase Polo-like Kinase 4 and provide a potential role for the kinase in regulating the distal tip complex. By combining these findings with a detailed distal tip interactome, I develop a hypothetical model of centriole assembly integrating our genetic understanding of centriole growth with my novel molecular mechanisms.
Section 1.1 – A historical perspective of centrosomes

Since the advent of cell theory by Schleiden and Schwann nearly 200 years ago (1838-1839), the ideas of self-replicating biological units and, thus, the basic requirement for cell division have captivated scientists. In 1887, advances in cytology allowed for the discovery of karyokineti
division – equal segregation of genomic material into two daughter cells – in mitotic nematode embryos by Theodor Boveri and in meiotic worm eggs by Edouardo Van Beneden (called pseudo-
karyokinesis due to its reductive nature)\(^1\). Independently, but simultaneously, they discovered that condensed chromosomes are aligned between a bipolar filamentous array and, subsequently, divided into 2 daughter cells. In his original study, Boveri discussed the organization of this astral array, defining the centrosome as the center of each pole. His findings led him to believe that the centrosome is “the true division organ of the cell, it mediates the nuclear and cellular division\(^1\).” Over the next 30 years, Boveri made several seminal discoveries regarding the centrosome and became the father of an enduring field of study that would expand well beyond cell division\(^3\).

Although the centrosome has many functions, most historical research focuses on the role of the centrosome during cell division. In 1890, David Hansemann observed cancer cells undergoing multipolar mitosis, as opposed to the normal bipolar mitosis\(^4,\;5\). This discovery led Boveri to hypothesize that supernumerary centrosomes during mitosis (cells with more than two centrosomes) would generate multipolar mitosis\(^3,\;5\). Boveri observed just that when performing classical dispermy experiments in which sea urchin eggs are fertilized with two sperm. Sea urchin eggs lack centrosomes and the sperm donates its centrosome to the embryo. When an egg is fertilized with two sperm, the resultant embryo contains double the number of centrosomes. Embryos with too many centrosomes underwent multipolar mitosis accompanied by unequal segregation of chromosomes\(^3,\;5-7\). Together with his observations that resultant embryos had “malfunctions”, Boveri postulated in 1914 that supernumerary centrosome-induced multipolar mitosis has the potential to generate aneuploidy and, potentially, cancer\(^3,\;5,\;8\). Since then, generations of scientists have studied the reliance on proper centrosome number and function during normal development and in preventing cellular transformation.

Centrosome number is governed by the centriole, the duplicating element at the core of the centrosome\(^9\). The centriole was first described in 1900 by none other than Boveri\(^3,\;10\). He reported
a prominent, densely stained granule at the center of the centrosome in 1895, but it was not until 5 years later that he described its duplicating nature and named it the centriole. When observing sea urchin embryos, Boveri saw separation of centrosomes during the 2-cell stage, prior to a second cleavage event. Since he knew only one centrosome had been donated to each cell by the sperm, he concluded that the centrosome needed to duplicate prior to the second mitosis. Modern studies have corroborated his interpretation, centrioles duplicate once per cell cycle ensuring that cells have two centrosomes as they enter mitosis. Each centrosome assembles one pole of the bipolar mitotic spindle. The need for duplication was obvious to Boveri, but not to others of his time. Without duplication of the genome or centrosomes, developing organisms would run out of materials essential for the execution of cellular divisions before they fully matured. His conviction to this idea carved his place as the father of the centrosome field, instead of his contemporaries. Though the cyclical nature of centriole duplication was first described in 1900, it was not until the modern age of genetic manipulation that we truly began to understand the mechanisms that underly this duplication cycle.

The centriole itself is a critical cellular organelle. Not only does it control the number of centrosomes, but centriole structure is critical for centrosome function and the assembly of other microtubule-based organelles like cilia and flagella. The first transmission electron micrograph of the centriole was published in 1954, when Fawcett and Porter observed the basal bodies of cilia in multi-ciliated cells (although they did not identify these structures as centrioles, but rather as “basal corpuscles”). Therefore, the first centriole micrographs are commonly attributed to de Harven and Bernhard in 1956, when the authors characterized centrioles in newts, chicken, mice, rats, and human cancer tissues. The study describes the conserved barrel shape of the centriole and establishes that the centriole is composed of microtubule bundles arranged in ninefold radial symmetry. As electron microscopy techniques improved, so did our understanding of the diversity of centriole structures. While most mammalian centrioles are made of triplet microtubule bundles, organisms like C. elegans and Drosophila have centrioles with singlet and doublet microtubules, respectively. Furthermore, surveys of different Drosophila tissues revealed diverse centriole architecture within an individual. Some tissues have long centrioles composed of microtubule triplets, while others have short centrioles made of microtubule doublets. It is this tissue-specific structural diversity within an individual that I believe highlights the complex nature of centriole assembly. This diversity shows that centrioles are not constructed from a singular
blueprint. Instead, the molecular mechanisms underlying centriole architecture must be plastic, responding to contextual cues to build the correct centriole for the cell.

Section 1.2 – Why do we need centrosomes?

Nearly 140 years since the centrosome was named, we continue to discover centrosome functions and find new ways that centrosomes contribute to development and homeostasis. No matter how many functions we find, one thing remains true: the core role of the centrosome is to construct specialized microtubule assemblies (Figure 1.1). The centrosome is the major microtubule organizing center in most animal cells\textsuperscript{17}. Microtubules nucleate from a proteinaceous cloud at the centrosome called the pericentriolar material. The meshwork pericentriolar material is studded with \(\gamma\)-tubulin ring complexes, which act as platforms for \(\alpha-/\beta\)-tubulin heterodimer recruitment, facilitating microtubule polymerization\textsuperscript{18}. Thus, centrosomes act as a microtubule hub

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{centrosome_diagram.png}
\caption{The centrosome is composed of two centrioles surrounded by a cloud of proteins called the pericentriolar material. Centrosomes organize microtubule-based machines by nucleating microtubules within the pericentriolar material. Growing microtubule ends (plus ends) point away from the centrosome, establishing inherent polarity. During mitosis, one centrosome organizes each of the two poles of the mitotic spindle to facilitate efficient cell division and accurate chromosome segregation. Model courtesy of Gregory Rogers.}
\end{figure}
to facilitate mitotic spindle assembly, generate specialized microtubule networks, and establish polarity in a cell\textsuperscript{17}. Additionally, centrioles act as basal bodies for cilia and flagella. In specialized cells, the centriole will dock at the plasma membrane and grow a microtubule projection, the axoneme, that acts as the central structure of cilia and flagella. These specialized organelles generate cellular motility, extracellular flow, and sense the external environment\textsuperscript{19, 20}. Below, I present an overview of centrosome functions, focusing primarily on its role as a microtubule organizing center.

**Sub-Section 1.2.1 – Interphase centrosome functions**

Although the most enigmatic function of the centrosome is construction of the bipolar mitotic spindle, the centrosome’s roles in interphase have become exceedingly evident. In a stereotypical epithelial cell, a single centrosomal microtubule organizing center is anchored near the nuclear envelope. This orients microtubules so that their growing plus ends point away from the centrosome and towards the cell’s periphery. The inherent polarity provided by this function is used by cells to generate and maintain polarity, organize other cellular organelles, and traffic cargo towards or away from the nucleus. Other cell types, such as neurons, heavily rely on the centrosome to generate specialized microtubule arrays to direct the morphology of the cell\textsuperscript{17}. Not surprisingly, Boveri hypothesized in 1887 that the centrosome is an enduring and permanent cellular organelle, not just a transient structure during mitosis\textsuperscript{1, 3}. The variety of centrosome functions certainly validates his theory.

The polarized microtubule array created by the centrosome allows for the directional movement of molecular motors throughout the cell\textsuperscript{17, 21}. Kinesin motors are generally plus end directed motors and, thus, move away from the centrosome and towards the cell’s periphery along the microtubule array\textsuperscript{21}. As kinesins transit microtubules, they are able to bind cellular cargo receptors (such as Gadkin:AP-1 or SKIP:Arl8) and associated cargo (such as endosomal vesicles or lysosomes, respectively)\textsuperscript{21-23}. Cargo-bound kinesins can continue to walk away from the nucleus and interact with trafficking machinery or other organelles. As such, kinesin-mediated transport plays a role in anterograde transport of materials and organellogenesis\textsuperscript{21, 24}. Conversely, dynein motors move toward the minus end of microtubules and participate in retrograde transport of materials. Since the minus ends are anchored at the centrosome, dynein motors can traffic cargo
towards the nucleus\textsuperscript{21}. By establishing microtubule polarity, the centrosome provides a roadmap that allows for the proper movement of cargo throughout the cell.

One of the most striking examples of this phenomenon is the trafficking of melanosomes—specialized vesicles containing the pigment protein melanin\textsuperscript{21, 25}. Studies in Xenopus and zebrafish showed that upon stimulation with hormones that promote cAMP production, perinuclear melanosomes move towards the periphery of a melanophore cell\textsuperscript{26, 27}. Conversely, reducing cAMP levels results in retrograde movement of melanosomes towards the centrosome\textsuperscript{27}. Subsequent \textit{in vivo} and \textit{in vitro} studies confirmed that changing the balance of motor activity promoted the directional transit along centrosome-derived microtubule arrays\textsuperscript{28, 29}. The trafficking of melanosomes allow pigment producing cells to protect skin stem cells from UV damage\textsuperscript{25}.

The polarity provided by the interphase microtubule array also assists in building the trans-Golgi network\textsuperscript{24, 30-32}. During mitosis, the Golgi vesiculates and must rebuild at the start of the next cell cycle. Although the Golgi can independently form cisternae, centrosomal microtubules are required to stack the cisternae into ribbons stereotypical of the Golgi apparatus. Cross-linking of Golgi-derived microtubules with centrosomal microtubules assists in the membrane fusion necessary to organize the Golgi ribbons into stacks\textsuperscript{30, 33}. Furthermore, these interactions establish a directional nucleus-centrosome-Golgi axis that creates higher order cell polarity. This axis points toward the leading edge of migrating cells and interferes with directionality when disrupted\textsuperscript{24, 32-34}. Additionally, this axis points towards lumens in tissues and cultured organoids, allowing cells to functionally use the established apico-basal polarity\textsuperscript{31, 35}. Meaning, without the ability to establish the nucleus-centrosome-Golgi axis, cells have reduced ability to traffic and secrete molecules\textsuperscript{33}. Additionally, without the ability to nucleate perinuclear microtubules, 3D organoid cultures struggle to form lumens\textsuperscript{35}.

Specialized cell types further co-opt centrosome derived polarity cues to direct traffic\textsuperscript{31}. For example, upon interacting with presented antigen, lymphocytes must quickly polarize toward the developing immunological synapse—the site where lymphocyte and antigen presenting cell make contact\textsuperscript{36}. To repolarize, dynein anchored at the immunological synapse grabs centrosomal microtubules and generates force that pulls them towards this site, thus pulling the centrosome\textsuperscript{37, 38}. The newly generated cytoskeletal hub helps to strengthen the synapse and ensures the directed effector functions of the lymphocyte. T-cells unable to polarize their centrosome to the immunological synapse cannot sustain downstream signaling and, as a result, are unable to
continue cytokine production\textsuperscript{37}. T cells unable to repolarize cannot perform their effector functions, such as cytolytic killing or B cell co-stimulation\textsuperscript{39, 40}.

Developing neurons, another specialized cell type, use centrosome-derived microtubules to direct axon extension\textsuperscript{31, 41}. Dissociated neurons cultured \textit{in vitro} project multiple neurites before selecting one to elongate and become an axon\textsuperscript{42}. Early studies showed that the centrosome and Golgi polarize towards the selected axon prior to extension\textsuperscript{43}. Additionally, centrosome-derived microtubules are essential for the elongation process\textsuperscript{44, 45}. Although it cannot be ruled out that the centrosome is merely responding to polarity cues, some have suggested that the positioning of the centrosome selects the axon. Further, centrosome polarization is associated with neuron migration in the developing cortex\textsuperscript{41}. Although, the necessity for the centrosome in this process cannot be experimentally disassociated from its role in neuron delamination during mitosis (discussed in \textbf{Sub-Section 1.2.2, page 15}).

Finally, the centriole plays another role in interphase cells outside of organizing the centrosome by acting as the basal body for cilia and flagella. Cilia and flagella are organelles that protrude from the main cell body and are composed of a long microtubule axoneme sheathed in plasma membrane\textsuperscript{19, 20}. Flagella are commonly used to propel cells, such as sperm. Cilia, on the other hand, come in two varieties: motile and non-motile. In \textit{Tetrahymena}, motile cilia line the outside of the cell and coordinate to promote movement. In mammals, motile cilia are commonly seen on multi-ciliated cells and are known to generate fluid flow outside of the cell, like moving mucus in airways\textsuperscript{20}.

Primary cilia, on the other hand, are non-motile cilia that act like antennas for the cell. They receive signals from extracellular cues, both chemical and mechanical (such as extracellular flow) and stimulate cellular responses\textsuperscript{46}. The most well-known signaling axis coordinated by primary cilia is Hedgehog signaling, which interprets developmental cues to trigger differentiation or entry into the cell cycle\textsuperscript{46-48}. Without a signaling molecule, the receptor Patched (Ptc) resides at the base of the primary cilium, preventing access of the G-protein-coupled receptor Smoothened (Smo)\textsuperscript{49, 50}. When a signaling molecule in the Hedgehog family (Hh) binds to Ptc, it translocates out of the cilium and into the plasma membrane. Smo can now enter the cilium and free Gli transcription factors to activate downstream pathways\textsuperscript{51-53}. Depending on the context, Hedgehog signaling can promote cell survival, differentiation, or proliferation. Proper regulation of this signaling axis is essential for organogenesis and neural patterning\textsuperscript{46}. Mutations in cilia proteins,
both those that localize to the basal body and along the axoneme, are known to cause polycystic kidney disease, polydactyly, and Left-Right patterning defects\textsuperscript{54}.

In cells with a single cilium, the oldest (mother) centriole docks to the plasma membrane to initiate ciliogenesis\textsuperscript{19}. From there, specialized vesicles are recruited to a structure on the centriole, called the distal appendages (see Figure 2.1, page 31), which promote remodeling of the plasma membrane to accommodate the morphological changes necessary for cilium extension\textsuperscript{55, 56}. The centriole microtubules will then begin to grow, pushing the membrane outwards and filling the center of the cilium. Most of this growth is facilitated by cilia-specific proteins allowing the centriole to retain its identity and, when the cilium eventually disassembles, the centriole is retained in the cell\textsuperscript{57}. The centriole plays a similar role in multi-ciliated cells as well, although these cells have specialized centriole assembly pathways that allow for rapid generation of hundreds of new centrioles to become basal bodies for each individual cilium\textsuperscript{58}. Since centrioles nucleate cilia, loss of centrioles causes ciliopathies and associated phenotypes\textsuperscript{54}.

While the centrosome is best known for its mitotic roles, the interphase centrosome plays diverse roles, especially in specialized cell types\textsuperscript{17}. The multitude of roles for centrosomes seem to rely on strict adherence to the proper structure of the centriole and centrosome. As such, centrosome-associated mutations commonly alter interphase centrosomes. Additionally, many of the cell types affected by these mutations have adapted the centriole to perform specialized functions. Therefore, identifying the etiology and pathogenesis of these diseases may reveal cell type specific requirements for centriole structures\textsuperscript{59}.

Sub-Section 1.2.2 Mitotic centrosome functions

While there are many interphase roles for the centrosome, the centrosome’s roles in mitosis are best characterized. During mitosis, the centrosome organizes the mitotic spindle, a microtubule machine that directs chromosome congression and subsequent segregation. The polarized microtubule array of the spindle allows for molecular motors to act upon the machine, underlying its function. This symphony of activity is conducted by the centrosome, without which shape, size, timing, and directionality of the mitotic spindle all suffer\textsuperscript{17}.

The normal mitotic spindle is a bipolar array which traps and directs the segregation of chromosomes into two daughter cells. At the center of each pole sits one centrosome. Therefore, it is essential that a cell has precisely two centrosomes as it enters mitosis\textsuperscript{60}. If cells have too many
or too few centrosomes, the number of spindle poles can change, compromising the fidelity of cell division\textsuperscript{61}. Proper duplication of centrioles underlies centrosome number and will be discussed in Section 2.2. Instead, this section focuses on how mitotic changes to the centrosome direct the assembly and function of the mitotic spindle.

Prior to entering mitosis, the two centrosomes reside near one another, creating a single microtubule organizing center during interphase. However, centrosomes must move away from each other towards opposite ends of the cell to generate a bipolar spindle during mitosis\textsuperscript{60}. This feat is mainly accomplished by the kinesin-5 motor Eg5\textsuperscript{62}. Eg5 is a homotetrameric motor in which 2 motor domains stick out from either side of a central rod, like a dumbbell\textsuperscript{63}. Since each centrosome nucleates microtubules with their plus-ends pointing outward, Eg5 tetramers will bind the closely spaced, antiparallel microtubule tracks between them\textsuperscript{64}. The two motor domain pairs, each bound to a microtubule emanating from a different centrosome, will processively walk towards the plus end of their respective microtubule. Since the tetramer is anchored together and cannot move in opposite directions at once, the force generated will instead be transduced to the microtubules, causing them to slide past one another\textsuperscript{63}. The sliding then creates pushing forces on the centrosome, forcing them away from each other\textsuperscript{64}. In cultured cells, centrosomes will stochastically space themselves far enough from each other to facilitate bipolarity\textsuperscript{65, 66}. When cells are treated with the Eg5 inhibitor, Monastrol, cells form a monopolar spindle, since the centrosomes are unable to separate\textsuperscript{67}.

In preparation for mitosis, centrosomes must also increase their microtubule nucleation capacity by expanding the size of their pericentriolar material, a process called centrosome maturation\textsuperscript{17}. The expanding pericentriolar material is built around an existing scaffold composed of the pericentriolar material components Pericentrin-like Protein (Plp; Pericentrin in humans), Spd-2 (Cep192 in humans), and Centrosomin (Cnn; CDK5RAP2 in humans; Spd-5 in \textit{C. elegans})\textsuperscript{18, 68}. Activation of the mitotic kinase Polo (Polo-like kinase 1 in humans) at the centrosome is primarily responsible for centrosome maturation in mitosis\textsuperscript{69}. Polo is recruited to the centrosome by Spd-2 and phosphorylates Cnn, creating a high affinity platform for the recruitment of additional Cnn\textsuperscript{69, 70}. Newly recruited Cnn is phosphorylated by Polo, generating a feed forward loop that results in the rapid expansion of the pericentriolar material\textsuperscript{69, 71}.

The increased pericentriolar material generates more binding sites for the microtubule nucleation machinery like XMAP215, TPX-2, and NEDD1 which can directly stabilize
microtubules and recruit γ-tubulin ring complexes\textsuperscript{72, 73}. Together, this dramatically increases the microtubule nucleating capacity of the centrosome during mitosis\textsuperscript{74}. Although not absolutely required for mitosis, microtubule nucleation from the centrosome ensures the efficient production of the bipolar mitotic spindle and the fidelity of chromosome segregation\textsuperscript{75, 76}.

The high microtubule density in the mitotic spindle, partially attributed to centrosome maturation, is used to generate the forces that power mitosis. Microtubules attach to chromosomal kinetochores to align chromosomes between the two centrosomes during metaphase. Cells rely on the inherent dynamic instability of microtubules, along with assistance from molecular motors, to push and pull the chromosomes into alignment. After alignment, the kinetochore microtubules also facilitate the force production necessary to generate isometric tension at the chromosomes\textsuperscript{60}. Sufficient tension on the kinetochore of each sister chromatid is necessary to satisfy the spindle assembly checkpoint and transit into anaphase\textsuperscript{77}. Thus, both the increased microtubule nucleation capacity and the bipolarity provided by the centrosome facilitate efficient chromosome segregation.

Additionally, the centrosome organizes the minus-ends of spindle microtubules, focusing the spindle poles and promoting proper microtubule flux\textsuperscript{60}. Most microtubules in the mitotic spindle are not directly anchored to the centrosome but are released from the centrosome or nucleated through different mechanisms (for example, the augmin/HAUS branching nucleation pathway)\textsuperscript{78}. Instead, microtubule associated proteins and molecular motors bridge spindle microtubules to centrosomal microtubules\textsuperscript{60, 79}. As a result, the spindle tapers towards the poles. Microtubule minus end associated proteins such as patronin (CAMSAP in humans), mud (NuMA in humans), and Asp (ASPM in humans) concentrate near the centrosome and establish parallel microtubule arrays between centrosomal and spindle microtubule populations. Minus end directed kinesin-14s (ncd in \textit{Drosophila}; HSET in humans) and dynein motor complexes then focus the microtubules to a tapered pole at the centrosome\textsuperscript{60, 62, 80-82}.

The centrosome also concentrates factors essential for poleward microtubule flux. Even after binding properly to a kinetochore, microtubules in the spindle are not static. Instead, both ends of the microtubules remain dynamic\textsuperscript{60}. At their kinetochore-attached plus ends, microtubules undergo assembly and disassembly, a process known as “Pac-man” flux. However, much of the disassembly occurs from the minus end, termed poleward flux. Poleward flux results in new tubulin subunits being added to the kinetochore-attached end and older subunits being lost from
the minus end while maintaining constant microtubule length, akin to actin treadmilling. The disassembly is mostly driven by kinesin-13 microtubule depolymerases: in *Drosophila* ‘Pac-man’ flux is primarily driven by Klp59C whereas poleward flux is driven by Klp10A (Kif2A in humans). While eliminating poleward flux increases spindle length, it is still unclear as to the exact role of microtubule flux in metaphase. Likely, flux prevents poleward ejection of chromosomes during error correction of improperly attached kinetochore microtubules. During error correction, microtubules are depolymerized on one side of the chromosome pair. If the attached microtubules continued to push unfettered, the chromosome would be forced away from the metaphase plate and towards the unattached side of the cell. However, if the pushing forces were tempered by microtubule flux, this effect may be decreased. Additionally, although disassembly of microtubules drives the movement of chromosomes towards each centrosome during anaphase A, flux rates are not proportional to the rate of chromosome movement suggesting this is not the primary role. By concentrating kinesin-13s, microtubule severing enzymes like katanin, and microtubule stabilizing proteins like patronin/CAMSAPs, the centrosome both tunes flux rates and promotes efficient anaphase.

Finally, centrosomes disassemble at the end of mitosis to reestablish proper microtubule nucleation capacity and cellular organization. As cells enter mitosis, kinases become activated to facilitate centrosome maturation. However, as cells exit mitosis, protein phosphatases dominate centrosome disassembly to reestablish the interphase centrosome. The protein phosphatase PP2A dephosphorylates an unknown target at the centrosome in telophase, likely Spd-2/Cep192 or Cnn/CDK5RAP2/Spd-5, to weaken the structural integrity of the pericentriolar material. Pulling forces on centrosome microtubules then pull the excess pericentriolar material proteins away from the centrosome. This leaves the centrosome with the core scaffold of pericentriolar material proteins that is maintained through interphase and will be required for centrosome maturation in the subsequent mitosis. Although excessive centrosome activity due to centrosome amplification is common in many cancers, it is yet unclear as to the effect of maintaining active centrosomes during interphase.

Along with coordinating spindle assembly, centrosomes also establish the directionality of cell division. To generate and maintain tissues, cells take cues from their microenvironment to inform when and where they must divide. As such, cells do not divide in random directions. Instead, the mitotic spindle is oriented along a specific axis to facilitate tissue growth and decide
cell fate\textsuperscript{89, 90}. For example, cells within a planar epithelium must divide symmetrically along that plane or they will be expelled from the tissue, resulting in smaller tissues and organs\textsuperscript{90}. Centrosomes are required to reliably orient the mitotic spindle along the designated axis\textsuperscript{89}.

Centrosomes do not only nucleate microtubules towards the chromosomes during mitosis; they also nucleate microtubules towards the cell periphery called astral microtubules (Fig. 1.1, page 11). The minus ends of these astral microtubules are anchored at the centrosome while their plus ends project into the cell cortex\textsuperscript{60}. Because of this, astral microtubules can be captured by microtubule binding proteins and molecular motors in the cortex. These captured astral microtubules can then polarize the spindle\textsuperscript{89, 90}. Several biochemical and physical mechanisms can, for instance, concentrate dynein complexes at the cell cortex along the division plane. There, dynein can pull astral microtubules towards the area of highly concentrated motors and orient the mitotic spindle along the division axis. In the previous example of a symmetrically dividing planar epithelium, dynein localizes to cell-cell junction signaling proteins\textsuperscript{91}.

Spindle orientation can also influence cell fate decisions by promoting the asymmetric distribution of cell fate determinants. Asymmetric cell division, that is a cell division giving rise to two daughter cells of different cell types, is a common mechanism to generate stratified epithelia and specialized cell types like neurons. Like symmetric divisions, capture and/or pulling of astral microtubules by proteins embedded in the cell cortex can orient the mitotic spindle\textsuperscript{92}. However, the signals used to concentrate dynein generally rely on polarization cues like apical-basal or anterior-posterior signals to divide along those polarized axes\textsuperscript{90}.

A classical example of asymmetric division occurs in the \textit{Drosophila} neuroblast, a neural stem cell that delaminates from the neuroectoderm during larval brain development. The neuroblast is a polarized cell type whose apical surface sits adjacent to the neuroectoderm layer\textsuperscript{93}. The apical surface of the cell is maintained by asymmetric localization of the Par complex: Bazooka (Baz; Par3 in humans), Par6, and atypical Protein Kinase C (aPKC)\textsuperscript{94, 95}. Opposite that, the cell fate determinant factors Numb, Prospero, and Brat localize to the basal side of the cell\textsuperscript{96}. If the neuroblast divides parallel to the neuroectoderm (symmetrically), these fate determinants will be equally segregated between them, giving rise to two new neuroblasts. However, if the neuroblast divides perpendicular to the neuroectoderm it will give rise to one neuroblast and one mother ganglion cell, which will eventually become a neuron. This is because perpendicular division results in deposition of cell fate determinants into the mother ganglion cell resulting in
cellular differentiation while the apical surface of the neuroblast is maintained adjacent to the neuroectoderm. A neuroblast will asymmetrically divide multiple times, depositing multiple mother ganglion cells on the same side of the precursor cell.

Centrosomes are essential for efficient neuroblast differentiation. Apically localized microtubule-binding proteins capture astral microtubules from one centrosome to orient the mitotic spindle. Baz, a member of the apical Par complex, recruits an adaptor protein, Inscurable, which recruits the microtubule binding Gai/Pins/Mud complex. Mud (NuMA in humans) can then capture astral microtubules to anchor the centrosome on the apical side of the neuroblast. However, that still does not explain why only one centrosome is captured on the apical side, while the basal centrosome localizes to the opposite side of the spindle.

Centrosomes do not nucleate microtubules during interphase in most Drosophila cells. The neuroblast, however, is a unique cell type in Drosophila because it retains a centrosomal microtubule organizing center during interphase. After mitosis, the daughter centrosome does not disassemble its pericentriolar material, but retains active Polo, and continues to nucleate microtubules. Thus, the younger centrosome is retained on the apical side of the neuroblast while the older centrosome, which loses its microtubule nucleation capacity, freely migrates to the basal side of the cell. During the subsequent mitosis, both centrosomes become active to generate the mitotic spindle and the oldest centrosome is deposited into the mother ganglion cell. The two centrioles comprising the apical centrosome then separate, giving rise to two centrosomes: a younger centrosome, which retains microtubule nucleation capacity, and an older centrosome, which loses its microtubule nucleation capacity. In collaborative work with the Basto lab, we discovered the first mechanism for inactivation of the older centrosome. We found that the key centriole duplication kinase, Polo-like Kinase 4 (Plk4), phosphorylates Spd-2 to remove pericentriolar material and inactivate the older centrosome. Without the ability to phosphorylate Spd-2, both centrosomes remain active, neuroblasts cannot establish proper spindle orientation, and fly brains do not develop properly.

Although we may simplify the centrosome’s mitotic role to creating a bipolar spindle, the centrosome orchestrates most aspects of mitosis. Because of this breadth of function, it is no surprise that the centrosome is essential for mitosis in most normal cells. However, a major gap in our knowledge is understanding how centriole and centrosome structure contributes to each of these roles, as well.
Section 1.3 – When good centrosomes go bad

Most genetic diseases caused by mutations in centosome genes affect specialized functions in unique cell types, like neural stem cells. Because the centosome is essential for efficient mitosis and development, mutations that interfere with general centosome functions are lethal. In this section, I discuss mechanisms that cells use to cope with centosome defects, how cells respond to these changes, and disease phenotypes associated with centrioles and centrosomes.

Sub-Section 1.3.1 – Cellular responses to centrosome defects

Genomic integrity is vulnerable during mitosis. Chromosomes must be equally divided into two daughter cells to avoid potentially lethal or, conversely, oncogenic errors. Dramatic centrosome disfunction, particularly changes to centrosome number, place cells at risk of chromosome mis-segregation. As such, cells have developed extensive mechanisms to detect and respond to the mitotic errors caused by centrosome disfunction. These mechanisms police the fidelity of mitosis to eliminate cells that have experienced, or may have experienced, genotoxic damage.

The spindle assembly checkpoint is likely the best-known mechanism of mitotic surveillance. The spindle assembly checkpoint prevents cells from transiting the metaphase-anaphase transition until all chromosomes are aligned at the metaphase plate by inhibiting the anaphase-promoting complex. The anaphase-promoting complex (also called the cyclosome) is a large E3 ubiquitin ligase that triggers mitotic progression by targeting cyclin B1 for proteolysis and enables sister chromatid separation by targeting securin to liberate the key protease separase. Separase is then able to cleave the cohesin complexes that prevent chromosome segregation by tethering sister chromatids together. Unattached kinetochores trigger the spindle assembly checkpoint by activating Mad2 to bind and sequester Cdc20, an anaphase-promoting complex activator. Once all chromosome pairs are attached to both sides of the bipolar mitotic spindle and the kinetochores are under proper tension, Cdc20 is freed to activate the anaphase-promoting complex. Normally, the spindle assembly checkpoint protects genomic integrity by providing cells with sufficient time to correct improperly attached kinetochores before completing mitosis.

Centrosomes are paramount to efficiently generating a bipolar mitotic spindle but are not absolutely required. Alternative microtubule nucleation mechanisms, both at the kinetochores...
and through microtubule branching by the augmin complex, can generate a microtubule network that will self-assemble into a mitotic spindle\textsuperscript{78, 114}. However, spindle formation is dramatically slowed without centrosomes\textsuperscript{115}. Therefore, the spindle assembly checkpoint protects cells without centrosomes from inefficient spindle formation by slowing mitotic progression until all chromosomes experience sufficient tension\textsuperscript{77}.

The spindle assembly checkpoint also protects cells from mitotic defects caused by having too many centrosomes, a phenomenon called centrosome amplification or having supernumerary centrosomes\textsuperscript{17}. As cells with supernumerary centrosomes enter mitosis, Eg5-mediated pushing forces will, ideally, move centrosomes as far apart as possible\textsuperscript{65}. As these centrosomes begin to nucleate microtubule asters, centrosome amplification causes the formation of multipolar spindles\textsuperscript{6, 116}. As a result, chromosomes can then be captured by any of these spindle poles, even more than two. Chromosomes attached to more than two spindle poles are subject to improper forces and, thus, struggle to satisfy the spindle assembly checkpoint\textsuperscript{60}. However, in some cases, cells will undergo multipolar cell division resulting in three daughter cells, which are often not viable\textsuperscript{65}. In this way, the spindle assembly checkpoint is the first line of defense against mitotic defects caused by centrosome loss or amplification, termed centrosome instability.

Even though the spindle assembly checkpoint tries to stall cells in mitosis until it is satisfied, cells do not remain in mitosis indefinitely\textsuperscript{77, 117}. Instead, cells that spend too long time in mitosis will either arrest or undergo apoptosis. The mechanisms that trigger this mitotic timing checkpoint are poorly understood, however the downstream effect is mediated through p53\textsuperscript{118}. On average, cells that remain in mitosis for more than 84 minutes trigger this checkpoint, even if they successfully undergo cell division. For reference, normal cells spend closer to 30 minutes in mitosis\textsuperscript{76, 119}. Downstream effectors of p53 vary depending on cell type and method used to trigger the checkpoint, but can result in cell cycle arrest, cellular senescence, or apoptosis\textsuperscript{106}. In response to long term mitosis, cell lines lacking p53 can undergo mitotic slippage, where cells stop attempting to generate a productive spindle and, instead, progress through mitosis without chromosome segregation\textsuperscript{117}. Mitotic slippage immediately doubles the ploidy of the resultant daughter cell, as well as doubling the number of centrosomes\textsuperscript{118, 120}. Some cell types can continue to divide after mitotic slippage, whereas others will arrest or undergo apoptosis\textsuperscript{120}.

While centrosome amplification can often result in cell death or arrest, cellular mechanisms exist to find a way around multipolar mitosis. Cells with supernumerary centrosomes can cluster
these centrosomes to two foci, resulting in a productive pseudo-bipolar spindle\textsuperscript{121}. The best-known mechanism of centrosome clustering is mediated by the kinesin-14 HSET, which binds to microtubules emanating from the centrosomes, crosslinking them and pulling the centrosomes towards one another similar to how Eg5 pushes centrosomes apart\textsuperscript{65}. Not surprisingly, cancer cells with supernumerary centrosomes commonly have high levels of HSET\textsuperscript{122}.

Although pseudo-bipolar spindles are more likely to generate viable daughter cells, they are extraordinarily prone to chromosome segregation defects and whole chromosome aneuploidy\textsuperscript{121}. As a multipolar spindle makes contacts with chromosomes, microtubules from multiple centrosomes can interact with the same sister chromatid. Centrosome clustering can then position those centrosomes at opposite sides of the spindle\textsuperscript{116}. If cells were to undergo chromosome segregation with a chromatid attached to opposite ends of the spindle, known as merotelic attachment, that chromatid will be trapped in a tug-of-war between those poles\textsuperscript{123}. As cells undergo cytokinesis, this tug-of-war will be randomly resolved, potentially resulting in the deposition of that chromosome into the wrong cell\textsuperscript{124}. Again, this results in rapid and dramatic genomic instability in the form of whole chromosome aneuploidy\textsuperscript{123}.

Even if that chromosome is segregated into the proper cell, issues can still arise. Because this chromosome did not congress with the rest of the genome, it can remain separate as the nuclear membrane reforms. This will result in the chromosome being sequestered into a micronucleus\textsuperscript{125}. Micronuclei contain some of the same protein components as the nucleus but are less likely to have the correct ratio of these components\textsuperscript{126}. Therefore, micronuclear import/export, transcription, and DNA replication is often defective\textsuperscript{126, 127}. Because of this, the chromosome within the micronucleus is often subject to additional genotoxic stress\textsuperscript{128}.

This is further compounded by the fact that the micronuclear membrane is more likely to rupture due to reduced nuclear lamina integrity\textsuperscript{126}. As the cytoplasm rushes into the ruptured micronucleus, slowly replicating DNA can experience double stranded breaks\textsuperscript{129, 130}. In fact, chromosome shattering and subsequent restitching, called chromothripsis, is common in micronuclei\textsuperscript{128}. Chromothripsis gives rise to random chromosomal rearrangements and duplications or deletions of regions of chromosomes\textsuperscript{131}. These genomic rearrangements can give rise to oncogenic translocations, loss of heterozygosity at deleted loci, and breakage-fusion-bridge cycles due to improper restitching of telomeres\textsuperscript{132}.
Sub-Section 1.3.2 – Selecting against centrosome instability

Since changes to centrosome numbers can give rise to many forms of genomic damage, cellular mechanisms designed to identify genomic damages are used to eliminate cells with centrosome defects. Most of these pathways depend on p53 activation and downstream signaling to eliminate cells with centrosome defects. p53 is a transcription factor considered the guardian of the genome. In response to most forms of DNA damage, p53 levels rise – either through inactivation of the E3 ubiquitin ligase Mdm2 or through direct phosphorylation of p53 – and it translocates to the nucleus. Depending on the type of damage, extent of damage, and the length of time it takes to repair this damage, p53 targets will slow the cell cycle, cause cell cycle arrest and senescence, or cause apoptosis. Because cells with centrosome defects have a propensity towards genomic instability, p53 will ensure centrosome homeostasis.

Additionally, genomic instability caused by centrosome instability directly generates selective pressure against centrosome instability. When p53-deficient cell lines – which do not arrest after centrosome instability – are experimentally depleted of centrosomes, proper centrosome number is reestablished after multiple generations. Intriguingly, when cell lines that normally maintain amplified centrosomes are depleted of their centrosomes through Plk4 inhibition, the cell population returns to the same state of centriole amplification after inhibitor wash out. Even though these cells did not have the normal two centrosomes, they had adapted to the new normal.

Furthermore, centrosome instability prolongs mitosis. As previously mentioned, prolonged mitosis is sufficient to induce a p53-mediated arrest, even if cells do not acquire DNA damage. Not only are classical p53 activation pathways not responsible for the arrest, but the mechanisms are different depending on whether centrosome loss or amplification are the culprit. A genome-wide CRISPR screen revealed that, in response to multiple prolonged mitoses due to centrosome loss, cells require the deubiquitinase activity of USP28 and the presence of 53BP1 to, somehow, activate p53 and trigger a p21-mediated arrest. This has been termed the mitotic surveillance checkpoint. Conversely, cells will arrest in G1-phase after centrosome amplification, even without 53BP1 or USP28. So, even though the downstream effect is the same, the mechanisms that sense changes to centrosome number are not understood.

Centrosome instability has also been shown to promote micronucleus formation. In addition to micronuclei being prone to chromothripsis and subsequent DNA damage-induced p53
stabilization, they are also prone to rupture\textsuperscript{126, 128, 131}. Membrane rupture allows access of cytoplasmic elements to the chromosome(s) housed within the membrane\textsuperscript{126}. Not only does this cause genomic damage, but cytoplasmic DNA is an innate immune trigger commonly used to identify viral infection\textsuperscript{140}.

Double stranded DNA viruses in the cytoplasm activate the cGAS-STING pathway, leading to cellular senescence and secretion of pro-inflammatory cytokines\textsuperscript{141}. This pathway is also co-opted by the cell to sense micronuclear rupture\textsuperscript{140}. Upon binding DNA, cGAS generates cyclic GMP-AMP (cGAMP) dinucleotides. cGAMP then binds to STING, forcing it to dimerize\textsuperscript{142}. Dimerized STING can activate NF-κB pathways and trigger the transcription of p16, leading to senescence\textsuperscript{140, 142}. Additionally, senescence associated secretory protein (SASP) cytokines, like IL-6 and IL-12 are produced, priming nearby cells to undergo senescence\textsuperscript{141}. SASP production \textit{in vivo} has been linked to many aging phenotypes and is generally thought of as deleterious to tissues\textsuperscript{143}.

Notably, centrosome amplification itself has been linked to a novel secretome. When naïve cells are cultured in conditioned media from centrosome amplified cells, they become more invasive. In the context of p53-deficient cancers, meaning they cannot arrest, this secretome promotes invasive behavior of cells that lack centrosome amplification if they are in the vicinity of a cell that has centrosome amplification\textsuperscript{144}. Including the SASP, this indicates that cells with centrosome instability can use paracrine signaling to alter their neighbors even if the cell experiencing centrosome instability is ultimately selected against\textsuperscript{140, 144}.

When considering the importance of the centrosome, it is essential to remember that the existence of the centrosome is not enough. Proper regulation of centrosome number and architecture underly all of these functions. Although we do not know the full consequences of centrosome structural aberrations, mutants that subtly alter its structure are implicated in disease and result in impaired viability in model systems\textsuperscript{59}. Furthermore, the diverse centrioles in different cell types suggest that centriole plasticity is essential for proper development and homeostasis\textsuperscript{15}. Understanding the diversity of centriole assembly outside of commonly used models will likely be the next great challenge of the field.
Sub-Section 1.3.3 Centrosome instability in disease

Since the first connection of centrosomes and cancer by Boveri in the early 1900’s, associations between centrosome instability and cancer have abounded\(^8\). Although these associations are common, models of centrosome instability over the past couple decades have given us mixed results regarding their link to cancer. Before discussing that, however, it is important to explore the impact of the centrosome during development.

Many studies in cell lines have shown the role of the centrosome in mitosis and brought about the belief that it is required for life. However, a landmark study in *Drosophila* testing this question yielded surprising results: flies without centrosomes were viable and even developed to adulthood, although they died soon after hatching because they lack cilia. Although cell division in these flies was slow and asymmetric cell division was unreliable, these flies looked morphologically normal. The story, however, is complicated by the fact that *Drosophila* embryos contain a maternal load of proteins, enough to give rise to centrosomes throughout embryogenesis even though the offspring itself genetically lacks the essential protein, Sas4. Not until larval stages do these flies actually lack centrioles\(^{145}\). Although surprising, this initial study challenged dogma regarding the essentiality of centrosomes and suggested that centrosomes could be altered in model organisms.

The next studies in flies focused on understanding centrosome amplification. Over expression of the key duplication kinase Plk4 is sufficient to cause centrosome amplification. Like the flies without centrosomes, flies can tolerate centrosome amplification without acquiring large-scale aneuploidy. This study revealed that flies have robust centrosome clustering mechanisms and strong spindle assembly checkpoints that ensure mitotic fidelity\(^{146}\). This model, however, allowed the researchers to ask a question originally posed by Theodor Boveri: Is centrosome amplification sufficient to cause cancer?

The researchers noticed one key difference between flies without centrosomes and flies with centrosome amplification that allowed him to answer that question: the unreliable asymmetric division of neuroblasts with amplified centrosomes resulted in an increase to neuroblast numbers\(^{145, 146}\). However, this increase was slight and did not result in the development of brain tumors in these flies. Instead, the researchers took larval brains from Plk4 overexpressing hosts and transplanted them into the abdomen of a naïve fly\(^{146}\). Normal brain tissue can survive in the abdomen of flies without over-proliferating\(^{147}\). In contrast, Plk4 overexpressing larval brains
continued to proliferate, forming tumors in the fly’s abdomen. Additionally, the researchers observed a few cases of metastasis to distant tissues such as the eye\textsuperscript{146}. Finally, there was direct evidence to support the idea that centrosome amplification is sufficient to cause cancer.

Not surprisingly, mouse models of centrosome amplification were more difficult to generate, even though all use Plk4 overexpression to achieve amplification. Unlike the fly, mouse models of centrosome amplification corroborated the original notion that centrosome homeostasis is required for proper development, as many of the mice died during development or shortly after birth. Surprisingly, though, these first groups found that centrosome amplification was not sufficient to form tumors\textsuperscript{148}. Instead, the first models of centrosome amplification displayed high levels of cell death due to severe aneuploidy. For instance, centrosome amplification in the developing brain caused reduced brain size (microcephaly) and neonatal death of the mice. Even when Plk4 overexpression in the brain was crossed to a p53-deficient background, mice did not develop brain tumors, but had progressive neural degradation due to aneuploidy in progenitor cell populations\textsuperscript{149}.

Other models of Plk4 overexpression showed much of the same. Overexpression of Plk4 in the developing epidermis was well tolerated. These cells continued to divide, albeit at a lower rate, and maintain the tissue\textsuperscript{150}. A follow-up study of Plk4 overexpression in the epidermis, however, showed that most mice died soon after birth because the skin could not establish proper barrier function. In the mice that survived, Plk4 was somehow no longer overexpressed and skin cells did not have rampant centrosome amplification. When these mice were crossed to conditional p53 knockout mice lacking p53 in the epidermis, they found that transient centrosome amplification in the skin during development accelerated tumor formation. As a caveat, however, p53-deficient mice already had a high rate of tumor formation\textsuperscript{151}. Together, all signs pointed towards centrosome amplification not being sufficient to cause tumorigenesis. Instead, centrosome amplification exacerbated genomic instability in already vulnerable cancers.

Some groups, however, were undeterred by these results and continued to pursue this question. The definitive answer came when one group developed a mouse that they could induce centrosome amplification in adulthood. Using a doxycycline-inducible promoter, long term Plk4 overexpression was induced in adulthood to study the effects of centrosome amplification on already developed tissues. For the first time, this group saw that centrosome amplification can cause cancer in mice with functional p53. Not only does amplification cause highly aneuploid
tumors, but it causes tumors in ~ 80% of mice. So, why did their model form tumors when other models of centrosome amplification cause death of the cells with amplification? This group speculates that this is because of low levels of Plk4 overexpression and modest centrosome amplification in their study. They believe that other models have too much amplification for cells to survive, but that the minor perturbation to mitosis afforded by having 1 or 2 extra centrosomes is tolerated by the cell but will cause tumorigenic aneuploidy. 

Examples of centrosome amplification can be seen in all spontaneous, solid tumor types. Additionally, many anti-mitotic treatments can generate tetraploid cells that have centrosome amplification. However, amplification is not the only problem in cancer. Our lab has recently found that centrosome loss occurs in localized prostate cancer. We also found that centrosome loss can generate many of the same genomic instability phenotypes as centrosome amplification. Centrosome loss was further able to generate genomic instability that resulted in transformation of a non-tumorigenic cell line. Further, centrosome loss is associated with multi-lobed nuclei, likely due to an inability to properly reform the nuclear envelope after cell division. However, no models of centrosome loss have been attempted in mammals.

On top of genomic instability, supernumerary centrosomes were found to promote invasive behavior and metastasis of cancer cells. Centrosome amplification increases the microtubule nucleation capacity resulting in the activation of Rac1 to promote the invasive behavior of cells. Furthermore, genomic instability due to mitotic disfunction is sufficient to promote metastasis. Researchers found that promoting chromosomal instability by altering the rate of microtubule disassembly during mitosis generates an increased micronucleus burden. This activates the cGAS/STING pathway to promote transcription of an inflammatory response that promotes metastasis.

We now understand that centrosome instability is intimately linked to cancer. Aneuploidy caused by centrosome instability can initiate tumor formation. Independent of aneuploidy, centrosome instability can directly promote metastasis. Additionally, cellular responses designed to fight chromosomal instability have been shown to promote metastasis. Finally, cells close to those that activate cGAS/STING can be influenced by the response that is secreted. Taken together, it is clear that centrosome homeostasis must be regulated to prevent cancer.

Mutations that effect centrosome function have also been found in individuals with developmental disorders. Mutations in core centriole, cilia, and centrosome genes are associated
with microcephaly, lissencephaly, polydactyly, primordial dwarfism, and many others\textsuperscript{46, 59}. Mouse models studying loss of these proteins have found that many of these mutations are directly causal. Most mechanisms of disease arise because these mutant mice struggle to generate specific sets of neurons from neuronal progenitor cells. For instance, Cep63-deficient mice have reduced centrosome duplication experience mitotic errors in neuronal progenitors causing p53-dependent apoptosis, resulting in microcephaly\textsuperscript{157}. Conversely, Cep83-deficient mice cannot properly anchor their centrosome to the apical cell surface in brain cortex progenitor cells. These cells then undergo excessive symmetric cell division, creating too many progenitor cells and not enough cortical neurons, resulting in smoothening of the brain cortex (lissencephaly)\textsuperscript{158}.

It remains unclear why neural development is so sensitive to centrosome disfunction, while other tissues remain unaffected. Likely, these tissues require specialized centrosome function that only take place in these tissues. Conversely, there may be less selective pressure against these mutations. Mutations that affect all cells, including the germ line, would be subject to more stringent evolutionarily pressure. This may also explain why there are no centrosome mutations that predispose an individual to cancer. Mutations that affect the mitotic functions of the centrosome would be lethal, therefore, they are selected against. Mammalian models of centrosome amplification point to this idea, as the offspring are non-viable\textsuperscript{151}.

Although this chapter touches on many of the key functions of the centrosome, it is essential to remember that most of these discoveries were made in cultured epithelial cells. There are certainly a plethora of complex functions unique to individual cell types that are yet to be discovered. Additionally, many facets of the centrosome’s organellogenesis still remain mysterious. Since centrosome function and centriole architecture are both diverse within an individual, centriole assembly mechanisms must be plastic to create cell type-specific structures and functions. Again, this point is highlighted by the numerous genetic diseases caused by mutations in centrosome proteins that only effect specific tissues. Until we can generate models to understand context-dependent centrosome function, analyzing how changes to centrosome architecture alter its function outside of mitosis will be difficult. However, gaining a more complete understanding of centriole assembly may help us to discover new functions of the centrosome and how form affects function.
CHAPTER 2 – Centriole assembly

The core component of the centrosome, the centriole, is a giga-dalton scale macromolecular complex considered one of the largest protein complexes in the cell\textsuperscript{159}. The centriole is a highly ordered barrel composed of unique proteins and unusual microtubule structures (Figure 2.1)\textsuperscript{12}. Centriole assembly is a multi-step process involving the hierarchical recruitment of these unique, evolutionarily conserved proteins within the correct temporal and spatial contexts\textsuperscript{160}. In this chapter, I discuss the molecular structure of the centriole and detail the steps of its biogenesis. To conclude the chapter, I focus on the overarching questions and guiding hypothesis that define the remainder of my dissertation.

Section 2.1 – Centriole structure

Key features of the centriole, both general ultrastructure and molecular composition, are well conserved throughout evolution\textsuperscript{12}. Many species have adapted the centriole to their specific needs, giving rise to structural variations and unique structures within the centriole\textsuperscript{15}. Below, I discuss some generalities of centriole structure and touch on a few species-specific variants. For simplicity, I focus on human centrioles, but will also touch upon unique features of \textit{Drosophila} centrioles since they are the model organism used in my thesis.

Sub-Section 2.1.1 – Centriole ultrastructure

Electron microscopists first described the barrel shaped centriole nearly 65 years ago\textsuperscript{13, 14}. Decades of electron micrographs and, recently, super resolution fluorescent microscopy images have painted a picture of detailed centriole architecture defined by the microtubules that make up the periphery of the barrel. Centriolar microtubules exist as bundles arranged in nine-fold radial symmetry that can be composed of singlet (ex. \textit{C. elegans}), doublet (ex. \textit{D. melanogaster}), or triplet (ex. \textit{H. sapiens}) microtubules. The microtubule composition within the bundles is also unique as the microtubules can diverge from the normal 13 protofilaments and the protofilaments are not always evenly spaced. In centriolar microtubule bundles, the inner most microtubule is called the A-tubule with the B- and C- tubules radiating outwards towards the centriole periphery. Additionally, mammalian centriolar microtubules are composed of unique tubulin paralogs – $\delta$-/e- tubulin – rather than the normal $\alpha$-/b- tubulin heterodimers that build cytoplasmic microtubules\textsuperscript{12, 15, 160}. The observation of unique microtubule compositions suggests that mechanisms at the
Centriole stabilize these structures instead of only relying on lateral interactions between protofilaments.

Centriole length is defined by the length of centriolar microtubules within the main centriole body\textsuperscript{161}. Centriole length is highly variable throughout evolution and within different cell types of a species. Human centrioles in tissue culture cells range between ~350 µm - ~550 µm long\textsuperscript{12}. In \textit{Drosophila} embryos, centrioles are ~180 µm long, whereas they can exceed 1 mm long in \textit{Drosophila} spermatocytes\textsuperscript{16}. Importantly, centriole length is highly consistent between different cells of a given cell type, suggesting that the length of the centriole is strictly regulated. Additionally, changes to centriole length interfere with their functions\textsuperscript{162}, suggesting that biological variation in length may confer unique functions to the centriole. For example, centrioles

---

**Figure 2.1** – The centrosome is composed of two centrioles surrounded by a proteinaceous cloud of pericentriolar material. The pericentriolar material recruits gamma tubulin ring complexes, allowing the centrosome to nucleate microtubules. The older mother centriole is structurally distinct from the younger daughter centriole. The mother centriole’s distal and subdistal appendages facilitate the unique functions of the mother centriole, like acting as a basal body during ciliogenesis. Figure courtesy of Gregory Rogers.
experimentally forced to become overly long are prone to fragment and generate centrosome amplification in the cell\textsuperscript{162}.

Unique molecular compositions within the centriole barrel establish polarity within the centriole\textsuperscript{12}. The centriole is sub-divided into a proximal and a distal region that are ultrastructurally unique. The proximal end of the centriole contains a unique structure called the cartwheel that confers ninefold radial symmetry to the centriole\textsuperscript{160}. The distal end is not only devoid of a cartwheel but the centriolar microtubules begin to taper as you reach the distal tip of the centriole, making the centriole thinner at the distal tip than at the proximal end\textsuperscript{163}.

Structural differences even exist between two centrioles in a single cell. During centriole duplication, a nascent centriole nucleates orthogonally off the proximal end of an existing centriole creating an attached pair of centrioles\textsuperscript{9}. The result is a younger daughter centriole growing off the side of an older mother centriole. In this configuration, structures specific to the mother centriole can anchor pericentriolar material to promote centrosome maturation\textsuperscript{18}. In mammals, only the oldest mother centriole has unique structures called distal and sub-distal appendages required for ciliogenesis, while daughter centrioles contain a cartwheel structure at their proximal end\textsuperscript{12}.

The cartwheel is another enigmatic structure originally observed by electron microscopy. In \textit{Drosophila}, the cartwheel resides at the proximal end of all centrioles within the lumen (only in daughter centrioles of human cells, as it is removed when the daughter centriole matures into a mother centriole)\textsuperscript{16}. The cartwheel appears as a central hollow tube with 9 spoke-like extensions pointing towards the centriole periphery. The centriole microtubules reside at the ends of these extensions\textsuperscript{15}. The cartwheel itself is composed of a single protein, Sas6, assembled into stacks of rings\textsuperscript{164, 165}. The cartwheel is essential for both centriole assembly and establishment of the centriole’s ninefold radial symmetry\textsuperscript{166, 167}.

Between the tips of the cartwheel and the microtubules sits another structure called the pinhead\textsuperscript{168}. Sas6 itself does not interact with microtubules, but instead binds to proteins within the pinhead that facilitate microtubule assembly at these sites\textsuperscript{169}. Two microtubule binding proteins reside within these pinheads, Cep135 and Sas4\textsuperscript{169, 170}. Both are important for centriole assembly and are thought to recruit microtubules to new centrioles. However, there is controversy regarding Cep135’s role, as it is not recruited to the centriole until after the microtubule wall is already built\textsuperscript{171}. 

In mammalian cells, mother centrioles have two unique structures at their distal ends that are essential for ciliogenesis. The distal and sub-distal appendages crown the distal end of the mother centrioles with ninefold radial symmetry\textsuperscript{56}. Recent super-resolution imaging has generated a clear picture of these structures. The distal appendages are curved assemblies that point upwards from the distal end of the centriole to form a basket-like shape. The sub-distal appendages, on the other hand, appear as wedges below the distal appendages\textsuperscript{172, 173}. In addition to their contributions to ciliogenesis, the sub-distal appendages nucleate microtubules and help prevent premature centrosome separation before mitotic entry\textsuperscript{174}.

Another mammal specific structure is the centriole linker, which tethers one mother centriole to another during the cell cycle. This prevents the two centrioles from migrating too far from one another during interphase, in principle limiting cells to a single functional centrosome. The linker, which is composed of the proteins C-NAP1 and Rootletin, is dissolved in late G2-phase prior to centrosome separation during mitosis\textsuperscript{175}.

Recent work using cryo-electron tomography (cryo-ET) of centrioles identified many new electron dense structures at the centriole. As the necessity for the technique would suggest, these are tiny structures that were previously unidentifiable with conventional electron microscopy methods. Although the function of these structures is unknown, they localize around the microtubule bundles and even within the lumen of individual microtubules\textsuperscript{163, 176}. The discovery of these new structures will help us understand fundamental questions about how the centriole is assembled and refine localization maps of characterized proteins within the centriole.

**Sub-Section 2.1.2 – Molecular composition of the centriole**

When considering the molecular composition of the centrioles, we tend to subdivide the centriole for ease of discussion. As discussed previously, the centriole has proximal-distal polarity. Additionally, when viewing the centriole in cross section, we divide it into 3 zones: the core, the bridge, and pericentriolar material (PCM) zones (\textbf{Figure 2.2})\textsuperscript{18}. Each of these zones contains unique sets of proteins that coordinate different functions of the centriole. However, these zones are not static or concrete, but can contain proteins and functions housed by other zones.

The core of the centriole constructs centriole architecture and is generated by proteins that control assembly. The core is built around the Sas6 cartwheel. Sas6 homodimerizes through its coiled coil domains, leaving two globular N-terminal domains. These globular domains can further
oligomerize so that 9 homodimers (18 monomers) form a ring with extended spokes pointing towards the microtubules. These rings can then stack atop each other, forming a multilayer cartwheel (Figure 2.3)\textsuperscript{165,166}.

The spokes of the cartwheel are interaction hubs for conserved proteins that recruit and stabilize the centriole microtubules. The essential centriole duplication factor, Ana2/Sas5/STIL, binds directly to Sas6 homodimers and provide multiple interacting domains for Sas4 and Cep135/Bld10\textsuperscript{169,177,178}. These pinhead proteins recruit the microtubules to the cartwheel, promote centriole growth, and stabilize the centriole microtubules. In human mother centrioles, which lose the cartwheel as they mature, Cep135 is especially important for stabilizing the now naked microtubules\textsuperscript{177}.

While the cartwheel is the definitive feature of the proximal centriole, other core zone proteins reside at the distal tip of the centriole. Notably, these proteins are the focus of my research, as they are responsible for centriole growth past the cartwheel.

An evolutionarily conserved set of proteins, which we termed the distal tip complex (DTC), cap the centriole tip to prevent growth and shrinkage of centriole microtubules\textsuperscript{179-183}. I discuss the DTC more in depth in Section 2.3 and Section 2.4. Briefly, Cep97, CP110, and Klp10A (Kif24 in mammals) regulate centriole growth through ill-defined mechanisms revolving around microtubule regulation\textsuperscript{184}. While they are implicated in preventing centriole growth associated with ciliogenesis, they are also required for growth of the centriole during its biogenesis.

The second zone is called the bridge zone. These proteins link the interior of the centriole to the exterior of the centriole. Many of these proteins extend between the microtubule bundles that make up the wall of the centriole barrel. The bridge proteins are implicated in pericentriolar material recruitment, centriole duplication, and centriole-to-centrosome conversion\textsuperscript{18}. 


Asl/Cep152 is a bridge protein that extend past the microtubule wall and can interact with cnn/Cdk5RAP2, Plp/Pericentrin, and Spd2/Cep192 to help form and organize the pericentriolar material\textsuperscript{68, 185-188}. Asl also coordinates centriole duplication by recruiting and regulating the essential duplication kinase Plk4\textsuperscript{189, 190}. Spd2/Cep192 displays similar localization patterns, although it is more implicated in centrosome maturation during mitosis in \textit{Drosophila}, rather than centriole duplication\textsuperscript{68, 70, 71}.

\textbf{Figure 2.2} – Centrosomal proteins reside in one of three zones built around the centriole. The inner-most zone is the core of the centriole, containing many proteins required for centriole assembly. The bridge zone contains proteins that traverse the microtubule bundles. Bridge zone proteins contain centriole duplication factors and recruit PCM proteins. Proteins in the pericentriolar material zone provide anchoring sites for gamma tubulin ring complexes and interact with other microtubule binding proteins. The lists on the right provide a general, but not exhaustive, list of proteins that exist within each of these zones. Hs – \textit{Homo sapiens}, Dm – \textit{Drosophila melanogaster}, Ce – Caenorhabditis elegans. Figure courtesy of Gregory Rogers.
Other bridge proteins reside throughout the entire centriole. Ana1/Cep295 and Ana3/RTTN both localize along the entire barrel and extend through the microtubule bundles. While they are implicated in centriole assembly and growth, it is unclear how these proteins contribute. Moreover, other proteins still are restricted between the cartwheel and the DTC, which we call the distal region of the centriole. An example of this is Rcd4/PPP1R35, which has also been implicated in centriole length regulation through an interaction with Ana3/RTTN.

The final zone is the PCM zone, which contains proteins that sit farther away from the centriole and function in microtubule nucleation. The bridge proteins Asl, Plp, and Spd2 all

Figure 2.3 – The proximal end of the centriole is composed of regular stacks of Sas6 cartwheels, structures that can self-assemble into ninefold radial symmetry. Sas4 and Ana2 localize to the spokes around the periphery of the Sas6 cartwheel. Sas4 recruits microtubules to these sites during procentriole formation, establishing the ninefold radial symmetry of the centriole microtubules.
interact with Cnn/Cep215 to scaffold the PCM\textsuperscript{70}. Cnn can then directly recruit $\gamma$-tubulin ring complexes to nucleate microtubules\textsuperscript{72}. The PCM zone also houses the mitotic kinase Polo/Plk1, which is responsible for PCM maturation by phosphorylating Spd2 and Cnn, creating high affinity binding site for additional Cnn\textsuperscript{69, 71}.

While this is not an exhaustive list of centriole components, it exemplifies the key structural components of the centriole and how they interact with each other to give rise to a functional unit. In collaborative work with the Rusan lab, we performed an extensive yeast 2-hybrid screen to map the interactions between centriole components. In total, we found $\sim$100 interaction, most of which were novel. While most remain uncharacterized, we discovered a mechanism by which novel Plk4 phosphorylation events on Cep135 control the diameter of Asl localization at the centriole\textsuperscript{197}.

**Section 2.2 – Centriole duplication**

Centrioles duplicate semi-conservatively to ensure cells enter mitosis with two and only two centrosomes\textsuperscript{9}. Like DNA replication, centriole duplication is intimately entrained to the cell cycle\textsuperscript{198}. While centriole duplication is evolutionarily conserved, I focus on *Drosophila* in this section to highlight some species-specific variation.

The centriole duplication cycle begins at the end of mitosis. During cell division, each cell receives one centrosome with two mature centrioles\textsuperscript{199}. Each of these mother centrioles have the full complement of proteins necessary to support centriole duplication in the subsequent cell cycle\textsuperscript{171, 200}. In *Drosophila*, the initial steps of centriole duplication occur at the end of mitosis (Figure 2.4). In human cells, however, much of this takes place at the beginning of S-phase\textsuperscript{9}.

First, a single spot is defined as the site of nascent daughter centriole assembly on the proximal end of an existing mother centriole. This spot, called the pre-procentriole, consists of the essential centriole duplication factors Plk4, Sas4, Ana2, and Sas6\textsuperscript{201-203}. The site of spot selection at the centriole is seemingly random but was suggested to occur due to liquid-liquid phase separation of Plk4\textsuperscript{204}. However, we currently do not understand this mechanism.

The next step is maturation of the pre-procentriole to a bona fide procentriole. The procentriole is characterized by the transformation of Sas6 from a spot to a cartwheel and recruitment of microtubules to the nascent procentriole\textsuperscript{178, 201, 205}. Again, the mechanisms driving procentriole development are still unknown.
As cells enter the subsequent mitosis, the next step of centriole duplication is maturation of the daughter centriole required to make centrioles competent to duplicate. This process, called centriole-to-centrosome conversion, occurs through the hierarchical recruitment of Cep135, then Ana1, then Asl\textsuperscript{171,200}. Without centriole-to-centrosome conversion, centriole duplication fails in the subsequent cell cycle because Asl is essential to recruit Plk4 to the centriole in late mitosis\textsuperscript{185,189,190}.

As centrioles progress through mitosis, the mother and daughter centrioles are still engaged with each other. That is, they are still physically linked to each other with the daughter touching the side of the mother\textsuperscript{206}. This configuration inhibits centriole duplication, likely by acting as a sink for centriole components or as a preferential recruitment site for duplication factors. Therefore, centrioles must disengage or physically distance themselves before the next centriole duplication cycle can begin\textsuperscript{200,207,208}.

Canonically, centriole disengagement occurs through the cleavage of PCNT by separase when it is activated during mitosis\textsuperscript{209-211}. However, disengagement can occur in cells with separase depletion, albeit delayed until G1 phase\textsuperscript{209}. I favor a disengagement mechanism primarily controlled by the physical distancing of the daughter centriole during centrosome maturation. As the pericentriolar material expands, the daughter centriole is pushed away from the mother and, after a certain distance, the mother centriole becomes competent to duplicate even when the daughter is still engaged\textsuperscript{212}. Furthermore, using inhibitors of Polo/Plk1, thus preventing centrosome maturation, prevents disengagement\textsuperscript{209}. Therefore, centrosome maturation is, at the very least, a prerequisite.

Additionally, the requirement for disengagement is still contentious for multiple reasons. When Plk4 is overexpressed, causing centriole amplification, you can see examples of mother centrioles with multiple daughters on their sides. Therefore, centrioles can form daughters while still engaged\textsuperscript{213}. Additionally, others have seen chains of linked grandmother-mother-daughter centrioles, suggesting the centrioles have gone through another duplication cycle without disengagement.
Figure 2.4 – Centriole assembly is coupled with the cell cycle. During mitosis, each daughter cell receives one centrosome with two centrioles, both of which are primed for duplication. In late mitosis, a molecular spot called the pre-procentriole defines the spot of new centriole assembly. The duplication components in the pre-procentriole (Plk4, Ana2, and Sas6) mature to form the Sas6 cartwheel. As cells enter S-phase, microtubules are recruited to the tips of the cartwheel, forming a bona fide procentriole. Around the same time components of the distal tip (Cep97 and CP110) are recruited to the growing end of the procentriole. Sometime during S/G2-phase, the procentriole will grow and develop into a daughter centriole. As cells enter mitosis, the daughter centriole recruits factors required for maturation into a duplication competent mother centriole (Cep135, Ana1, and Asl), a process called centriole-to-centrosome conversion. As cells divide, one centrosome is inherited by each daughter cell containing two duplication competent centrioles.
Sub-Section 2.2.1 – Stabilizing the pre-procentriole

Plk4 is the master regulator of centriole duplication. It is both necessary for centriole duplication and overexpression is sufficient to cause centriole amplification\textsuperscript{214-216}. While Plk4 phosphorylates many centrosomal substrates, we do not yet understand how, or if, most of them contribute to centriole duplication. The major exception is Plk4 phosphorylation of Ana2. We and others have discovered that multiple phosphorylation events on Ana2 (and the human homolog STIL) dictate the first steps of centriole duplication (Figure 2.5)\textsuperscript{9}.

Plk4 phosphorylates Ana2 along its entire length and we have provided mechanistic evidence as to the roles of these phosphorylation events. First, phosphorylation of the C-terminal STAN (STil/ANa2) domain of Ana2 creates a high affinity binding site for Sas6. Mutation of these sites to non-phosphorylatable alanine residues prevents the recruitment of Sas6 to the centriole and subsequent centriole assembly\textsuperscript{178, 201, 205}. Next, phosphorylation of the N-terminus of Ana2 stabilizes the pre-procentriole on the side of the mother centriole, allowing maturation to a bona fide procentriole\textsuperscript{202, 203}. Finally, phosphorylation of the central region of Ana2 disrupts binding with Plk4\textsuperscript{205}. Surprisingly, the latter discovery allowed us to further address a major question in our field: Why do daughter centrioles only form on the side of existing mothers instead of other locations throughout the cell?

The first studies trying to answer this question suggested that Ana2 was recruited to the mother centriole by binding directly to Plk4, which is already brought to the mother centriole by Asl\textsuperscript{178, 189}. However, the tool they used to determine this incorrectly influenced their interpretations – their Ana2 deletion mutant not only prevented an interaction with Plk4, but also prevented its interaction with another duplication factor, Sas4\textsuperscript{202}. When using a phospho-mimetic mutation of Ana2’s central region which cannot bind Plk4, but can bind Sas4, we found that Ana2 could still be recruited to the centriole without Plk4 binding. However, we also found that Plk4’s kinase activity was still required for recruitment of Ana2 to the centriole\textsuperscript{205}. These discoveries suggested that we needed to revise our understanding of the first steps of centriole duplication.
Figure 2.5 – In *Drosophila*, centriole duplication begins at the end of mitosis when Plk4 is activated at the centriole. (A) Plk4 is initially recruited to the centriole by Asterless. (B) Plk4 then interacts with centriole-resident Sas4 at the pre-procentriole. Ana2 is brought into proximity of Plk4 by binding to Sas4. (C) Plk4 is then activated, where it phosphorylates Ana2 on multiple sites. (D) First, the phosphorylation of Ana2 on S38 stabilizes the pre-procentriole. Subsequent phosphorylation of Ana2’s STAN domain recruits Sas6 to the site of the pre-procentriole. Figure courtesy of Gregory Rogers.
We therefore hypothesized that phosphorylation of Ana2 by Plk4 would increase its affinity for a ligand at the centriole, thus promoting pre-procentriole formation on the mother centriole. Therefore, we screened bridge zone proteins at the centriole to find this binding partner. We found that Sas4 bound more tightly to Ana2 when kinase active Plk4, but not a kinase dead version, is present\textsuperscript{202}. Interestingly, we also found that Ana2 becomes phosphorylated by Plk4 at Ser38, a phosphorylation event essential for centriole duplication, only in the presence of Sas4\textsuperscript{201, 202, 205}.

Phosphorylation of Ana2 S38 had already been identified as required for centriole duplication\textsuperscript{201, 205}. Therefore, we investigated the mechanistic requirement for S38 phosphorylation during pre-procentriole assembly. We found that S38 phosphorylation increases Ana2’s affinity for Sas4. Using super-resolution microscopy studies, we also found that phosphorylation of S38 is required for pre-procentriole stabilization on the side of the mother centriole\textsuperscript{202}. Another study corroborated this finding in human cells using Fluorescence Recover After Photobleaching (FRAP) to show that phosphorylation of the homologous residue in STIL reduces turnover of STIL at the centriole\textsuperscript{203}. Taken together, we concluded that centriole duplication and assembly is restricted to the side of an existing mother centriole because that is where Sas4 is present to stimulate a unique and required phosphorylation event.

Major gaps in our knowledge of centriole duplication still exist, especially regarding how the site of pre-procentriole recruitment is selected and how the pre-procentriole matures to form the highly ordered procentriole. While this is one of the major focuses of our lab and my research, this is not the major focus of this thesis. Instead, I am interested in downstream steps that govern centriole growth and assembly.

Section 2.3 – Centriole assembly

Centriole assembly can be divided into two temporally and spatially distinct steps. First, procentriole assembly includes construction and growth of the cartwheel and recruitment of microtubules to the cartwheel. Second is growth of the distal end of the centriole past the cartwheel\textsuperscript{9, 160}. Growth of centriolar microtubule bundles is slow and processive, unlike microtubule growth throughout the cell\textsuperscript{217, 218}. Unique centriolar growth factors impart multiple layers of regulation to these microtubules at the distal tip of the centriole.
Sub-Section 2.3.1 – Cartwheel assembly

The centriole cartwheel is composed of multiple stacks of Sas6 rings that are regularly spaced, providing a scaffold for the proximal centriole to be built\textsuperscript{165}. As discussed above, little is known about how the cartwheel emerges from the pre-procentriole spot on the side of the centriole. However, the procentriole is first observable by electron microscopy at the start of S phase\textsuperscript{219}. Since there are no reports of naked cartwheels without microtubules, it is likely that cartwheel assembly and procentriole assembly occur simultaneously.

One recent landmark study provided the first evidence for how assembly of the proximal end occurs. Although it highlights little about the mechanisms by which it is constructed, it does provide evidence for how length of the proximal centriole is regulated. The authors generated transgenic \textit{Drosophila} expressing Sas6-GFP under the control of its own promoter. They then performed live imaging of embryos using 3D-Structured Illumination microscopy to visualize centrioles in super-resolution. Using this technique, they analyzed changes to Sas6-GFP intensity to analyze cartwheel growth rates\textsuperscript{220}. Notably, \textit{Drosophila} embryos rapidly undergo multiple S phase and mitotic cycles without intervening Gap phases\textsuperscript{221}. Therefore, analysis of pre-procentriole accumulation of Sas6 without procentriole formation should be negligible in this system.

They found that decreasing the genetic dosage of Plk4 by half resulted in slower cartwheel growth without changing the final intensity of Sas6 acquired. Whereas doubling the genetic dosage of Plk4 increased growth rate but resulted in less Sas6 and, thus, shorter cartwheels. Using FRAP, they also found that Sas6 is incorporated into the growing cartwheel from the proximal end, near the existing mother centriole\textsuperscript{220}. Interestingly, Plk4 does not directly phosphorylate Sas6 in \textit{Drosophila}, suggesting that Plk4 is generating these changes by phosphorylating another component of the pre-procentriole.

While the authors focus on the implications of Plk4 protein level oscillations during development, I would rather speculate on the molecular assembly\textsuperscript{220}. Plk4 phosphorylation of Ana2 recruits Sas6 to the procentriole\textsuperscript{178}. However, we do not know the extent of Ana2 phosphorylation required for procentriole assembly. Perhaps only 50\% of Ana2, which exists as a tetramer, is normally phosphorylated to efficiently recruit enough Sas6\textsuperscript{222}. However, when the dosage of Plk4 is increased, too much Ana2 is phosphorylated and binds Sas6 too tightly. This may prevent proper Sas6 reassembly into cartwheels, generating shorter centrioles. Conversely, by reducing the genetic dosage of Plk4 it would take longer to overcome the threshold of Ana2
phosphorylation necessary for Sas6 recruitment and reassembly. Eventually, however, the threshold is surpassed, leading to the normal amount of Sas6 recruited. In this way, Plk4 could tune the amount of “active” Ana2 at the centriole to promote proper cartwheel assembly\textsuperscript{220}.

Recruitment of microtubules to the cartwheel is much better understood as it is controlled by one protein: Sas4/CPAP. Sas4/CPAP is originally recruited to the pre-procentriole but is then reorganized to the spoke region within the cartwheel\textsuperscript{12}. Sas4/CPAP has 2 known microtubule binding domains, the PN2/3 domain and the MBD (microtubule binding domain), both of which contribute to recruitment of microtubules. Furthermore, the PN2/3 domain was shown to promote centriole growth by stabilizing centriole microtubules\textsuperscript{223}. Overexpressing the human CPAP is a reliable method to generate overly long centrioles\textsuperscript{162}.

Even though overexpression can promote centriole over-elongation, it is unclear if Sas4/CPAP normally contributes to growth of the microtubules past the cartwheel. Conflicting data regarding Sas4/CPAP localization further confounds understanding of Sas4/CPAPs role in distal centriole growth\textsuperscript{215, 223-226}. Furthermore, since Sas4/CPAP is required for centriole duplication and microtubule recruitment, knockdown studies that could address this are not a viable option\textsuperscript{202, 203}. As discussed below, many proposed mechanisms of centriole growth rely on the assumption that Sas4/CPAP normally promotes growth. However, this assumption has yet to be validated.

Sas4/CPAP contains another unique region within its PN2/3 domain named the LID domain. The LID domain drapes across the top of the distal-most β-tubulin of the centriole microtubules and prevents microtubule polymerization. Combined with its ability to stabilize centriolar microtubules, Sas4/CPAP’s PN2/3 domain contributes to the processivity of centriole microtubule growth\textsuperscript{224}.

Seemingly concurrent with Sas4/CPAP-mediated microtubule recruitment, however, is the recruitment of the distal tip complex (DTC) to the distal end of the centriole\textsuperscript{12}. The DTC consists of proteins that are unique to the distal tip of the centriole and coordinate centriole length\textsuperscript{160, 179, 225}. Importantly, the DTC promotes timely growth of the centriole past the cartwheel\textsuperscript{160}. Since the DTC seems to dominate these activities, the role of Sas4/CPAP in distal centriole assembly is not clear.
Sub-Section 2.3.2 – Distal centriole assembly

Like cartwheel assembly, growth of the distal end of the centriole is a highly regulated process that occurs with distinct timing\textsuperscript{12}. Conflicting information, however, has made it difficult to understand when this occurs. Originally, centriole growth was thought to occur during a nascent centriole’s first S/G2-phase\textsuperscript{217,219}. However, recent super-resolution studies suggest that centriole growth occurs during mitosis\textsuperscript{227}. Regardless of the timing, the factors required for centriole growth generally localize to the centriole during early S phase\textsuperscript{12}.

Additionally, distal centriole growth research suffers from unclear definitions regarding bona fide centriole growth. Therefore, I would like to preface this section with my views on centriole growth before discussing mechanistic specifics. The main phenotype observed when researchers describe centriole over-elongation is growth of individual microtubules past the distal tip\textsuperscript{225}. However, these microtubule offshoots are not indicative of bone fide centriole growth. Instead, these phenotypes describe dysregulation of microtubule dynamics. While this is informative in genetic studies that identify growth regulators, the microtubule offshoot phenotype confuses our understanding of molecular mechanism. To me, bona fide centriole growth involves collective growth of the entire barrel. The premise of this distinction revolves around the disparate functions of the distal tip complex, which must both inhibit spurious microtubule growth and promote centriole elongation\textsuperscript{181, 182, 228}. When discussing \textit{Drosophila} models, I will point to specific contexts that show each of these phenotypes and dissect them as unique.

Even though we know some of the factors that participate in centriole growth, a clear picture has not yet emerged. Regardless of specific mechanisms, there is consensus that centriole growth is driven by stabilization of centriolar microtubules. Sas4/CPAP is implicated in distal centriole elongation because it stabilizes microtubules \textit{in vitro} and overexpression of Sas4/CPAP promotes the formation of microtubule offshoots\textsuperscript{224,225}. Since Sas4/CPAP stimulates growth, many of the proposed mechanisms associated with other proteins revolve around regulating Sas4/CPAP.

The conserved centriolar proteins Ana3/RTTN, Ana1/Cep295, and Rcd4/PPP1R35 are all required to build full length centrioles. Without any one of these factors, centrioles are shorter than they should be. While the physical interactions between these proteins are necessary for their functions, they have no a clear effect on microtubule stability. Instead, experimental data focus on the necessity of these proteins for Sas4/CPAP overexpression-induced microtubule elongation\textsuperscript{191-196}. However, we still lack a mechanistic understanding of why these proteins are essential for
Sas4/CPAP to elongate microtubules. Since these proteins localize throughout the distal centriole and extend through the microtubule wall, perhaps they are actually the proteins stabilizing the microtubules and Sas4/CPAP overexpression merely recruits more of these proteins.

Another conserved centriole growth factor is Centrobin (Cnb). Even though the protein is evolutionarily conserved, the mechanisms of action are different in different species\textsuperscript{229}. In human tissue culture cells, Cnb is required to recruit CPAP to centrioles\textsuperscript{230, 231}. However, Cnb mutant \textit{Drosophila} still have centrioles, suggesting that the essential centriole duplication factor Sas4 localizes to centrioles. Regardless, centrioles in Cnb mutant \textit{Drosophila} spermatocytes are still shorter. Electron micrographs of these centrioles reveal that the C-tubules within the microtubule bundles are not properly constructed\textsuperscript{232}. Interestingly, the spermatocyte is one of the only cell types in \textit{Drosophila} that has triplet microtubules, and thus a C-tubule\textsuperscript{233}. So, even though Cnb is ubiquitously expressed in \textit{Drosophila} cells, it plays a cell type-specific role in spermatocyte centriole assembly. Not only does this highlight a mechanism of centriole structural variation within an individual, but the general requirement of Cnb in human centrioles, which are composed of triplet microtubules, suggests evolutionary conservation and generalization of this mechanism\textsuperscript{15}.

Much of our understanding of centriole growth revolves around proteins that localize to the distal tip of the centriole\textsuperscript{160}. The distal tip, itself, is a planar surface at the distal end of the centriole where microtubule bundles collectively stop growing\textsuperscript{217}. However, we lack a mechanistic understanding of how proteins at the distal tip regulate centriole growth. Additionally, many of these proteins are not evolutionarily conserved and may provide specialized functions to mammalian centrioles, such as regulating ciliogenesis. Since these proteins may not contribute to a general understanding of centriole growth, I will touch on them first before focusing on the proteins more relevant to my project.

The first is a centriole distal appendage protein, Cep120. Cep120 stabilizes microtubules \textit{in vitro} and is required for assembly of full-length centrioles\textsuperscript{234, 235}. Cep120 preferentially localizes to daughter centrioles, through its interaction with the accessory protein Talpid3, where it prevents premature centriole maturation\textsuperscript{234, 236}. Later, Cep120 also promotes distal appendage formation on mature, mother centrioles\textsuperscript{236}. The mechanism of Cep120-mediated centriole growth is thought to rely on its interaction with CPAP and the accessory protein SPICE\textsubscript{1}\textsuperscript{236-238}.

Ofd1, on the other hand, is a centriole growth inhibitor whose knockdown results in centriole elongation. Interestingly, Ofd1 depletion-mediated centriole elongation is restricted to
growth of the distal end of the centriole\textsuperscript{239}. Other phenotypes associated with centriole growth, such as overexpression of CPAP, result in both proximal and distal elongation\textsuperscript{225}. However, the mechanism by which Ofd1 prevents centriole growth is unknown. Since Ofd1 is required for distal appendage assembly, knockdown may hypothetically free distal appendage proteins like Cep120 to promote centriole growth\textsuperscript{239, 240}. In this case, the distal and sub-distal appendages would, themselves, act as centriole growth inhibitors.

While the above mechanisms of centriole length regulation are not conserved, a core set of distal tip resident proteins called the distal tip complex (DTC) have contributed to maintaining centriole length throughout evolution. The DTC is first recruited to the distal tip of the centriole during procentriole assembly\textsuperscript{160}. Normally, the DTC prevents growth and shrinkage of centriole microtubules through antagonistic activities\textsuperscript{183}. However, the DTC also coordinates timely centriole growth to ensure proper centriole assembly\textsuperscript{181, 182}. These seemingly disparate functions of the DTC make understanding distal centriole growth both complicated and intriguing.

The core components of the DTC, Cep97 and CP110, were first identified as inhibitors of ciliogenesis. Both are unique to the centriole distal tip, knockdown of either protein in mammalian cells causes premature ciliogenesis, and overexpression of CP110 inhibits normal ciliogenesis\textsuperscript{179}. A third component of the DTC, Kif24, is a kinesin-13 microtubule depolymerase that prevents aberrant ciliogenesis\textsuperscript{184}. Furthermore, Cep97 and CP110 must be removed from the distal tip of the centriole before microtubules of the ciliary axoneme can grow\textsuperscript{241}. Because of this, the DTC was originally thought of as a cap that prevents all microtubule growth at the distal centriole.

Since CP110 removal is required for growth of the ciliary axoneme, much of what we know regarding Cep97 and CP110 regulation revolve around their removal from the centriole distal tip\textsuperscript{179}. For example, Kif24 knockdown led to spurious ciliogenesis by removing CP110 from the centriole distal tip\textsuperscript{184}. CP110 levels are regulated by ubiquitination and deubiquitination by SCF\textsuperscript{CyclinF} and USP33, respectively, to promote timely CP110 degradation and ciliogenesis\textsuperscript{242, 243}. Additionally, there are multiple mechanisms to traffic Cep97 and CP110 away from centriole\textsuperscript{244, 245}. For instance, the distal tip resident protein Cep104 binds Cep97/CP110 and carries it away from the centriole by binding EB1 and tracking along the growing tips of cytoplasmic microtubules\textsuperscript{246}.

Since a major role of the mammalian DTC is to inhibit ciliogenesis, it is difficult to disentangle the implication of DTC function in regulating centriole growth. While CP110 or Cep97
knockdown promoted the formation of microtubule offshoots from centriole distal tips, we do not know if the microtubule growth was driven by ciliary microtubule growth factors or other mechanisms\textsuperscript{179, 225}. Flies, on the other hand, do not rely as extensively on cilia as mammals\textsuperscript{247}. So, many of the studies looking at the distal tip complex in \textit{Drosophila} have been most informative in understanding its function in centriole growth.

Like in mammals, both Cep97 and CP110 knockout flies display a microtubule offshoot phenotype, even in cells that do not form cilia. However, when observing these centrioles, there is an obvious transition between main centriole body and microtubule offshoot. Even though the microtubule offshoot phenotype would suggest that centrioles are longer, the body of the centriole is actually shorter\textsuperscript{181, 182}. Additionally, Klp10A mutant flies, which lack the kinesin-13 with homologous function to Kif24, display dramatically elongated centrioles. In this case, the entire centriole body is longer and displays expansion of both the proximal and distal ends\textsuperscript{228}. These phenotypes highlight the need for clear distinction between microtubule offshoots and bona fide centriole growth.

The phenotypes associated with Cep97 and CP110 knockout also highlight an important duality of the DTC. Normally, the DTC functions to prevent growth and shrinkage of the centriole microtubules\textsuperscript{179}. However, the DTC is also required for centriole growth. In these mutant flies, we see that centrioles do not grow even though centriole microtubules grow too long\textsuperscript{181, 182}. The role of the DTC may rely on these disparate functions, to impart processivity to distal centriole growth and ensure collective growth of the centriole microtubules to maintain a planar distal end.

Furthermore, observations made in \textit{Drosophila} S2 cells challenge the canonical function of the DTC. The studies characterizing the DTC’s role in preventing ciliogenesis suggested that the DTC acts as a cap to prevent all microtubule growth at the centriole. However, Klp10A knockdown in S2 cells promotes centriole microtubule elongation past the distal tip. If the DTC formed a cap that prevented microtubule dynamics, Klp10A knockdown should have no effect since the microtubules could not grow. Furthermore, we see that Cep97 or CP110 RNAi causes Klp10A-dependent centriole disassembly in S2 cells (\textbf{Figure 2.6})\textsuperscript{183}. Together, this suggests that microtubules at the distal tip are dynamic, but that Cep97 and CP110 protect the centriole by defining a limit past which Klp10A-induced microtubule depolymerization cannot pass.
Even though we lack mechanistic evidence explaining centriole growth, evidence from genetic studies cause me to present a model as such (Figure 2.7). Centriole microtubules are dynamic; however, the distal tip complex performs antagonistic functions to prevent growth and shrinkage. Klp10A prevents microtubule offshoot formation by depolymerizing microtubules that grow past the DTC\textsuperscript{183, 228}. Cep97 and CP110 promote timely and collective growth of the distal centriole microtubules and protect the centriole by stopping depolymerization of microtubules\textsuperscript{181, 182}. However, this model lacks a factor that physically interacts with microtubules at the distal tip. Although Cep97 and CP110 promote centriole growth, there is no obvious mechanism for them to
stabilize microtubules at the distal tip. Furthermore, if the normal function of the DTC is to prevent growth and shrinkage of the centriole, that function be modulated to promote timely growth.

Section 2.4 – How do centrioles grow?

There are still many gaps in our knowledge as to how centrioles grow. However, the most pressing questions revolve around a mechanistic understanding of microtubule regulation at the distal tip. First, how are microtubules stabilized within the DTC? Second, how are DTC proteins regulated to permit timely centriole growth? To answer these, we pursued the overarching hypothesis that an unidentified microtubule binding protein exists within the DTC whose function is modulated by the known DTC proteins to balance microtubule stability and growth.

First, I discuss the discovery of a new centriole growth factor, Cep104, previously found to regulate ciliogenesis. Second, I find that Plk4 phosphorylates components of the distal tip...
complex and then refine a mechanism of Plk4 activation. From there, I speculate as to how phosphorylation of the DTC could modulate functions of the proteins and tip the balance towards centriole growth.
CHAPTER 3 – Cep104 is a member of the distal tip complex

Centrosome function requires proper assembly of the centriole. Centriole architecture is generated through coordinated processes of multiple complexes at the centriole\(^1\). As centrioles grow, the microtubule bundles that compose the ultrastructure of the barrel extend past the distal end of the cartwheel\(^2\). These microtubules grow processively before halting and maintaining a constant length defined by a conserved set of proteins called the distal tip complex (DTC)\(^{180, 215, 248}\). The DTC itself performs counteracting functions to prevent growth and shrinkage of microtubules at the distal tip\(^3\). In *Drosophila*, the kinesin-13 microtubule depolymerase Klp10A prevents growth of microtubules past a limit defined by the DTC\(^{183, 228}\). Conversely, Cep97 and CP110 cooperate to preserve length by preventing disassembly of microtubules below the DTC\(^{181, 182, 225}\). Although the DTC is required to maintain proper length, it localizes to the procentriole prior to centriole growth\(^1\). Therefore, a major gap in our understanding is how the centriole grows while the inhibitory distal tip complex is present.

In mammals, Cep97 and CP110 are also required to prevent spurious ciliogenesis\(^{179}\). Normally, Cep97 and CP110 create an inhibitory cap at the distal tip of the centriole that must be removed prior to ciliogenesis\(^{179, 184}\). However, mutant *Drosophila* lacking Cep97 and/or CP110 do not undergo aberrant ciliogenesis\(^{181, 182}\). Instead, somatic cells lacking Cep97 or CP110 have shorter centrioles with individual microtubules jutting from their distal ends. Therefore, *Drosophila* is an ideal model to study centriole growth independent of potentially confounding ciliogenesis defect phenotypes.

Although genetic models show that Cep97 and CP110 cooperate to promote centriole growth, it is yet unclear how they mechanistically function, since neither directly interacts with microtubules\(^{179, 181, 182}\). Likely, the DTC exerts its activity through a microtubule binding protein that resides at the distal tip. However, there were no microtubule binding proteins known to reside at the distal tip of the centriole when we started this project. We, therefore, hypothesize that an unknown microtubule binding protein resides at the distal tip of the centriole to promote centriole growth.

To that end, we identify a *Drosophila* homolog of Cep104, a distal tip resident protein with a microtubule binding TOG domain\(^{246}\). Although Cep104 knockdown does not alter centriole growth in humans\(^{246}\), we observe shorter centrioles in *Drosophila* S2 cells depleted of Cep104.
Furthermore, we show that Cep104 overexpression results in excessively long centrioles. We then reveal how Cep104 localizes to the distal tip using a structure-function approach. Finally, we find that Cep97 is required for Cep104 localization to the centriole. Taken together, we propose a hypothetical model of Cep104’s role in centriole growth.

Section 3.1 – Identifying Drosophila Cep104

In humans, Cep104 is a distal tip resident protein implicated in ciliogenesis. It consists of an N-terminal jelly roll (JR) domain known to bind Cep97, a downstream coiled coil (CC) which facilitates homotypic interaction, a microtubule binding TOG domain, a Zinc Finger Array (ZnF) known to bind CP110 and Nek2, and a C-terminal EB1-binding SxIP motif (Figure 3.1A). Cep104 was shown to promote ciliogenesis by binding EB1 and trafficking Cep97 away from centrioles along microtubule +TIPs, thus removing the inhibitory cap to ciliogenesis. The necessity of Cep104 for ciliogenesis was also confirmed in CRISPR mutant zebrafish. Studies in Tetrahymena, however, show that Cep104 is required to maintain microtubule structure at the ciliary tip, but Cep104 loss does not prevent ciliation. However, Cep104 has not been implicated in regulating centriole length.

To identify a Cep104 homolog in Drosophila, the D. melanogaster proteome (flybase.org) was searched against the human amino acid sequence. The top hit was an uncharacterized gene, CG10137, which is 47% similar to human Cep104 and has 31% identity. Evolutionary alignment of CG10137 with other species showed that homology clusters in the major functional domains of Cep104, confirming that CG10137 is a Drosophila homolog of Cep104 (Figure 3.1.A and Appendix A). Using the evolutionary analysis, we defined the bounds of Drosophila Cep104’s functional domains as follows: Jelly Roll (JR, aa. 1-167), TOG domain (TOG, aa. 454-729), and Zinc Finger Array (ZnF, aa. 805-941). Pairing the evolutionary alignment with coiled-coil prediction software, we defined the bounds of the coiled coil (CC, aa. 202-318). The major difference between Drosophila Cep104 and other species analyzed is that Drosophila Cep104 lacks an EB1 binding SxIP motif. For the remainder of the chapter, I will refer to Drosophila Cep104 as Cep104, while other species will be designated as discussed.
Figure 3.1 – (A) The domain structure of *Drosophila* Cep104 (CG10137) matched to the human homolog. Cep104 is composed of an N-terminal Jelly Roll domain, predicted coiled coil, microtubule-binding TOG domain, and a C-terminal Zinc Finger array. *Drosophila* Cep104 lacks the EB1-binding SxIP domain found in human Cep104. (B) GFP-tagged Cep104 transgene localizes as two spots at S2 cell centrioles (Plp), indicative of a distal tip marker. Insets are 4x zoom. (C) Time-lapse imaging of S2 cells expressing GFP-Cep104 and TagRFP-EB1 shows that *Drosophila* Cep104 does not microtubule +TIP track, consistent with the fact that it lacks the SxIP site.
We next wanted to validate that Cep104 localizes to the distal tips of centrioles in *Drosophila*. To do this, we expressed a full length GFP-tagged transgene of Cep104 in *Drosophila* S2 cells. Immunofluorescence for centrosomes (Plp) in these cells showed that GFP-Cep104 localizes as two distinct spots at the centrosome (Figure 3.1.B). This is indicative of distal tip complex proteins, as one spot localizes to the distal tip of the mother centriole and a second spot localizes to the daughter centriole’s distal tip. Finally, because Cep104 lacks the conserved SxIP sequence found in other species, we wanted to ensure that it cannot +TIP track on microtubules. Time-lapse imaging of S2 cells co-expressing GFP-Cep104 and TagRFP-EB1 shows that Cep104, in fact, does not colocalize with EB1 as it +TIP tracks. Instead, Cep104 is seen as two distinct spots at the centrosome (Figure 3.1.C).

Section 3.2 – Cep104 is a bona fide member of the distal tip complex

We next wanted to see if Cep104 co-localizes with the DTC. To answer this, we first needed to generate antibodies against DTC proteins. We generated polyclonal antibodies against bacterially-expressed fragments of Klp10A (MBP-Klp10A 1-204), CP110 (MBP-CP110 326-549), and Cep97 (His6-Cep97 514-806). Western blots to validate these antibodies were performed from cells depleted of that protein by RNAi or RNAi-depleted cells expressing an RNAi-resistant GFP-tagged transgene (Figure 3.2.A). Antibodies were then used for immunofluorescence in S2 cells co-stained with Plp to mark centrosomes (Figure 3.2.B). Both Cep97 and CP110 localized to two distinct spots at the centrosome, indicative of distal tip localization. As Klp10A is not unique to the distal tip of the centriole, staining for Klp10A showed a more global distribution with apparent localization to individual microtubules and the midbody in cytokinetic cells.

These new tools allowed us to co-localize GFP-tagged Cep104 with the other components of the distal tip complex using 3D-Structured Illumination Microscopy (3D-SIM). We see that GFP-tagged Cep104 co-localizes with both Cep97 (Figure 3.3.A) and CP110 (Figure 3.3.B). We then wanted to validate co-localization of endogenous Cep104 with the DTC by generating an antibody using bacterially-expressed fragments of Cep104 (MBP-Cep104 730-941). However, when attempting to validate the antibody, we were unable to detect endogenous Cep104 by western blot even though we could detect a GFP-tagged transgene (Figure 3.4.A). Efficacy of RNAi-induced knockdown was also validated by depleting Cep104-GFP in cells stably expressing an
inducible transgene (Figure 3.4.B). This suggested that Cep104 is either not present in S2 cells or expressed at undetectably low levels.

Even though we were unable to detect Cep104 by western blot, RNAseq data in S2 cells suggest that Cep104 is expressed (collaborator’s data not shown). Therefore, we proceeded to test the effect of Cep104 depletion on centriole length. Because centrioles are so small (only around 180 nm long in S2 cells when fully grown\(^\text{183}\)), centriole length measurements are difficult. Therefore, we used 3D-SIM to acquire super-resolution images of S2 centrioles stained with Cep97 to guide our centriole length measurements. As a proxy for centriole length, we measured

---

**Figure 3.2** – Developing antibodies to study the distal tip complex in *Drosophila*. (A) Western blots of specified distal tip complex protein from S2 cell lysates with control RNAi (Ctrl) or depleted of the specified gene without or with replacement with RNA-resistant GFP-tagged transgenes. (B) Immunofluorescence images of endogenous distal tip complex proteins in S2 cells. Plp is used as a mother centriole marker. Insets are 4x zoom.
the distance between the two Cep97 spots of mother-daughter centriole pairs. Line scans through the center of these two spots were generated and distance was measured from peak intensity to peak intensity and outer shoulder to outer shoulder (defined as 20% of the total fluorescence intensity) (Figure 3.5.A). Surprisingly, Cep104 depletion resulted in a shorter distance between the two Cep97 spots (Figure 3.5.B and C). Average Peak-to-Peak distance decreased by 9.91% (215 nm in Control to 193.7 nm in Cep104 RNAi) while Shoulder-to-Shoulder distance decreased by 5.72% (417.9 nm in Control to 394 nm in Cep104 RNAi). Although modest, growth of the distal centriole in S2 cells is already small, as most of the centriole is made up of the Sas6 cartwheel183. Additionally, technical limitations may also have limited our ability to observe larger distances. Finally, unlike Cep97 or CP110 knockdown in S2 cells which results in apparent centriole loss (see Figure 2.6, page 49), Cep104 depletion did not alter centriole number (Figure 3.5.D).
Figure 3.5 – Cep104 knockdown results in shorter centrioles. (A) Representative image and line scan graph detailing Peak:Peak and Shoulder:Shoulder measurements. Centrioles were stained with Plp and distal tips were stained with Cep97. (B and C) Dot plot showing mean±SD distance of (B) Peak:Peak or (C) Shoulder:Shoulder measurements between Cep97 spots in cells with control (Ctrl) or cep104 RNAi. n > 50 centrioles, Welch’s t-tests used to determine significance ** p < 0.01. (D) Bar graphs showing proportion of cells with <2, 2, or >2 centrioles after RNAi treatment. 100 cells per replicate, n = 3, bars represent mean±SEM, two-way ANOVA performed on distributions, n.s. not significant.
Figure 3.6 – Cep104 overexpression causes centriole elongation. (A) Overexpression of GFP-tagged transgenes in S2 cells. Bars represent mean±SEM, n = 3 replicates of 100 cells each, significance determined by two-way ANOVA and Tukey’s T-test, ** p < 0.01 * p < 0.05 (B and C) Dot plot of distance between Cep97 spots in cells expressing transgene for 5 days. (B) Peak:Peak distance and (C) Shoulder:Shoulder distance. Bars represent mean±SD, n > 50 centrioles, significance was determined by Welch’s t-test *** p < 0.001 **** p< 0.0001 (D) Gallery of 3D-SIM images from Cep104-GFP overexpression. Cells were immunostained with Plp to denote centrioles and Cep97 to identify distal tips.
Since Cep104 depletion results in shorter centrioles, we next asked if Cep104 overexpression would cause centrioles to elongate. Klp10A depletion in S2 cells causes centriole elongation, however, these centrioles are unstable and fragment, resulting in centriole amplification (see Figure 2.6, page 49). Therefore, we first used centriole number as an assay to test Cep104 overexpression induced centriole growth. Additionally, because large fluorescent tags can alter protein function, we tested both N-terminal (GFP-Cep104) and C-terminal (Cep104-GFP) GFP fusion constructs. Overexpression of Cep104-GFP, but not GFP-Cep104, resulted in a small, but significant, increase in the proportion of cells with more than the normal 2 centrioles (Figure 3.6.A). Additionally, 3D-SIM analysis of centrioles in Cep104-GFP overexpressing cells revealed that overexpressed Cep104 results in centriole elongation (Figure 3.6.B and C). Average Peak-to-Peak distance increased by 14.69% (210.4 nm in GFP-expressing control to 241.3 nm in Cep104 overexpression), while Shoulder-to-Shoulder distance increased by 8.28% (448.1 in control to 485.2 in Cep104 overexpression). Additionally, we see that Plp localizes as an elongated, U-shape in cells overexpressing Cep104 compared to a circle around the mother in control cells (Figure 3.6.D). This is indicative of a phenotype in Klp10A mutant spermatocytes where proximal proteins, such as Plp, begin to extend up the barrel of the over-elongated centriole.

To summarize, Cep104 localizes to the distal tips of centrioles. Although only slight differences, 3D-SIM analysis shows that Cep104 depletion leads to centriole shortening, while Cep104 overexpression causes centriole growth. Additionally, it appears that N-terminally tagged GFP-Cep104 does not influence centriole length, but does localize to the distal tip, making it ideal for localization studies. Taken together, we conclude that Cep104 is a bona fide member of the centriole length regulating DTC.

Section 3.3 – Cep104 interacts with Cep97 to target distal tips

Because Cep104 is a novel component of the DTC, we next wanted to understand how it is recruited to the distal tip of centrioles. Centriole assembly begins in S-phase when microtubules are recruited to the Sas6 cartwheel. The DTC is then recruited to the procentriole soon after that. It is currently unknown how the DTC identifies and targets the distal tips of centrioles; therefore, we investigate the role of Cep104 in DTC establishment.
Figure 3.7 – Cep104 requires the Jelly Roll and Coiled Coil to properly localize to the distal tip. (A) Immunofluorescence images of GFP-tagged Cep104 mutants expressed in S2 cells, Plp is stained as a centrosome marker. (B) Co-localization of GFP-Cep104 with Plp spots was quantified to show the percent of centrioles with Cep104 localization. n = 3 Error bars represent ±SEM. Significance determined by One-way ANOVA and Tukey’s Test (**** p<0.001 compared to Full-Length) (C) Of centrioles with Cep104 localization, the proportion of centrioles with 2 distinct spots was determined. n = 3 Error bars represent ±SEM. Significance determined by One-way ANOVA and Tukey’s Test (**** p<0.001 compared to Full-Length)
Next, we wanted to understand which domains of Cep104 are required to target centrioles. Therefore, S2 cells were transfected with GFP-tagged deletion mutant transgenes (Figure 3.7.A), then centrosomes were immunostained for Plp to assess colocalization (Figure 3.7.B). We see that neither the TOG domain nor the ZnF are required for distal tip localization (Figure 3.7.C and D). Constructs lacking the JR domain or the CC, however, have reduced localization to the centriole (Figure 3.7.C). Loss of JR shows severely reduced centriole localization. In order to assess distal tip localization, GFP-Cep104 was classified as either one or two spots. We cannot say if cells with one spot are localized to the distal tip, however, cells with two distinct spots likely localize to the distal tip. Even at centrosomes where the Cep104 mutant localizes, both the JR and CC mutants show severely reduced proportions of centrosomes with two distinct spots (Figure 3.7.D). Therefore, we conclude that the JR is required for Cep104 localization to the centriole while the CC is required for distal tip localization. However, further analysis using 3D-SIM is required to validate this conclusion.
To understand how these fragments promote Cep104 localization to the distal tip, we next assayed which centriolar proteins these regions interact. First, we surveyed a selection of centriolar proteins involved in procentriole assembly (Sas6, Ana2, and Sas4), centriole-to-centrosome conversion (Cep135, Ana1, and Asl), and the DTC itself (Cep97, CP110, Klp10A, and Cep104) to assess whether they could interact with Cep104 (Figure 3.8)12. We co-expressed GFP-tagged Cep104 mutant proteins with V5-Cep104 in S2 cells and performed GFP immunoprecipitations (IPs). Western blots for V5 revealed that Cep104 only interacts with Cep97 and itself at the centriole (Figure 3.8). This is in contrast with human Cep104, which also interacts with CP110.

We then wanted to understand which regions of Cep104 bind these targets. So, we generated GFP-tagged Cep104 fragments to perform GFP IPs in cells co-expressing full length V5-tagged targets (Figure 3.9.A and 3.10.C). First, we saw that Cep104 molecules primarily interact through their CCs (Figure 3.9.B). Mutants lacking the CC showed dramatically reduced binding to V5-Cep104, while adding the CC to the TOG domain restored binding to V5-Cep104 (Figure 3.9.B, lane 8 versus lane 7). Surprisingly, the JR also binds to V5-Cep104 on its own,

Figure 3.9 – Cep104 self-associations occur primarily through its coiled coils (A) Structural maps of Cep104 mutants with corresponding amino acid breakpoints and domain structure. (B) GFP-tagged Cep104 mutant proteins were co-expressed with V5-Cep104 in S2 cells followed by α-GFP IP from pre-cleared lysates (Inputs). n = 3
albeit weakly (Figure 3.9.B, lane 3). Cep104 binding to Cep97 was approached differently, as Cep104 deletion mutants never fully ablated Cep97 binding, suggesting a larger interface on the protein. (Figure 3.10.A). Therefore, we generated Cep104 mutants starting with the JR, which showed some binding with Cep97 and adding the subsequent domains (Figure 3.10.C). We saw that V5-Cep97 binds maximally to a complex interface of Cep104 that spans from the JR though the CC and the unstructured linker that precedes the TOG domain (aa. 1-453, Figure 3.10.B).

Using the same methods, we mapped the DTC interactome (data not shown) and confirmed these data by Yeast 2-Hybrid197 (collaborator’s data not shown). Together, we assembled a map of the DTC interactome to guide our understanding of its molecular assembly (Figure 3.11). First, Cep104 forms homotypic interactions through its JR (aa. 1-167) and homotypic interactions through its CC (aa. 202-318). Cep104 interacts with Cep97’s central region and C-terminus (aa. 352-806) through a complex interface that spans its JR, CC, and linker domain (aa. 1-453). Next, Cep97’s C-terminus (aa. 514-806) can interact with the N-terminus of CP110 (aa. 1-325). Finally, CP110 N-terminus (aa. 1-325) can interact with Klp10A’s N-terminus (aa. 1-201). The interaction map points to a central role for Cep97 in bridging the microtubule binding Cep104 with potential regulatory functions of CP110. This is congruent with observations that Cep97 is stably incorporated into the centriole after recruitment, whereas CP110 dynamically exchanges with the cytoplasm181. CP110 may also guide Klp10A localization to the site of the DTC to initiate catastrophe if microtubules grow past the distal tip183.

Because the centriole localizing regions of Cep104 interact with Cep97, we hypothesized that Cep97 may recruit Cep104 to the centriole. To test this, we knocked down DTC proteins using RNAi and expressed our GFP-Cep104 transgene in S2 cells to assess localization (Figure 3.12). While knocking down CP110 or Klp10A did not prevent localization of GFP-Cep104 to the centriole, knocking down Cep97 did. As a caveat, Cep97 depletion prevents centriole growth potentially confounding this conclusion. However, depletion of CP110 also blocks centriole growth without impairing Cep104 localization183.
**Figure 3.10** – Cep97 and Cep104 interact via Cep104’s N-terminus. (A and B) GFP-tagged Cep104 mutant proteins were co-expressed with V5-Cep97 in S2 cells followed by α-GFP IP from pre-cleared lysates (Inputs). n = 3 (A) GFP-tagged Cep104 fragments from Figure 3.7A. (C) Structural maps of Cep104 mutants used in panel B with corresponding amino acid breakpoints and domain structure.

**Figure 3.11** – Interaction map of the distal tip complex. Lines indicate fragments or regions of proteins where interaction was determined by co-IP only (black lines) or co-IP and Yeast 2-Hybrid (red lines). Abbreviations: JR – Jelly Roll, CC – Coiled Coil, ZnF – Zinc Finger array, LRR – Leucine Rich Repeat.
Figure 3.12 – Cep97 is required for proper localization of Cep104. Immunofluorescence images of cells expressing GFP-Cep104 after 12 day RNAi depletion of the indicated protein. Centrosomes are denoted by Plp. Insets are 2.5x zoom.

Figure 3.13 – Developing TOG mutants to disrupt microtubule binding. Microtubule co-sedimentations of purified Cep104 TOG domains with in vitro polymerized microtubules. S = supernatant (unbound) P = pellet (microtubule bound).
Section 3.4 – Does Cep104 bind microtubules to regulate centriole length?

In order to gain mechanistic insight into Cep104’s ability to promote centriole growth, we next wanted to ask which domains of Cep104 are required to promote growth. To that end, we are developing mutants of Cep104 lacking the CC, TOG, and ZnF to test in our centriole growth assay. Additionally, since we hypothesize that Cep104 needs to bind microtubules to promote centriole growth, we are testing point mutants in the TOG that may abrogate microtubule binding. TOG domains bind microtubules through their conserved heat repeats (HRs), therefore, we made point mutants in individual HRs and tested their ability to bind microtubules in vitro (Figure 3.13)\textsuperscript{254}. Although none of the point mutants completely abrogate microtubule binding, it appears that mutants in HR-B (A531E/V532E) show reduced co-sedimentation. Further testing is required to determine the extent that these mutations prevent this interaction.

Herein we introduce a new component of the Drosophila distal tip complex, Cep104. Cep104 is required for proper centriole growth in S2 cells and overexpression of Cep104 is sufficient to cause excessive centriole growth. We find that Cep104 localizes to the distal tip of the centriole by binding to Cep97 through its conserved JR and CC domains. Furthermore, we map the DTC interactome to inform future studies of DTC function. We believe that Cep104 is a missing piece that may help to explain why Cep97 and CP110 are required for centriole growth.

Section 3.5 – Modification and regulation of the distal tip complex

While Cep104 appears to promote centriole growth, questions regarding how the centriole grows when the inhibitory DTC is present remain. Even though Cep104 can promote centriole growth when overexpressed, evidence in flies suggests that endogenous Cep104 is present at the distal tip even when centrioles are not growing\textsuperscript{246} (collaborator’s data not shown). We hypothesize that phospho-regulation of the DTC is likely responsible for converting DTC functions to promote timely growth. This would allow for the activation of growth factors or deactivation of inhibitory DTC components when necessary for centriole growth.

This is not unfounded as studies have found that CP110 and Klp10A are phosphorylated by different kinases. Klp10A is phosphorylated in its motor domain by Casein Kinase 1α to reduce its ability to depolymerize microtubules\textsuperscript{255}. Whereas CP110 is a Cdk2 substrate\textsuperscript{180}. Additionally, Tau Tubulin Kinase 2 is required for Cep97/CP110 removal during ciliogenesis\textsuperscript{256}. However, none of these phosphorylation events have been linked to centriole growth. As we develop a mechanistic
understanding of centriole length maintenance, the next step to understanding centriole growth will likely take the form of regulation of these essential proteins. Recent evidence suggests that centriole growth occurs during mitosis, potentially pointing to mitotic kinases as centriole growth regulators. Because the essential centriole duplication kinase, Polo-like kinase 4 (Plk4), is active during mitosis\(^\text{213}\), I tested whether Plk4 could phosphorylate the DTC. I found that, in fact, Plk4 can phosphorylate DTC proteins can be in vitro (Figure 3.14). Also, previous screens containing DTC components showed phosphorylation of Cep97 by Plk4 in vitro\(^\text{178}\). Moreover, CP110 phosphorylation by Plk4 in human cells has been linked to centriole biogenesis, albeit not, specifically, growth\(^\text{257}\). Although we do not yet know what the function of these phosphorylation events are, the interaction mapping in this study will provide a framework for how phosphorylation may affect interactions within the DTC.

**Figure 3.14** – Klp10A is phosphorylated by Plk4 in vitro. GST-tagged Klp10A fragments (See Figure 3.11, page 84) were bacterially expressed and purified, then incubated with purified Plk4 kinase domain (1-317) and radioactive γ\(_{\text{32}}\)-P-ATP to test Plk4’s ability to phosphorylate Klp10A. Reactions were then resolved by SDS-PAGE and exposed to film to examine radioactive decay (autoradiograph). Experiment performed by Dr. Daniel Buster.

Section 3.6 – Materials and methods

**Cell culture, transfection, and RNAi**

*Drosophila* S2 cells (Invitrogen) were cultured in Sf-900II SFM media (Life Technologies) as previously described\(^\text{258}\). dsRNA and DNA plasmids were transfected into S2 cells by nucleofection as previously described\(^\text{205}\). For immunoprecipitation assays, 40 μg dsRNA was
transfected on day 0 and day 4. 2 µg of DNA was transfected on Day 4, concurrent with dsRNA transfection. For immunofluorescence microscopy and immunoblotting assays, cells were transfected with 40 µg dsRNA on days 0, 4, and 8. 2 µg of DNA was transfected on days 4 and 8, concurrent with dsRNA tranfection. For all experiments, gene expression was induced with CuSO₄ on Day 5 and cells were maintained in CuSO₄ throughout the remainder of the experiment. Control dsRNA was generated as previously described²⁰⁵. dsRNA targeting Klp10A UTR, CP110 UTR, and Cep104 UTR were synthesized from PCR product of 3’ UTR sequences of ESTs fused to the T7 RNA polymerase promoter sequence: 5’-TAATACGACTCACTA. dsRNAs targeting Cep97 was synthesized from the PCR product of the T7 RNA polymerase promoter sequence fused to 5’-3’ UTR fusion constructs in which the Cep97 coding region was deleted from the EST by mutagenesis. In vitro dsRNA synthesis was prepared as previously described²⁰⁵.

**Bacterial expression, protein purification, and antibody generation**

Expression and purification of recombinant protein was performed as previously described²⁰⁵. Briefly, BL21 DE3 (New England Biolabs) grown to log phase were induced with 0.4 mM IPTG then temperature shifted from 25˚C to 14˚C and incubated overnight. Cells were then lysed and incubated with the appropriate resin (MBP-tagged proteins with amylose resin (New England Biolabs), GST-tagged proteins with glutathione resin (GOLDBIO), His₆-tagged proteins with HisPur resin (Thermo Fisher Scientific)). Resin was washed by gravity flow, eluted, and dialyzed into PBS. Protein was then conjugated to KLH and injected into either 2 chicken (His₆-Cep97-514-806), 3 rats (MBP-Klp10A-1-201), 2 guinea pigs (MBP-CP110-326-549), or 2 rabbits (GST-Cep104-730-941) by Pocono Rabbit Farm and Labs. Antibodies were then affinity purified by incubating antiserum with GST-tagged versions of each protein fragment conjugated to Affi-gel 10 resin (BIO-RAD). Cep97 antibodies were further immunodepleted against BL21 DE3 lysate conjugated to Affi-gel 10 resin and flow-through was collected.

**Immunoblotting**

Cells were lysed in Lysis Buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, and 0.1 mM PMSF), concentrations were determined by Bradford, followed by addition of Laemmli sample buffer. Extracts were boiled for 5 min and stored at -20°C. Samples of equal total protein were resolved by SDS-PAGE, transferred onto nitrocellulose (GE Healthcare), probed with primary and secondary antibodies, and scanned on a LiCor Odyssey CLx imager (Li-Cor Biosciences). Primary antibodies used for western blotting include rat anti-Klp10A
(this study, 1:1000), guinea pig anti-CP110 (this study, 1:1000), chicken anti-Cep97 (this study, 1:1000), rabbit anti-Cep104 (this study, 1:1000), mouse anti-V5 monoclonal (Thermo Fisher Scientific, 1:3000), mouse anti-GFP monoclonal JL8 (Thermo Fisher Scientific, 1:3000), and mouse anti-α-tubulin monoclonal DM1A (Thermo Fisher Scientific, 1:3000). Antibody were diluted in western blocking buffer (5% milk in PBS-0.1% Tween-20). Host animal specific IRDye 800CW secondary antibodies (Li-Cor Biosciences) were prepared according to the manufacturer’s instructions and used at 1:3000 dilution.

**Immunofluorescence microscopy**

S2 cells were spread on concavalin A-coated glass bottom plates/cover slips and fixed in ice cold methanol for 15 minutes or 10%Paraformaldehyde in HL3 (5 mM HEPES, pH 7.2, 5 mM Trehalose, 10 mM NaHCO3, 70 mM NaCl, 5 mM KCl, 20 mM MgCl2, 115 mM Sucrose) + 1 mM EGTA for 12 minutes. Cells were washed with PBS-0.1% Triton X-100 and blocked in slide blocking buffer (5% normal goat serum in PBS-0.1% Triton X-100) for 30 minutes at room temperature. Primary antibodies were diluted in blocking buffer and slides were incubated overnight at 4°C (rabbit anti-Plp (1:3000), rat anti-Klp10A (1:1000), guinea pig anti-CP110 (1:3000), chicken anti-Cep97 (1:5000), rabbit anti-Cep104 (1:1000), rat anti-Asl (1:1000)). After 3 washes with PBS-0.1% Triton X-100, cells were incubated at room temperature with Hoechst 33342 (Thermo Fisher Scientific, 3.2 μM final concentration) and secondary antibodies diluted in blocking buffer for 30 minutes (anti-host animal AlexaFluor 488 (Thermo Fisher Scientific, 1:1500), anti-host animal Rhodamine Red-X (Jackson Immunoresearch, 1:1500), and anti-host animal Cy5 (Jackson Immunoresearch 1:1500)). Slides were then washed 3 times with PBS-0.1% Triton X-100 and mounted with homemade mounting medium (PBS, 90% glycerol, and 0.1 M propyl gallate) for deconvolution microscopy or Prolong Glass (ThermoFisher) for slides for 3D-SIM. Deconvolution microscopy was performed on the DeltaVision Core system (GE Healthcare) equipped with an Olympus IX71 microscope, 100x objective (NA 1.4), and a CoolSnap HQ2 CCD camera (Photometrics). Images were acquired and processed with softWoRx v7.00 (GE Healthcare). 3D-Structured Illumination Super-Resolution microscopy was performed using a Zeiss ELYRA S1 (SR-SIM) microscope using solid-state (405/488/561/642 nm) laser lines, a 63x objective (NA 1.4), 3 pattern rotations, and an EM-CCD camera (Andor iXon). Images were acquired with Zeiss ZEN Black 2.3 software followed by SIM reconstruction, channel alignment, and maximum intensity projection. Protein colocalization and centriole counts were determined...
manually from micrographs. Centriole length measurements were performed in Fiji (ImageJ) and
statistics and graphs were prepared in GraphPad Prism 8 (GraphPad).

Constructs

To generate S2 cell expression constructs, full-length cDNAs Cep97 (DGC EST library
1.0), CP110 (DGRC, RE58503), Klpl0A (DGC EST library 1.0), and Cep104 (DGC EST library
1.0)) were PCR amplified (Phusion polymerase, ThermoFisher) and subcloned into pMT/V5 His
C vector containing in-frame coding sequences for EGFP or V5 under the control of a
metallothionein inducible promoter. PCR-based site-directed mutagenesis was used to make
deletion mutants. To generate bacterial expression constructs, fragments of full-length cDNAs
were PCR amplified and subcloned in-frame into pMal-c2x (MBP-tagged constructs), pGEX-6p2
(GST-tagged constructs), or pET28A (His6-tagged constructs).

Immunoprecipitation assays

Immunoprecipitation assays were performed as previously described105. Recombinant,
purified GFP-Binding Protein fused to human Fc domain was bound to magnetic Protein A
Dynabeads (ThermoFisher), and then cross-linked with DMP. Coupled Dynabeads were stored in
PBS-0.1% Tween 20 at 4˚C. Prior to use, beads were equilibrated in IP buffer (50 mM Tris, pH
7.2, 125 mM NaCl, 1 mM EGTA, 1 mM DTT, 0.5% Triton X-100, 1x SigmaFast Protease
inhibitors, 0.1 mM PMSF, and 1 μg/mL SBTI). Transfected cells were harvested and lysed in IP
buffer, concentration was determined by Bradford assay, and lysates were diluted to 5 mg/mL.
Lysates were then clarified by centrifugation at 10,000 xg for 5 minutes at 4˚C and inputs were
made from pre-cleared lysates in Laemmli Buffer. GBP-coated beads were rocked with lysates for
30 min at 4˚C, washed four times by resuspending beads in 1 mL IP buffer, transferred to a new
tube during the final wash, then boiled in 2x Laemmli sample buffer.

Microtubule co-sedimentation

Microtubule co-sedimentations were performed as previously described259. Briefly,
porcine tubulin was polymerized BRB80 buffer containing 1 mM GTP and 1 mM DTT using
increasing concentrations of Paclitaxel (final concentration 20 µM). Purified His6-TOG domain
and mutants were incubated with polymerized microtubules for 20 minutes and sedimented for 30
minutes at 70,000 xg. Supernatant was removed and microtubule pellet was resuspended in an
equal volume of 2x Laemmli sample buffer. Then equal volumes of supernatant and pellet were
resolved by SDS-PAGE to assess co-sedimentation.
CHAPTER 4 – Plk4 homodimerization precedes relief of autoinhibition

Cells must duplicate centrioles once and only once per cell cycle to ensure they enter mitosis with two centrosomes. Precisely regulated mechanisms exist to promote timely centriole duplication and prevent over-duplication – a process known as centriole amplification. Polo-like Kinase 4 (Plk4) is central to mechanisms promoting centriole duplication and, thus, is considered the master regulator of centriole duplication. Plk4 is both required for duplication and overexpression is sufficient to cause centriole amplification. Therefore, many of the mechanisms governing centriole duplication are centered around maintaining Plk4 levels and controlling Plk4 activity to ensure that duplication is spatially and temporally regulated.

Many salient mechanisms that regulate Plk4 involve its ability to self-regulate. Prominently, Plk4 activation results in its own destruction. Plk4 autophosphorylation of a regulatory element downstream of its kinase domain generates a phospho-degron that is recognized by the SCF \( \text{Slimb} \) E3 ubiquitin ligase (\( \beta \)-TrCP is the F-box in humans). Plk4 activation results in its degradation during most of the cell cycle, keeping levels below the threshold needed for centriole assembly. In late mitosis, however, Plk4 levels rise to promote centriole duplication. The PP2A subunit Twins (Tws) becomes dominant at the end of mitosis, allowing dephosphorylation of Plk4’s phospho-degron preventing recognition by SCF \( \text{Slimb} \) and subsequent proteasomal degradation. High levels of Plk4 can then phosphorylate substrates at existing centrioles to trigger duplication during the next cell cycle.

Plk4 is not always active, however. When a Plk4 molecule is first translated, it is made in an autoinhibited state that prevents activation. While the mechanism of autoinhibition is poorly understood, it requires a linker downstream of the kinase domain (Linker 1 or L1) to maintain this state. Relief of autoinhibition occurs when two Plk4 molecules homodimerize. Plk4 homodimerizes through its unique tandem Polo box domain consisting of Polo box 1 (PB1) and Polo box 2 (PB2). Dimerized Plk4 molecules can then activate their partner in trans through a mechanism which requires their PB3 domains. The mechanism underlying relief of autoinhibition is still poorly understood as well, however we currently lack the tools to dissect dimerization from the other functions of PB1/2.
Herein, we develop a Plk4 mutant that blocks dimerization while retaining the ability of PB1/2 to bind other known targets and be ubiquitinated by Slimb. Using this tool, we confirm that homodimerization is required for efficient activation of Plk4 and uncover a new mechanism that regulates Plk4 levels. Surprisingly, we find that dimerization is required for Plk4 to bind the

**Figure 4.1** – Developing a Plk4 Dimerization Mutant. (A) Cartoon diagraming the domain structure of Plk4. DRE – Downstream Regulatory Element, PB – Polo box (B) Ribbon representation of *Drosophila* PB1/2 dimers highlighting the (B’) interaction interface. Contribution of individual amino acids and the mutations to be made on the (B’’) α-helix and (B’’’) β-strand. (C) GFP IPs performed from pre-cleared lysates from S2 cells co-expressing Plk4-GFP or myc-tagged Plk4 mutants.
activator Asterless. Finally, we temporally dissect the steps required for relief of autoinhibition and update an existing model of Plk4 activation.

Section 4.1 – Developing a Plk4 dimerization mutant

Plk4 homodimerization occurs through its conserved tandem Polo box domain (PB1/2). Crystal structures of PB1/2 from multiple species, including *D. melanogaster*, *C. elegans*, and *H. sapiens*, have been determined and revealed a conserved mechanism of homodimerization (Figure 4.1.B)\(^{266,267}\). Polo box domains consist of a β-sheet of 6 anti-parallel β-strands with an intervening α-helix positioned behind the sheet. Plk4 homodimerization is coordinated by residues from the α-helix and 6\(^{th}\) β-strand at the C-terminus of each PB2, creating a composite β-sheet from the 12 β-strands of both molecules with anti-parallel α-helices underneath (Figure 4.1.B').

To generate the dimerization mutant, we mutated the residues on these different regions to either change their charge or add bulkiness to the residue. Dimerization mutant 1 (DM1, Figure 4.1.B'') targets 4 residues on the α-helix (I573H, A576D, L577A, V579D) and dimerization mutant 2 (DM2, Figure 4.1.B''') targets 4 residues on the β-strand (V590D, I592T). We then tested the dimer mutants’ abilities to bind wild-type Plk4 by performing co-immunoprecipitations (IPs). GFP-tagged wild type Plk4 (WT) was co-expressed in S2 cells with myc-tagged mutants followed by GFP IPs. While WT-myc co-IPs with Plk4-GFP, neither dimerization mutant nor a mutant with all 6 amino acid mutations bind Plk4-GFP (Figure 4.1.C).

Next, we wanted to understand how the dimerization mutations effect Plk4 function. Homodimerization is required for Plk4 activation and *trans*-autophosphorylation of the phosphodegron\(^{264}\). Therefore, Plk4 mutants that cannot dimerize should have increased levels compared to their wild type counterparts. To test this, we expressed Plk4 mutants in cells and performed western blots to assay for Plk4 levels (Figure 4.2.A). When expressed at low levels, WT is efficiently degraded and, thus, undetectable by western blot. Kinase-dead Plk4 (KD), however, is dramatically stabilized because it cannot generate its phospho-degron. When the dimerization mutants are expressed in cells, we see that levels of mutant Plk4 are higher than WT but are not stabilized as much as KD (Figure 4.2.A). Additionally, we see that the dimer mutants run as a single, fast-migrating, unphosphorylated band by SDS-PAGE. This suggests that homodimerization mutants have reduced capacity to autophosphorylates, but still have some basal level of activity. Since each of the dimerization mutants were stabilized to similar levels,
we decided to continue future experiments with DM2 in an attempt to minimize confounding variables since it contained fewer mutations than DM1.

Previously, it was shown that kinase dead Plk4 can be trans-autophosphorylated when co-expressed with wild type Plk4, allowing it to be recognized and degraded by SCF\textsuperscript{Slimb} \textsuperscript{264, 265}. Therefore, we used the same assay to see if co-expression of Plk4 with DM2 can promote DM2 destruction (Figure 4.2.B). We, again, saw that Plk4 WT can trans-autophosphorylate Plk4-KD, resulting in a band shift upwards and KD degradation. However, WT cannot trans-autophosphorylate or promote the destruction of DM2, consistent with the idea that DM2 is a bona fide dimerization mutant (Figure 4.2.B). Therefore, we conclude that homodimerization promotes efficient activation and destruction of Plk4 but is not absolutely required.

Section 4.2 – Plk4 can activate independent of homodimerization

Previous studies have shown that relief of Plk4 autoinhibition is normally stimulated by homodimerization\textsuperscript{264, 265}. However, when Plk4 concentrations are sufficiently high, Plk4 deletion mutants lacking PB1/2 have kinase activity without dimerization\textsuperscript{197, 270} (see Figure 3.14, page 68). Therefore, we wanted to test the activity of Plk4 DM at extreme concentrations to determine if it can activate without dimerization.

---

**Figure 4.2** – Plk4 dimerization mutants have reduced activity. (A) Western blots of whole cell lysates from S2 cells expressing myc-tagged Plk4 mutants. Graph of normalized myc band intensity relative to kinase dead. n = 3, one way ANOVA and Tukey’s multiple comparisons test were used to determine significance, **** p < 0.0001
When Plk4 is active, it becomes hyperphosphorylated and can be detected as a slower migrating band when resolved by SDS-PAGE. Using a metallothionein promoter, we can tune the expression of transgenes by changing the concentration of CuSO4 in the media\textsuperscript{258}. Therefore, we tested increasing expression of Plk4 mutants and looked for band shifts by western blot to assay Plk4 activity (Figure 4.3.A). Slower migrating species of Plk4 WT are present at low expression levels, indicative of dimer-induced activation. Plk4 KD, however, never band shifts – appearing as a single, faster migrating, unphosphorylated species at high CuSO4 concentrations. At low expression levels, Plk4 DM2 appears as an unphosphorylated band, however, dramatic overexpression of DM2 results in a band shift. Additionally, we see that DM2, but not KD, can generate a phospho-degron and recognized by SCF\textsuperscript{Slimb} as evident by co-IP (Figure 4.3.B). Therefore, dimerization is not absolutely required for activation of Plk4, but dimerization appears to increase the efficiency of Plk4 activation.

However, if dimerization is required for relief of autoinhibition and activation, how is Plk4 DM2 activated? To answer this, we first observed Plk4 localization in S2 cells. We observed that Plk4 KD and Plk4 DM2, but not Plk4 WT, form large puncta in cells that do not colocalize with centrioles (Figure 4.3.C). We believe that it is within these puncta, where Plk4 concentrations are much higher than in the cytoplasm, that Plk4 DM2 is activated. Again, this behavior is similar to activation of Plk4 mutants lacking PB1/2 \textit{in vitro}\textsuperscript{197}.

While Plk4 predominantly regulates its levels by homodimerization and trans-autophosphorylation, the ability of Plk4 to aggregate and activate may be an auxiliary mechanism to prevent Plk4 accumulation in cells. Conversely, previous research has shown that Plk4 can undergo liquid-liquid phase separation (LLPS) at the centriole to activate and promote centriole duplication\textsuperscript{204}. Since LLPS occurs above concentration thresholds, we may be creating an artifactual condensate by overexpressing Plk4 mutants that are not regulated by auto-destruction. Regardless, we believe that we have found a novel, dimerization-independent mechanism of Plk4 activation that can limit Plk4 levels. While the purpose of this study is to dissect dimerization’s role in Plk4 activation, it is important to consider this secondary mechanism of activation as a potential caveat in each of the subsequent experiments.
Section 4.3 – Plk4 dimerization is required to bind Asterless

In cells, Plk4 is not autonomously regulated. Instead, Plk4 activators and inhibitors are required to shepherd Plk4 to the centriole, shield it there, and promote its proper activation. In *Drosophila*, many of these tasks are controlled by different domains on the essential centriole gene Asterless (Asl)\(^{189, 271}\). Asl is an extended coiled coil protein that resides in the bridge zone of the centriole, connecting the centriole core to the pericentriolar material\(^{272}\). Previously, our lab and others have found that Asl recruits Plk4 to the centriole prior to centriole duplication\(^{189, 190, 270, 271}\). Additionally, we have found that Asl is required for the efficient activation of Plk4, as Asl knockdown results in Plk4 running as a single unphosphorylated band on a western blot\(^{190, 270}\). Since Asl is a known Plk4 activator, we wanted to know how Asl regulates our dimerization mutant.

We therefore expressed Plk4 in cells depleted of Asl and assayed for Plk4 levels. As expected, Asl depletion results in the stabilization of WT, which runs as a single, unphosphorylated band (Figure 4.4.A). This is consistent with previous findings that show Asl is required for efficient Plk4 activation and destruction. Additionally, Asl depletion does not influence KD levels. Surprisingly, however, Asl depletion does not alter levels of DM2, suggesting Asl does not activate DM2. Additionally, we find that DM2 levels are similar to WT levels after Asl depletion.

Asl function is predicated on the idea that it brings 2 Plk4 molecules together to catalyze homodimerization at low Plk4 concentrations\(^{190}\). Therefore, we tested the idea that overexpression of Asl would be sufficient to promote homodimerization of Plk4, even when it can, itself, not dimerize. Asl has two Plk4 binding regions that perform different functions. Asl has been experimentally dissected into 3 regions (Asl-A, -B, -C). Asl-C is the centriole targeting domain and is sufficient to cause centriole amplification when overexpressed, but Asl-A has a more subtle role\(^{189, 190, 270}\). Asl-A binds to Plk4 and, in turn, is phosphorylated by Plk4\(^{270}\). Unphosphorylated Asl-A is a Plk4 activator, while phosphorylated Asl-A is a Plk4 inhibitor. Since Asl-A is known to stimulate Plk4 activity, we asked if Asl-A could force DM2 to dimerize with Plk4 and stimulate activity.
To do this, we performed co-IPs of Plk4-GFP with either myc-tagged WT or DM2 in the presence or absence of V5-tagged Asl-A. Since it was thought that Asl-A stimulates kinase activity by bringing 2 Plk4 molecules together, we expected Asl-A to force dimerization of DM2 with WT. However, we saw that Asl-A cannot overcome the need of Plk4 to autonomously

**Figure 4.3** – A new mechanism of Plk4 activation. (A) Expression of GFP-tagged Plk4 mutants at various CuSO₄ concentrations. Arrows indicated band shifts. (B) GFP IPs from cells expressing GFP-tagged Plk4 mutants at high concentrations. (C) Immunofluorescent images from cells expressing Plk4-GFP mutants. Plp used to stain centrosomes. Scale Bar – 4 µm
To test why, we next asked if Plk4 DM2 could bind Asl-A. IPs were performed in cells expressing Plk4 mutants and, although DM2 could bind endogenous Asl-A, it had dramatically reduced binding compared to WT or KD when normalized to Plk4 levels (Figure 4.3.C). Therefore, we tested if DM2 could bind Asl-A. V5-tagged Asl-A was co-expressed with Plk4 mutants in cells depleted of endogenous Asl. We found that, indeed, Plk4 DM2 was unable to bind Asl-A (Figure 4.5.A). However, this was surprising to us, as Asl-A is known to bind a basically charged groove created by Plk4 PB1/2 dimers that is not disrupted by the DM2 mutations. So, if Asl-A were to promote dimerization of 2 Plk4 molecules by binding the groove of each monomer, then this function should be preserved in our mutants. Therefore, rather than forcing dimerization of Plk4 in solution, we hypothesize that Asl-A promotes Plk4 activation by binding to dimers in solution and relieving autoinhibition. Along with the observation that Plk4 cannot efficiently activate without Asl, we conclude that homodimerization alone is not sufficient to activate Plk4. Instead, dimers need an activating protein, Asl, before they will trans-autophosphorylate.

To further dissect this mechanism, we next wanted to understand the specific role of Asl-A in Plk4 activation. Therefore, we made a mutant in Plk4 previously shown to disrupt Plk4’s interaction with Asl-A. Indeed, the R490E mutation that changes the charge of the basically charged interface, cannot bind to Asl-A (Figure 4.5.A), but does not interfere with Plk4’s ability to homodimerize (Figure 4.5.B). Additionally, like Plk4 DM2, R490E is nominally stabilized and runs as a single de-phosphorylated band (Figure 4.5.C). Taken together, we believe that Asl binding is required to facilitate homodimerization-induced Plk4 activation.
Section 4.4 – Is Plk4 homodimerization required to relieve autoinhibition but dispensable for Plk4 catalytic activity?

In order for Plk4’s kinase domain to become fully active, it is thought that two steps must happen. First, relief of Linker 1 (L1) enforced autoinhibition\textsuperscript{261, 264}. Second, phosphorylation of Plk4’s T loop. However, the contribution of homodimerization to each of these steps is not understood. The current model assumes that homodimerization relieves autoinhibition and Plk4 molecules phosphorylate their partner’s T loop in \textit{trans}\textsuperscript{264, 265}. While Plk4 WT can phosphorylate KD T loop in \textit{in trans}, these studies were at high concentrations of Plk4, which, we saw was able to overcome the need for normal dimerization control mechanisms. Therefore, we plan to use our separation of function mutants to dissect the role of homodimerization in each of these steps.
After Plk4 becomes active, relief of autoinhibition is reinforced by phosphorylation of L1. L1 phospho-mimetic mutants (L1PM) are unable to maintain autoinhibition and, as a result, do not require canonical mechanisms for relief of autoinhibition\textsuperscript{264}. For instance, Plk4 L1PM no longer requires PB3 to activate in cells. Therefore, we have mutated our DM2 and Asl-binding R490E mutants to also contain the L1PM. By relieving autoinhibition independent of dimerization (L1PM/DM2), we can test the ability of Plk4 to perform \textit{cis}-autophosphorylation of its T loop in S2 cells and \textit{in vitro}. To that end, we have developed a phospho-specific antibody towards Plk4’s T loop (\textbf{Figure 4.6}) and validated it \textit{in vitro}.

Finally, we can test the role of Asl-binding during Plk4’s activation cycle. L1PM/R490E mutants of Plk4 will allow us to determine if Asl binding is merely required for relief of autoinhibition or if it also plays a role in activating Plk4 kinase activity.

\textbf{Figure 4.5} – Plk4 must dimerize to bind Asterless (A) GFP IPs from pre-cleared lysates from S2 cells depleted of endogenous Asl and co-expressing V5-AslA and GFP-tagged Plk4 mutants. (B) GFP IPs from pre-cleared lysates from S2 cells co-expressing Plk4-R490E-myc and GFP-tagged Plk4 mutants. (C) Western blot from S2 cells expressing myc-tagged Plk4 mutants.

\textbf{Section 4.5 – A revised model for Plk4 activation}

While the physical mechanisms enforcing Plk4 autoinhibition remain elusive, we created dissection of function mutations to polish a model of Plk4 activation and challenge assumptions from previous reports. First, Plk4 is made in a monomeric, autoinhibited state\textsuperscript{261, 264}. Plk4 homodimerization occurs through the C-terminus of the tandem polo box, however, that is not
sufficient to activate Plk4\textsuperscript{266, 267}. Instead, homodimerization creates a composite surface upon which Plk4 activators, such as Asl can bind\textsuperscript{267}. Plk4 activators can then coordinate with PB3 to relieve Plk4 autoinhibition\textsuperscript{268, 269}. Once autoinhibition is relieved, Plk4 dimers can then autophosphorylate\textsuperscript{265}. While they appear to be capable of phosphorylating in \textit{trans}, it is not essential, as open Plk4 can also autophosphorylate in \textit{cis}.

In the absence of dimerization or Asl, Plk4 can use an alternative pathway of activation. By clustering in the cytoplasm, Plk4 can activate to generate a phospho-degron. The idea of local aggregation is consistent with a recent report that Plk4 is found in the cytoplasm as monomers/dimers even though it is capable of phase separating under certain conditions\textsuperscript{273}. We believe that this aggregation mechanism may be a way to prevent local concentration of Plk4 outside of the centriole.

Finally, Asl-A is phosphorylated by Plk4 after it activates Plk4\textsuperscript{270}. In this way, Plk4 generates its own inhibitor. The requirement for Asl to trigger homodimerization-mediated relief of autoinhibition suggests that all active Plk4 is immediately inhibited, regardless of the concentration of Asl in the cell. This may provide an additional level of regulation that prevents centriole amplification, even during mitosis when normal centriole duplication is occurring. Meaning that even when dimerized Plk4 is being dephosphorylated and will, thus, not be degraded, it is still inactive since it is obligatorily bound to Asl-A\textsuperscript{263}.

The discovery that Plk4 homodimerization is not sufficient to activate the kinase underlies the spatial regulation of Plk4. Localization of Plk4 inhibitors and activators to the centriole can concentrate inactive Plk4 and stimulate rapid activation to initiate the centriole assembly pathway. Conversely, if active Plk4 was recruited to the centriole, cells risk spontaneous Plk4 concentration and \textit{de novo} centriole assembly in the cytoplasm. Therefore, the requirement for Plk4 activators can ensure that new centrioles only assemble on the side of existing centrioles and prevent centrosome amplification due to \textit{de novo} centriole assembly.

\textbf{Section 4.6 – Materials and methods}

\textbf{Cloning and dsRNA generation}

PCR-based site-directed mutagenesis (Phusion polymerase, Thermo Fisher) was used to make point mutants in previously generated Plk4 (GFP and myc) constructs in the pMT/V5 His C vector. pFastBac1 constructs were made sequentially by first using restriction cloning (SpeI-NotI)
(New England Biosciences) to insert Plk4 cDNA C-terminally tagged with V5 and His6 tags. Next Maltose Binding Protein (MBP) with a Precission Protease site was PCR amplified from pMal-c2x and cloned into pFastBac1 Plk4-V5-His6 using megaprimer mutagenesis. Control dsRNA or dsRNA targeting Asl was synthesized from PCR product fused to the T7 RNA polymerase promoter sequence: 5’-TAATACGACTCACTATAGG. In vitro dsRNA synthesis was prepared as previously described205.

**Cell culture, transfection, and RNAi**

*Drosophila* S2 cells were cultured in Sf-900II SFM media (Life Technologies) as previously described258. dsRNA and DNA constructs were transfected into S2 cells by nucleofection, as previously described205. Cells were transfected with up to 2 µg of DNA. During RNAi, cells were transfected with 40µg dsRNA on days 0, 4, and 8. DNA constructs were transfected concurrent with dsRNA on days 4 and 8. Gene expression was induced with 0.25 mM CuSO4 for 48 hours unless otherwise stated.
**Antibody generation**

Plk4 phospho-Tloop antibodies were raised in rabbits against synthesized phospho-peptide [ ] (Pocono Rabbit Farm and Laboratories). Serum was then affinity purified against the phospho-peptide and further immuno-depleted against non-phosphorylated peptide.

**Cell lysates and immunoblotting**

Cells were lysed in Lysis Buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, and 0.1 mM PMSF), concentrations were determined by Bradford, followed by addition of Laemmli sample buffer. Extracts were boiled for 5-10 min and stored at -20°C. Equal total protein amounts of whole cell lysate were resolved by SDS-PAGE, transferred onto nitrocellulose (Amersham), incubated in blocking buffer (5% milk in PBS-0.1% Tween-20), probed with primary and secondary antibodies, and scanned on a LiCor Odyssey CLx imager (LiCor Biosciences). Primary antibodies used for western blotting include rat anti-Cep135 (1:1000, McLamarrah et al., 2018), guinea pig anti-Slimb (1:1000, Klebba et al., 2015), rat anti-Asl (1:1000, Boese et al., 2018), mouse anti-V5 monoclonal (1:3000, Life Technologies), mouse anti-myc (1:3000, Cell Signaling), mouse anti-GFP monoclonal JL8 (1:3000, Clontech), and mouse anti-α-tubulin monoclonal DM1A (1:3000, Sigma-Aldrich). Antibody were diluted from 1:1000-1:3000 in blocking buffer. Plk4-pTloop immuno-blotting was performed using different blocking (5% BSA in TBS-0.1% Tween-20) and washing buffers (TBS-0.1% Tween-20) were used. Primary antibody host-specific IRDye 800CW or IRDye 680CW secondary antibodies (Li-Cor Biosciences) were prepared according to the manufacturer’s instructions and used at 1:3000 dilution.

**Immunoprecipitation assays**

IPs were performed as previously reported\textsuperscript{105}. GFP-binding protein (GBP) was fused to the Fc domain of human IgG (pIg-Tail) (R&D Systems), tagged with His\textsubscript{6} in pET28a (EMD Biosciences), expressed in *E. coli* and purified on HisPur Cobalt resin (Fisher) according to manufacturer’s instructions. Purified GBP was bound to magnetic Protein A Dynabeads (ThermoFisher), and then cross-linked by incubating with 20 mM DMP in PBS, pH 8.3 for 2 hours at room temperature followed by quenching with 0.2 M ethanolamine, pH 8.3 for 1 hour at room temperature. Coupled Dynabeads were stored in PBS-0.1% Tween-20 at 4°C. Prior to use, beads were equilibrated in IP buffer (50 mM Tris, pH 7.2, 125 mM NaCl, 1 mM DTT, 0.5% Triton X-100, 1x SigmaFast Protease inhibitors [Sigma], 0.1 mM PMSF, and 1 μg/mL SBTI).
Transfected cells were harvested and lysed in IP buffer, concentration was determined by Bradford, and lysates were diluted to 5 mg/mL. Lysates were then clarified by centrifugation at 10,000 xg for 5 minutes at 4°C and inputs were made from pre-cleared lysates in Laemmli Buffer. GBP-coated beads were rocked with lysates for 30 min at 4°C, washed four times by resuspending beads in 1 ml IP buffer, transferred to a new tube during the final wash, then boiled in 2xLaemmli sample buffer. Inputs and IPs were then analyzed by western blot.

**Bacterial expression and protein purification**

His\textsubscript{6}-tagged fusion constructs were cloned into pET28, then transformed into BL21 DE3 (NEB) strain of *E. coli*. Liter cultures (LB+ with kanamycin) were grown to around OD\textsubscript{600} 0.6 (8-10 hrs) at 23°C then induced with 0.1 mM IPTG for 16-18 hour at 14°C. Bacterial pellets were then frozen at -80°C. Baculovirus-derived pellets were prepared in Sf9 cells according to manufacturer’s protocols (Gibco). Pellets were thawed, resuspended in Wash buffer (25 mM Tris-HCl pH 8.0, 300 mM NaCl, 1% Triton X-100, 0.1 mM β-mercaptoethanol, 0.1 mM PMSF, and 10 mM Imidazole), and lysed by passing through an Avestin homogenizer. His\textsubscript{6}-tagged constructs were then immobilized on NiNTA resin (Roche) and washed on a 20-mL chromatography column with 60 mL Wash buffer, 20 mL High Salt Wash (25 mM Tris-HCl pH 8.0, 500 mM NaCl, and 0.1 mM β-mercaptoethanol, and 10 mM Imidazole), and 20 mL Heat Shock Protein removal buffer (25 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM MgCl\textsubscript{2}, and 10 µM ATP). 8 protein fractions were eluted from resin by incubating in Wash Buffer + 200 mM imidazole for 5 minutes each. Finally, purified protein was concentrated, exchanged into appropriate running buffer, and resolved by SDS-PAGE to test for purity.

**Immunofluorescence microscopy**

S2 cells were spread on concanavalin A-coated glass bottom plates and fixed in ice cold methanol for 12 minutes. Cells were washed with PBS-0.1% Triton X-100 and blocked in slide blocking buffer (5% normal goat serum in PBS-Triton X-100) for 30 minutes at room temperature. Primary antibodies were diluted in blocking buffer and slides were incubated for 1 hour at room temperature (rabbit anti-Plp (1:3000), mouse anti-myc (1:3000)). After 3 washes with PBS-0.1% Triton X-100, cells were incubated at room temperature with Hoechst 33342 (Life Technologies, 3.2 µM final concentration) and secondary antibodies diluted in blocking buffer for 30 minutes (anti-rabbit Rhodamine Red-X (1:1500), anti-mouse AlexaFluor 488 (1:1500)).Slides were then washed 3 times with PBS-0.1% Triton X-100 and mounted with homemade mounting medium.
(PBS, 90% glycerol, and 0.1 M propyl gallate). Deconvolution microscopy was performed on a DeltaVision core system (GE Healthcare) equipped with an Olympus IX71 microscope, a 100x objective (NA 1.4), and a cooled charge-coupled device camera (CoolSnap HQ2; Photometrics). Images were acquired with softWoRx vxx software (GE Healthcare).
CHAPTER 5 – Towards a molecular understanding of centriole assembly

Centriole assembly is a complex, multi-step process with cell-type specific adaptations that allow tissues to build their perfect centrioles. Although years of genetic studies have identified around a dozen proteins that contribute to centriole growth, we still lack a mechanistic view of how these proteins interact to promote centriole biogenesis. Therefore, research at the molecular level is the essential next step to create a clear picture of centriole assembly.

The distal tip complex (DTC) is an evolutionarily conserved set of proteins that coordinates centriole assembly at the distal tip\(^{179, 180, 215, 248}\). In *Drosophila*, Cep97 and CP110 are both required for centriole growth and to prevent microtubule projections from jutting past the distal tip\(^{181, 182}\). Contrarily, Klp10A is required to disassemble microtubules that grow past the distal tip\(^{183, 228}\). In *Drosophila* S2 cells, CP110 also prevents Klp10A-mediated disassembly of centrioles\(^{183}\). However, it is unclear how these proteins coordinate these functions to maintain centriole length and promote timely growth.

To that end, we have pursued a molecular understanding of centriole growth at the distal tip by tackling two essential problems. First, even though the DTC coordinates microtubule stability at the distal tip, the DTC lacks known microtubule stabilizing proteins. Herein, we identify Cep104 as a novel microtubule binding DTC component that promotes centriole growth.

Second, during most of centriole biogenesis, the role of the DTC is to prevent growth and shrinkage of the centriole\(^{225}\). However, the DTC also promotes centrioles growth\(^{181, 182}\). Therefore, the function of the DTC proteins must change to allow and promote centriole growth. We have discovered that the mitotic kinase Plk4 phosphorylates DTC proteins. Since recent studies show that centriole growth occurs during mitosis\(^{227}\), we hypothesize that DTC phosphorylation by Plk4 is permissive to centriole growth. Furthermore, we find that Plk4 must bind Asl to stimulate its kinase activity, challenging dogma suggesting that Plk4 dimerization is sufficient for its activation\(^{265}\).

Section 5.1 – A hypothetical model of centriole growth at the distal tip

The distal tip complex is recruited to the procentriole in early S phase, concurrent with microtubule recruitment by Sas4\(^9\). Prior to centriole growth in mitosis, Sas4’s microtubule capping LID domain prevents microtubule polymerization and centriole growth\(^{224}\). As an additional
mechanism, the kinesin-13 microtubule depolymerase Klp10A is prevents spurious microtubule growth past the DTC\textsuperscript{183}. Furthermore, Cep97 and CP110 knockdown in Drosophila S2 cells destabilizes the centriole, causing its destruction, suggesting that the DTC contributes to procentriole stability\textsuperscript{183} (Figure 2.6, page 49).

Since CP110 interacts with the microtubule binding region of Sas4, CP110 may be required for Sas4 to bind to the tips of microtubules with its LID domain\textsuperscript{197}. Together, CP110 and Sas4 could stabilize procentriole microtubules and structure prior to centriole growth past the cartwheel\textsuperscript{225}. Since Cep97 is required to stabilize CP110 protein levels at the distal tip\textsuperscript{179, 181}, both Cep97 and CP110 knockdown could destabilize centrioles through the same Sas4-mediated mechanism.

Next, microtubules must grow past the cartwheel to form the distal centriole during mitosis. Likely, this is caused by stabilization or polymerization of microtubules. However, Sas4 localizes to the cartwheel and is not available to stabilize microtubules at the distal tip\textsuperscript{215}. Since Cep104 is a DTC microtubule binding protein and overexpression is sufficient to cause centriole elongation, we speculate that Cep104 stabilizes microtubules during distal centriole growth\textsuperscript{246} (Figure 5.1).

TOG domain containing proteins like Cep104 are, generally, microtubule polymerases that bring soluble tubulin heterodimers into proximity of growing microtubule tips\textsuperscript{259}. Interestingly, they perform this function using multiple TOG domains to bind the microtubule lattice and soluble heterodimer simultaneously. Cep104, to my knowledge, is the only TOG protein with only a single TOG domain. However, Cep104’s coiled coil domain may overcome this limitation by promoting dimerization of two Cep104 molecules – creating a single functional unit with multiple TOG domains. Thus, preventing dimerization of Cep104 could be a mechanism to disrupt microtubule polymerization at the distal tip without compromising Cep104’s microtubule binding, which would leave microtubules susceptible to destabilization.

Even though overexpression of Cep104 can promote centriole elongation, Cep104 localizes to the distal tip when centrioles are static\textsuperscript{246}. Therefore, Cep104 must also abide by DTC mechanisms that prevent centriole growth and shrinkage. So, the presence of Cep104 alone is not sufficient to cause centriole growth during mitosis. Instead, mitosis-specific mechanisms likely manipulate the DTC to promote centriole growth. Since kinases dominate nearly every aspect of mitosis\textsuperscript{274}, we hypothesize that phosphorylation of the DTC is permissive to centriole growth.
Plk4, the master regulator of centriole duplication\textsuperscript{216}, is a mitotic kinase that can phosphorylate the DTC. Because Plk4 is essential for centriole biogenesis\textsuperscript{214}, knockdown of Plk4 causes centriole loss, limiting our ability to detect Plk4 functions outside of centriole duplication – in fact, we published two of the only reports detailing outside functions\textsuperscript{105, 197}. Although phosphorylation could cause many changes within the DTC, I favor a mechanism that promotes Cep104 dimerization to activate microtubule polymerization at the distal tip.

Cep97 is required for Cep104 to localize to the centriole by binding a region overlapping Cep104’s dimerization domain. Therefore, Cep97-mediated recruitment of Cep104 to the centriole may be a mechanism to inhibit Cep104 dimerization and ensure Cep104 does not promote centriole growth. Therefore, Plk4 phosphorylation of the DTC could activate Cep104 to promote polymerization. Additionally, we found that Plk4 can phosphorylate Klp10A in its motor domain – a known mechanism of kinesin inactivation\textsuperscript{255}. Therefore, the combination of activating a polymerase and deactivating a depolymerase could promote centriole growth during mitosis.

Centrioles maintain their length after centriole growth, never growing or shrinking again\textsuperscript{217}. If Plk4 phosphorylates the DTC to promote growth, why then do centrioles not grow during every mitosis? We hypothesize that the availability of Plk4 activators physically limits centriole growth. Since we found that dimerization is not sufficient to activate Plk4, we predict that localization of activators plays a critical role in Plk4 function. Therefore, regions of the centriole devoid of Asl, for instance, would not have active Plk4. Since Asl is enriched around the proximal end of the centriole, the DTC of a fully grown centriole may be spatially protected from Plk4 phosphorylation and deactivation.

Finally, after centrioles grow during mitosis, they must actively maintain that length\textsuperscript{183}. Unlike in the procentriole, Sas4 is not available at the distal tip of fully grown centrioles to stabilize microtubules. Therefore, we hypothesize that Cep104 binds to microtubules at the DTC to prevent growth and shrinkage. That could explain why centrioles are shorter when Cep104 is depleted from cells. Like the speculative mechanism of stabilization, Cep97 and CP110 could then promote Cep104 binding stably to centriole microtubules to prevent disassembly of the distal centriole. Furthermore, the existence of a microtubule binding protein stabilizing the distal tip could explain how Klp10A-mediated catastrophe of overly long microtubules is rescued by the DTC instead of depolymerizing the main body of the centriole\textsuperscript{183}. 
The necessity of Cep97 and CP110 binding to the microtubule stabilizing protein could also explain the cell type-specific variability of centriole length within an individual\textsuperscript{233}. By coordinating the binding affinity of these proteins for Cep104, for instance, cells could tip the balance towards microtubule assembly without needing to rely on changing protein levels of Cep104. As such, post-translational modifications of the modulatory DTC proteins could influence length without, potentially, compromising microtubule stability by completely ablating microtubule binding.

In order to understand centriole assembly, a molecular mechanism of microtubule stability at the distal tip should likely be the first priority. Without that understanding, it will be difficult to

\textbf{Figure 5.1} – Model of Cep104 in the DTC. Cep104’s TOG domain stabilizes the distal tips of centriolar microtubules to promote growth and prevent Klp10A-mediated disassembly of the centriole body. Cep104 is functionally tethered to the rest of the DTC through its N-terminus, which interacts with Cep97.
dissect molecular changes that promote centriole growth. However, I believe that microtubule binding proteins like Cep104 will be the key to finally dissecting the functions of DTC proteins at the centriole.
APPENDICES

Appendix A – Cep104 evolutionary alignment

Evolutionary alignments were performed on Clustal Omega using data acquired from uniport.org (Xenopus laevis, Danio rerio, Mus musculus, and Homo sapiens) or flybase.org (Drosophila melanogaster). Highlighted regions of Drosophila Cep104 correspond to functional domains defined in this study. Gray – Jelly Roll, Blue – Coiled Coil, Green – TOG, and Purple – ZnF.

<table>
<thead>
<tr>
<th>Species</th>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
<td>Drosophila</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
<td>Drosophila</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
<td>D. MELANOGASTER</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
<td>D. MELANOGASTER</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
<td>D. MELANOGASTER</td>
</tr>
</tbody>
</table>

D. MELANOGASTER

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. MELANOGASTER

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. RERIO

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. RERIO

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. RERIO

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. RERIO

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. RERIO

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. RERIO

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. RERIO

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>

D. RERIO

<table>
<thead>
<tr>
<th>Alignment</th>
<th>Highlighted Regions</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. MELANOGASTER</td>
<td>APNGSLTSHDIIIFQQPAK</td>
</tr>
<tr>
<td>D. RERIO</td>
<td>TRFCP-FPQEI11LQAERCRK</td>
</tr>
<tr>
<td>X. LAEVIS</td>
<td>MPQKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>H. SAPIENS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
<tr>
<td>M. MUSCULUS</td>
<td>MPHKLGFVVSSSHEDGFSARELMIHAVTSV</td>
</tr>
</tbody>
</table>
### Appendix B – Primer sequences

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cep104 dsRNA</td>
<td>TAATACGACTCACTATAGGGgccaaatccctgccccagc</td>
<td>TAATACGACTCACTATAGGGggccagagcatagcgcc</td>
</tr>
<tr>
<td>Cep104 UTR</td>
<td>TAATACGACTCACTATAGGGtaatcgacttatggcc</td>
<td>TAATACGACTCACTATAGGGGaanacggatagtggg</td>
</tr>
<tr>
<td>GFP-Cep104 dsRNA</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>Cep104-GFP</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>V5-Cep104</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>GFP-Cep104-JR</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>GFP-Cep104-CC-TG</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>GFP-Cep104-ZnF</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>GFP-Cep104-TOG</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>GFP-Cep104-deltaZnF</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>GFP-Cep104-deltaJR</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgtaatttgacttctgccttgctatattcccggg</td>
</tr>
<tr>
<td>His-Cep104-714-941</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgccgccatcaccatcaccat</td>
</tr>
<tr>
<td>GST-Cep104-714-941</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgccgccatcaccatcaccat</td>
</tr>
<tr>
<td>Cep104-deltaZnF-GFP</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgccgccatcaccatcaccat</td>
</tr>
<tr>
<td>GFP-Cep104-201</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgccgccatcaccatcaccat</td>
</tr>
<tr>
<td>GFP-Cep104-318</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgccgccatcaccatcaccat</td>
</tr>
<tr>
<td>GFP-Cep104-453</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgccgccatcaccatcaccat</td>
</tr>
<tr>
<td>Cep97 UTR Fusion</td>
<td>CCGGGTACCgccgccatcaccatcaccat</td>
<td>CCGGAATTCgccgccatcaccatcaccat</td>
</tr>
<tr>
<td>--------------</td>
<td>-------</td>
<td>----------</td>
</tr>
</tbody>
</table>
BIBLIOGRAPHY


68. Conduit, P.T. *et al.* Centrioles Regulate Centrosome Size by Controlling the Rate of Cnn Incorporation into the PCM. *Current Biology* 20, 2178-2186 (2010).


266. Slevin, L. et al. The Structure of the Plk4 Cryptic Polo Box Reveals Two Tandem Polo Boxes Required for Centriole Duplication. Structure 20, 1905-1917 (2012).


