Mechanistic Dissection of the Centriole Duplication and Ciliogenesis Proximity Interaction Maps

by

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ABSTRACT

The centrosome is the main microtubule-organizing center in animal cells, participating in a variety of cellular processes from cell polarization to cell division. The core of the centrosome consists of two cylindrical microtubule-based structures termed centrioles, which recruit a matrix of associated pericentriolar material. Importantly, centrioles function as basal bodies for nucleating the formation of flagella and cilia, including the primary cilium, a sensory organelle for receiving and transmitting developmentally important signaling pathway. Centrosome and cilium dysfunction are associated with a variety of human diseases including cancer and ciliopathies. To develop new diagnostic and therapeutic approaches for these diseases, it is essential to elucidate the mechanisms that regulate the biogenesis and function of the centrosome/cilium complex, which requires the identification of the parts list for the centrosome and mapping the interaction among these parts.

In this thesis, we focused on functional characterization of two novel centrosome proteins, CEP103 and CCDC66, which we identified as proximity partners of known centriole duplication and ciliogenesis proteins. CEP103 is upregulated in early differentiation of multiciliated epithelial cells and has high proximity to the de novo centriole duplication protein CCDC67. Supporting these earlier finding in our laboratory, we showed that CEP103 localizes to the centrosome and in part to satellites in a subset of cells. High resolution imaging experiment identified CEP103 as a proximal protein of the PCM, with partial co-localization with centriole fiber proteins. CEP103 has a dynamic localization patter during cell cycle, where it localizes to the spindle poles and microtubules during mitosis and central spindle during telophase. In vivo and in vitro experiments defined CEP103 as a microtubule associated protein. Analyses of the CEP103 truncation mutants revealed that the C terminal half of the protein was required for its localization to the centrosome and microtubules. Proximity dependent biotinylation of CEP103 revealed its extensive interactions with centrosome and centriolar satellite proteins. In o-localization and co-immunoprecipiation experiments, we showed that CEP103 interacts with CCDC67 and CEP63, two centriole duplication proteins that mediate formation of procentrioles during initiation phase. Work from our laboratory identified CCDC66 as a component

of the centrosome, cilium and microtubules with functions during cilium formation and trafficking. To gain insight into the molecular mechanism of these functions, we performed immunoprecipiation experiments with the candidate interacting partners of CCDC66 identified by BioID pulldowns and showed that CCDC66 forms a complex with the ciliogenesis proteins PCM1 and CEP290. In structure-function experiments using truncation mutants, the retinal degeneration mutant form of CCDC66 did not interact with PCM1, suggesting that its interaction with PCM1 and proper function at the centrosome is important for retinal development. Together, our work identified new regulatory proteins of centriole duplication and cilium formation and contributed to our understanding of the mechanisms of centrosome/cilium biogenesis and function.

ÖZET

Sentrozom hayvan hücrelerinde hücre kutuplaşmasından hücre bölünmesine, çeşitli hücresel süreçlere katılan ana mikrotübül organize merkezidir. Sentrozomun merkezi sentrozomla ilişkili perisentriyolar materyali bağlayan mikrotübül tabanlı iki silindrik yapı olan sentriyollerden oluşmuştur. Önemli şekilde, sentriyoller flagellum ve sil organellerinin, gelişimsel olarak önemli sinyal yolaklarında rol oynayan bir duyu organeli olan primer sil de dahil olmak üzere, oluşumunda bazal yapılar olarak rol oynarlar. Sentrozom ve sil yapılarının çalışmalarının bozulması kanser ve siliyopatiler dahil olmak üzere birçok çeşitli insan hastalığıyla ilişkilidir. Bu hastalıklara karşı yeni tanı ve tedavi yaklaşımları geliştirmek için, sentrozom/sil kompleksinin biyogenezi ve çalışmasını düzenleyen mekanizmaları aydınlatmak gereklidir. Bunun için de sentrozomu meydana getiren parçaların ve bu parçalar arasındaki etkileşimlerin tespiti gereklidir.

Bu tezde, sentrozom ve sil biyogenezinde rol oynadığı bilinen proteinlere yakın proteinler olarak belirlediğimiz iki yeni sentrozom proteini olan CEP103'ün ve CCDC66'nın fonksiyonel karakterizasyonuna odaklandık. CEP103'ün birden fazla sil oluşturan epitel hücrelerinin farklılaşması sürecinde anlatımı artmaktadır ve sıfırdan sentriyol üretiminde rol oynayan sentriyol duplikasyon proteini CCDC67'ye proksimite göstermektedir.CEP103'ün sentrozoma ve bazı hücrelerde sentriyolar satellitlere lokalize olduğunu gösteren, laboratuvarımızdaki CEP103 ile ilgili daha önceki keşifleri destekleyen. bilgilere ulaştık. Yüksek çözünürlükte aldığımız mikroskop görüntüleri, CEP103'ün, sentriyol iplik proteinleri ile kısmen ortak lokalizasyonu olan proksimal bir perisentriyolar materyal proteini olduğunu gösterdi. CEP103 mitozda iğ ipliklerine ve mikrotübüllere; telofazda ise mitotik köprüye lokalize olduğu dinamik bir lokalizasyon desenine sahiptir. In vivo ve in vitro deneyler, CEP103'ün mikrotübül ilişkili bir protein olduğunu belirledi. CEP103'ün farklı bölgesini temsil eden mutant parçaların analizi, proteinin karboksil ucunun sentrozom ve microtübül lokalizasyonu için gerekli olduğunu gösterdi. CEP103'ün proksimite bağımlı biyotinlenmesi deneyleri proteinin sentrozom ve sentriyolar satellitlerle olan geniş etkileşim ağını ortaya çıkardı. Lokalizasyon ve bağışıklık çöktürme deneyleri, CEP103'ün öncül sentriyollerin oluşumunda rol oyanyan iki sentriyol duplikasyon proteini olan CCDC67 ve CEP63 ile

etkileştiğini gösterdi. Laboratuvarımızdaki deneyler, CCDC66'yı sil oluşumu ve trafiğinde rol oynayan bşr sentrozom,sil ve mikrotübül proteini olarak belirledi. Bu fonksiyonların moleküler mekanizmalarına ışık tutmak amacıyla, BioID çöktürme deneyleri ile belirlediğimiz CCDC66'nın aday etkileşim partnerleri ile yaptığımız bağışıklık çöktürme deneylerinde, CCDC66'nın sil biyogenezi proteinleri olan PCM1 ve CEP290 ile etkileştiğini gösterdik. CCDC66'nın farklı bölgelerini temsil eden parça mutantları ile yaptığımız yapı fonksiyon deneylerinde retina dejenerasyondan sorumlu mutant parçanın PCM1 ile etkileşmediğini keşfettik. Bu sonuç, CCDC66'nın PCM1 ile etkileşiminin retina gelişiminde önemli olduğunu gösterdi. Kısacası, yaptığımız çalışma sentriyol duplikasyonu ve sil oluşumunda düzenleyici rol oynayan yeni proteinler keşfetti ve sentrozom/sil biyogenezinin mekanizmalarını anlamamıza katkıda bulundu.

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I thought I wasn't gonna make it but here it is… Three years of graduate school at Koc University comes to an end. The whole story started when I first listened to one of Julie Theriot's ibiology seminars during my sophomore year at Istanbul University. Cell skeleton couldn't be more interesting! With their rapid assembly and disassembly rates, cytoskeletal proteins act as hyper active super heroes in the cell. Always at front, actin cytoskeleton formed waves of filaments as they pushed the front end of the cell. By forming alot bigger structures than their individual selves, tubulin monomers endowed the Stentor its big mouth covered with cilia with which it could capture prey such as free-flowing bacteria. Evolution of such complexity, also brought some dangers, however. Actin cytoskeleton is mercilessly exploited by intracellular parasites such as Listeria and Rickettsia, giving you bad diarrhea while viral particules conviently walked their way into the cell nucleus on microtubule tracks to overwrite bits of pages of your genetic information. They are still mind-boggling and amazing though. All parties with their precise and awe-inspiring ways drew us into the sun-light lacking world of biology laboratories where we learned more about nature without feeling the sun and the breeze on our faces.

So the first thank you goes to Julie Theriot with whom I have not had the chance to meet. Thank you for giving great seminar talks. You are THE JULIE THERIOT of cell biology. Second big thanks goes to my advisor Elif Nur Firat Karalar, who undoubtedly excited me even more on the biology of centrosome/cilium complex and microtubules. Learning how to drive in a field with somebody as excited as herself was priceless. I'd also like to thank my thesis commitee members Umut Şahin and Nurhan Özlü for accepting to be my juries.

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TABLE OF CONTENTS

LIST OF FIGURES

NOMENCLATURE

GAMMA TURC	Gamma Tubulin Ring Complex
gPRA	Generalized Progressive Retinal Atrophy
CCDC	Coiled coil domain containing
DEUP1	Deuterosome Assembly Protein 1
PCM	Pericentriolar Material
C-NAP1	Centrosomal Nek2 Associated Protein
LRRC45	Leucine Rich Repeat Containing 45
MTOC	Microtubule Organizing Center
CEP	Centrosomal Protein
SCLT1	Sodium Channel and Clathrin Linker 1
FBF1	Fas Binding Factor 1
ODF2	Outer Dense Fibers of Sperm Tails 2
TCHP	Trichoplein Keratin Filament Binding
CP110	Centriolar Coiled-Coil Protein 110
PCNT	Pericentrin
CDK5RAP2	CDK5 Regulatory Subunit Associated Protein 2
PLK	Polo Like Kinase
PCM-1	Pericentriolar Material 1

TZ	Transition Zone
NPHP	Nephrocystin
MKS	Meckel-Gruber Syndrome
BBS	Bardet-Biedl Sydrome
IFT	Intraflagellar Transport
RTK	Receptor Tyrosine Kinase
CV	Ciliary Vesicle
GEF	Guanine Nucleotide Exchange Factor
ALI	Air Liquid Interphase
CTL	Cytotoxic T Lymphocyte
GFP	Green Fluorescent Protein
NGS	Next Generation Sequencing
RD	Retinal Degeneration
HELA	Henrietta Lacks
ciRNA	Circular RNA
PEG	Polyethylene Glycol
BIRA	Biotin Protein Ligase
BIRA*	Promiscuous Biotin Protein Ligase
DNA	Deoxyribonucleic Acid

SIRNA	Short interference RNA
RNA	Ribonucleic Acid
RPE1	Retinal Pigment Epithelial 1 Cells
COP1	Coat Protein Complex 1
KIF	Kinesins Gene Family
HSAV1	Salvador
MST2	Mammalian Sterile 20-Like Kinase 2
NEK2	Never in Mitosis A-Related Kinase 2
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
CPAP	Centrosomal P4.1-Associated Protein
HEK293T	Human Embryonic Kidney 293 T antigen cells
BIOID	Proximity-Dependent Biotin Identification
CNN	Centrosomin
SPD-5	Spindle Defective Protein 5
MCHP	Primary Autosomal Recessive Microcephaly
ZNF335	Zinc Finger Protein 335

1 INTRODUCTION

Centrosomes are the main microtubule organizing centers in animal cells. In a resting cell, microtubules nucleated by the centrosome function in important cellular processes such as cell polarity, intracellular trafficking and cell motility. In a proliferating cell, the centrosome nucleates and organizes the bipolar spindle that segregates the chromosomes equally to daughter cells during mitosis. Centrosome is composed of two centrioles, and pericentriolar material (PCM) which is recruited by and around the centrioles. Vertebrate cells also have small electron dense granules termed centriolar satellites that localize around the centrosome in a microtubule dependent manner (Barenz et al., 2011; Tollenaere et al., 2015).

Centrioles are microtubule-based organelles that are about 250 nm in diameter and 500 nm in length in animal cells and they share an evolutionarily highly conserved nine fold symmetry (Bornens and Piel 2002)). Centrioles are situated orthogonally to one another with their proximal ends embedded at the pericentriolar material while their distal ends grow outwards from the proximal lumen. The two centrioles of the centrosome are structurally and biochemically different from each other. The older “mother” centriole, but not the younger “daughter” centriole, bears distal appendages that interact with the plasma membrane during primary cilium formation and sub-distal appendages that anchors a subset of microtubules (Tanos, Yang et al. 2013, Huang, Xia et al. 2017). The differences between the mother and daughter centrioles are likely due to the semiconservative nature of centriole duplication during cell cycle.

The pericentriolar material is a protein matrix recruited by the centrioles and its main function is nucleation of microtubules through gamma-tubulin ring complexes. Gamma TURC complex assembles microtubule networks by inducing the polymerization of alpha- and beta- tubulin (Kollman, Merdes et al. 2011). Over the years, proteomics analysis of the centrosomes isolated from different cells and organisms identified the biochemical composition of the PCM. Superresolution microscopy analysis of a handful of PCM proteins revealed a surprisingly highly

ordered spatial organization of matrix where proteins are organized in concentric rings around the centrioles.

Like DNA replication, centriole duplication occurs once per cell cycle to ensure the correct segregation of both the genetic material and the centrioles (Nigg 2007). Deregulation of centriole duplication causes centriole number abnormalities and errors in segregation of genetic material (Tachibana, Gonzalez et al. 2005). Centriole number aberrations are a conspicuous phenotype of cancers (Kerketta, Ghosh et al. 2017). Centrioles are also essential at early stages of animal development (Klotz, Dabauvalle et al. 1990) Although centrioles are required for mammalian development, some organisms like insects still develop in the absense of centrioles into adulthood but they exhibit sensory problems that prevent them from finding food and water, causing early death of the organisms (Basto, Lau et al. 2006).

In this thesis, we aimed to biochemically characterize two centrosomal proteins CEP103 and CCDC66. CEP103 was initially identified as a gene, the expression of which was upregulated in multiciliated mouse tracheal epithelial cells (Hoh, Stowe et al. 2012). It was later on identified as a proximity partner of the centriole duplication protein CCDC67 (DEUP1) (Firat-Karalar, Rauniyar et al. 2014). CCDC66 was first identified as a canine generalized progressive retinal atrophy (gPRA) gene. Screening of the Shapendoes breed of dogs affected by the disease showed that the CCDC66 gene was mutated in all individuals carrying the disease (Dekomien, Vollrath et al. 2010).

Chapter two includes a detailed introduction to the centrosome/cilium complex biology where biogenesis, maintenance of the centrosome/cilium complex, centrosome/cilium complex related diseases, in vitro reconstitution attempts of the centrosome organelle and evolution of the centrosome is discussed in further detail. Chapter three covers information related to CEP103, our findings and discussions regarding the protein. Chapter four includes the materials and methods used to explore both CEP103 and CCDC66 proteins. Finally Chapter five covers information related to CCDC66, our findings and discussions regarding the protein.

2 LITERATURE REVIEW

2.1 CENTROSOME

Centrosomes function as the main microtubule organizing centers in animal cells. In an interphase cell, microtubules emanating from the centrosome function in critical cellular processes including intracellular trafficking, cell polarity and cell motility. In a cycling cell, the centrosome nucleates and organizes the bipolar mitotic spindle that mediates equal segregation of chromosomes to daughter cells.

At the core of the centrosomes are two centrioles, which recruit the pericentriolar material (PCM) (Figure 1). Vertebrate cells also have small electron dense granules termed centriolar satellites that localize around the centrosome in a microtubule and molecular motor- dependent manner (Barenz et al., 2011; Tollenaere et al., 2015). Centrioles are microtubule-based cylindirical structures that are about 250 nm in diameter and 500 nm in length in animal cells. Remarkably, the centrioles have an evolutionarily highly conserved nine fold symmetry. Centrioles are situated orthogonal to one another with their proximal ends closing together at the pericentriolar material while their distal ends stretch outwards from the proximal lumen. Fine variations between the two centrioles of a single centrosome is also an evident characteristic that distinguish the two centrioles from each other in terms of structure an function. On its distal end, the older centriole termed “mother centriole” bears a set of protein complexes called the distal- and subdistal appendages, reminiscent of a crown and these structures mediate docking of the centriole to the plasma membrane during ciliogenesis and anchoring a subset of microtubules, respectively. These structures are not present in the younger centriole termed “daughter centriole”, which is characterized by the presence of daugher centriole proteins such as Cep120 and Neurl4 (Winey and O'Toole 2014).

The pericentriolar material (PCM) is a matrix of proteins that are spatially organized in concentric rings around the centrioles (Figure 1). The almost complete proteome of the PCM1 was identified through the mass spectrometric analysis of the centrosomes isolated from different cells and organisms. The main function of the PCM1 is the

nucleation and organization of the microtubules, assigning the centrosome MTOC function. Microtubule nucleation ability of the PCM is mediated by gamma tubulin ring complexes (γ-TURC), which assemble microtubules through its longitudinal interaction with alpha- and beta- tubulin (Kollman, Merdes et al. 2011).

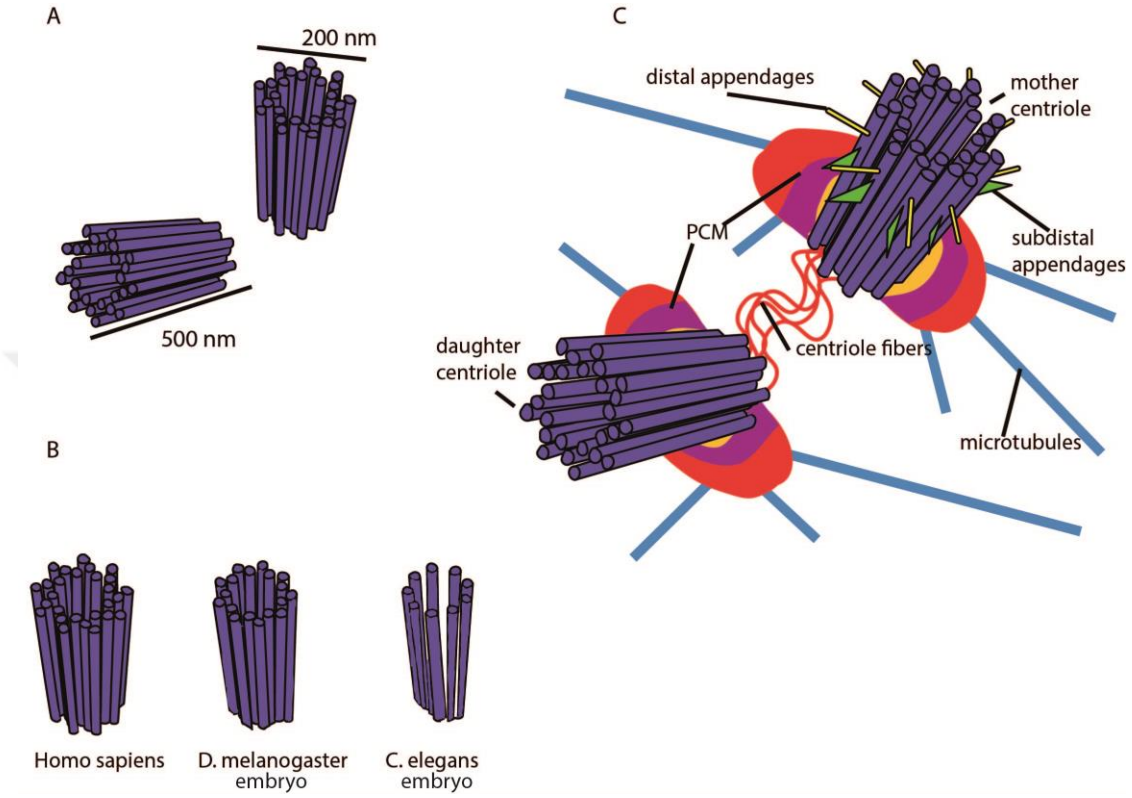


Figure 2.1-1. Anatomy of the Centrosome A) The centrosome is composed of barrel shaped centrioles that are 200 nm in diameter and 500 nm in length in animal cells. B) Mammalian centrioles have radially organized nine-fold symmetrical microtubule triplets. However, there are variations in the organization of microtubules across species. While *Drosophila melanogaster* embryos have microtubule doublets, *C. elegans* embryos have a singlet microtubules. C) Mother centriole bears distal and sub-distal appendages on its distal end with daughter centriole do not have these structures. Distal appendages mediate docking of the mother centriole to the plasma membrane during ciliogenesis and subdistal appendages anchor a subset of microtubules. The two centrioles are linked to one another through centriole fibers and are embedded in the protein meshwork called pericentriolar material (PCM). PCM proteins are organized in concentric rings of different diameters around the centrioles. These distinct concentric rings are shown as different colors where red shows the protein assembly of highest diameter while yellow shows the protein assembly of smallest diameter. PCM assigns the microtubule organizing center function to the centrosome.

2.1.1 CENTRIOLES

Centrioles are one of the largest macromolecular protein complex assemblies in cells, which are about 250 nm in diameter and ranging between 200 nm and 500 nm in

length in different cell types and organisms (Bornens 2002). Centrioles are microtubule-based cylindirial structures and there are two orthogonally-oriented centrioles at the core of the centrosome. Importantly, centriolar microtubules are extensively modified at the posttranslational level including acetylation, detyrosination and polyglutamylation and these modifications make centrioles cold resistant, stable and resistant to depolymerizing reagents such as nocodazole (Janke and Bulinski 2011). Supporting the stable nature of these structures, fluorescence recovery after photobleaching (FRAP) experiments demonstrated that tubulin recovery rate of the centrioles was very low (Pearson, Giddings et al. 2009).

Centrioles have a remarkable evolutionarily conserved nine fold symmetrical structure. Mammalian centrioles are composed of nine radially arranged triplet microtubules, which has a complete microtubule (A-tubule) of 13 protofilaments and the two partial microtubules of 10 protofilaments (B-tubule and C-tubule). B and C tubules are arranged in a way to share 3 protofilaments with the adjacent tubule to complete the protofilament number to 13 (Guichard, Hachet et al. 2013). Although the nine fold symmetrical organization of the centrioles is conserved across species, there are structural variations in the microtubule scaffold of the centrioles. While the mammalian centrioles have 9-fold symmetrical triplet microtubules, *Drosophila melanogaster* and *Caenorhabditis elegans* embryos have 9-fold symmetrical doublets and singlet microtubules, respectively (Bettencourt-Dias and Glover 2007). The schematic comparison of centrioles with microtubule triplets, doublets and singlets is shown see Figure 1B.

Given that centrioles are microtubule-based structures, they are inherently polar structures where microtubule minus ends are at the proximal end and plus ends are at the distal end of the centrioles. While centioles are composed of nine fold symmetric microtubule triplets at their proximal ends, their distal end is composed of nien fold symmetric microtubule doublets (Li, Fernandez et al. 2012). The two centrioles are tethered together in their proximal ends by connecting fibers that are composed of C-NAP1, Rootletin and LRRC45 (Fry, Mayor et al. 1998, Yang, Liu et al. 2002, He, Huang et al. 2013). Connecting fibers provide cohesion between the two centrioles during interphase to maintain the single MTOC of the resting cells (Nigg and

Stearns 2011). As the cell proceeds into S and G2 phases, the proteinaceus fibers connecting the two centrioles are phosphorylated by Nek2 and degraded (Rellos, Ivins et al. 2007). Free and duplicated centrioles are then pushed away from one another by kinesin motors to perform as individual spindle poles of the dividing cell (Tanenbaum and Medema 2010). Premature splitting of the centrioles associated with C-NAP1 mutations is associated with the Seckel like syndrome in *Bos taurus* (Floriot, Vesque et al. 2015). Interestingly, centriole splitting prior to mitosis is physiologically important for responding to chemo-stimuli in white blood cells (Schliwa, Pryzwansky et al. 1982).

The two centrioles of the centrosome, termed the mother and daughter centrioles, are different from each other in their age, structure and function. While the older mother centriole assembles two cell cycles ago, the younger daughter centriole assembles in the previous cell cycle. Most functions of the centrosome are mediated by the mother centriole that include docking of the centrioles to the plasma membrane during ciliogenesis, recruitment of the pericentriolar material that mediates microtubule nucleation and organization and anchorage of microtubules. The mother centriole, but not the daughter centriole, has distal and sub-distal appendages, which mediate key mother centriole functions during ciliogenesis and organization of microtubule network. Assembly of both of these structures requires ordered recruitment of a subset of proteins. While CEP89, CEP83, SCLT1, CEP164 and FBF1 are recruited in a hierarchical fashion to form the distal appendages (Tanos, Yang et al. 2013), ODF2, CCDC68, CCDC120, TCHP,CEP170 and NINEIN are recruited in a similar way to form the subdistal appendages (Huang, Xia et al. 2017). Distal appendages confer the ability to the mother centriole to dock to the plasma membrane to initiate ciliogenesis and to organize the microtubule network through anchoring a subset of mirotubules (Tanos, Yang et al. 2013). Moreover, they mediate secretory lysosome release from the immunological synapses (Stinchcombe, Randzavola et al. 2015). Subdistal appendages are important for anchoring a subset of microtubules and coordination of ciliary beating pattern in motile multiciliated cells (Gorgidze and Vorobjev 1995, Kunimoto, Yamazaki et al. 2012).

2.1.2 PERICENTRIOLAR MATERIAL

Pericentriolar material (PCM) is a matrix of proteins recruited by the centrioles to form the centrosome and its main function is nucleation and organization of microtubules, which assigns the centrosome MTOC function. Unlike other organelles, PCM is not membrane bound. Electron microscopy analysis of the PCM identified this protein-based structure as a electron dense amorphous homogenous mass around the highly ordered centrioles. While EM studies could not reveal the structural organization of the PCM, they definitively showed the microtubules originated from the PCM and thsu assigned the MTOC function of the centrosome to the PCM (Gould and Borisy 1977). Analysis of various PCM proteins using superresolution microscopy identified the pericentriolar material of interphase human and Drosophila cells as a highly ordered structure in which proteins are spatially organized as molecular fibers or toroids extending out from the centriole wall (Fu and Glover 2012, Sonnen, Schermelleh et al. 2012). In human cells, the C terminus of Pericentrin (PCNT) is localized closer to the centriole wall, while the N terminus extends out to the cytoplsmic face. Like PCNT, CEP152 is also organized as a molecular fiber in the PCM. Other PCM components such as CEP192, CDK5RAP2, gamm-tubulin and CEP120 are organized as concentric rings of different diameters (Lawo, Hasegan et al. 2012). An independent study also identified a very similar organization of the PCM proteins of *Drosophila* embryos, highlighting the conserved structural features of the matrix across species (Mennella, Keszthelyi et al. 2012).

PCM is built to have its highly ordered organization in two interdependent pathways in mammalian cells (Woodruff, Wueseke et al. 2014). In the first pathway, termed the scaffolding pathway, the elongated PCNT structure organizes the inner and outer layers of PCM. In the second pathway, Cep192, Cep215 and gamma-tubulin together functions in PCM matrix formation. During mitosis, PCM expands and matures through the activity of Polo Like Kinase 1 (PLK1) (Lane and Nigg 1996) and Aurora A Kinase (Hannak, Kirkham et al. 2001). Once PCNT is phosphorylated by PLK1, it recruits other PCM components including gamma Tubulin, CEP192 and GCP-WD (Lee and Rhee 2011) and explands the PCM into a large matrix.

2.1.3 CENTRIOLAR SATELLITES

Centriolar satellites are 70 – 100 nm array of electron dense granules that localize around the centrosome in vertebrate cells. Although centriolar satellites can be ubiquitously found across vertebrate species; their localization, molecular composition and numbers varies in different cell types. In fibroblasts and epithelial cells, majority of the centriolar satellites are clustered around the centrosome and the minority of them are scattered in the cytoplasm (Kubo, Sasaki et al. 1999). In ciliated epithelial cells of the mouse trachea and oviduct, centriolare satellites had an apical localization at the base of the cilia. Interestingly, in intestinal and renal epithelial cells that do not have a cilium, centriolar satellites also had a similar apical localization pattern.

To date, about 30 proteins have been identified as satellite proetins. Interestingly, all these proteins except for PCM1 (pericentriolar material 1) localizes both to the satellites and the centrosome/cilium complex (Tollenaere, Mailand et al. 2015). PCM1 interacts will all other satellite proteins and its depletion and knockout results in lack of satellies (Hori and Toda 2017). Therefore, PCM1 is defined as the molecular marker to label and identify centriolar satellties. Also, overexpression of the centriolar satellite scaffold PCM1 leads to formation of aggregates that are not delineated by a membrane (Balczon, Bao et al. 1994, Dammermann and Merdes 2002, Kubo and Tsukita 2003). Recent evidence implicates centriolar satellites in critical functions not only for the maintenance of centrosome and cilium complex like ciliogenesis and centriole duplicaiton but also for other cellular processes including autophagy and neurogenesis (Firat-Karalar, Rauniyar et al. 2014, Tollenaere, Mailand et al. 2015). Although these lines of evidence identifies these structures as ubiquitous regulators of the centrosome/cilium complex, their molecular mechanism of action is not known.

2.2 CILIA

Cilia are evolutionarily conserved microtubule-based structures that extend out form the apical surface of vertebrate cells across species ranging from nematodes to protozoa. They are broadly classified as motile cilia and immotile primary cilia based on their number, structural organization and motile behavior.

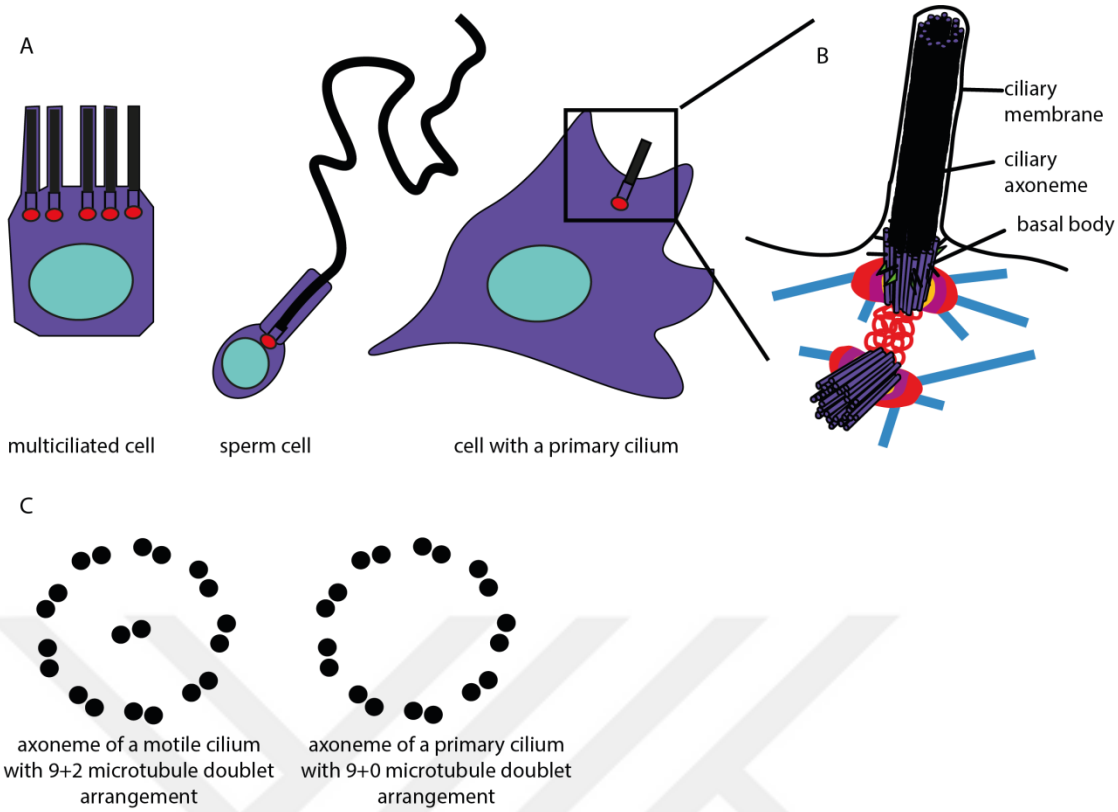


Figure 2.2-1. Cilia differ in form and function. A) Multiciliated cells of the respiratory tracts are a good example to show the role of motile cilium. Motile cilia of respiratory tract can help clearance of mucus out of the airways and the lungs. Defects in the function of motile cilia can cause coughing and sneezing phenotypes in mice and can even lead to deadly soft-tissue infections (Kunimoto, Yamazaki et al. 2012). Sperm cells also carry a motile cilium called flagellum which propells the cell towards chemical cues. Unlike motile cilia, primary cilia are immobile and they execute sensory functions such as the photoreceptor sensory cilium (Khanna 2015) or cholangiocyte cilium that can sense mechanical stress (Masyuk, Masyuk et al. 2006). B) Anatomy of a cilium. A cilium consists of a basal body, ciliary axoneme and ciliary membrane. Although this composition is universal, detailed structural variations in the axoneme of motile and primary cilium exists. C) Axoneme of a motile cilium is generally composed of 9 microtubule doublets arranged in 9 fold symmetry and a single microtubule doublet centered in the middle.

2.2.1 MOTILE CILIUM

While many eukaryotic cells generally possess one cilium, some unicellular organisms such as Paramecium and multicilliated vertebrate cells bear dozens of motile cilia that beat in a synchronized fashion to create directed flow of the extracellular fluid. In contrast to the motile cilia that are present in high numbers, sperm cells have a single motile cilum, termed flagellum that bead to confer motility to these cells. Defects in the structure and function of motile cilia are associated with primary ciliary dyskinesia, which are characterized by chronic airway diseases, infertility and defects in brain

development and laterality (Brooks and Wallingford 2014). In mammals, motile cilia are found in the fallopian tube where they beat to push the egg towards the endometrium (Croxatto and Ortiz 1975), on epithelial cells of the respiratory tract where they help clear the mucus filled with air pollutants and air borne particles (Ganesan, Comstock et al. 2013) and in brain ventricles where ciliary beating partitions and conveys cerebrospinal fluid (CSF) (Faubel, Westendorf et al. 2016).

Ciliary axoneme grows from the basal body and shares the same conserved nine fold symmetry of the centrioles. In addition to the circumferentially arranged 9 set of microtubule doublets, the axoneme of the motile cilia also contain a pair of central microtubules that are important of directional ciliary beating (Brooks and Wallingford 2014) and such organization is abbreviated as 9+2 pattern. Although most motile cilia microtubules are arranged in 9+2 pattern, a subset of motile cilia has different microtubule scaffold arrangements. For example, nodal cilia have 9+0 microtubule doublet arrangement (Pennekamp, Menchen et al. 2015). Nodal cilia of the node cells determine the left-right symmetry of the body and defects in its assembly and function cause “situs inversus”, which is characterized by the reversed localization of several internal organs in the body (Nonaka, Shiratori et al. 2002). In addition to their different axonemal arrangement, motile cilia contain dynein motor proteins connecting the microtubule doublets of the axoneme and these motors power the axonemal beating.

2.2.2 PRIMARY CILIUM

The primary cilium is immotile and it is present as one copy in almost all cells in the human body except for the multiciliated epithelial cells and the flagellum. The immotile primary cilia are sensory organelles that receive and transmit signals including light, chemicals, proteins and mechanical stimuli from the extracellular environment. Among the signaling cascades regulated by primary cilia are Hedgehog, Wnt and PDGFR pathways (Schneider, Clement et al. 2005, Liu, Chen et al. 2014)). The importance of cilia in these signaling pathways are highlighted by the manifestation of ciliary structure and function defects as ciliopathies, which are characterized by multitude of developmental symptoms affecting retina and kidney among others.

Early ultrastructural studies of the primary cilia by electron microscopy defined it as a vestigial organelle protruding from the cell surface. The rediscovery of the primary cilia as a sensory organelle happened when a genetic screen in mice that aimed at characterizing neural tube closure defects linked to Hedgehog signaling defects identified mutations in Intraflagellar Transport components including IFT88 and IFT172 (Huangfu, Liu et al. 2003). Since this discoverey, the functional relationship between primary cilia and Hedgehog signaling has been dissected at the mechanistic level. Most Hedgehog effectors localize to the primary cilium where they transduce the signals. In unstimulated cells, the seven transmembrane containing receptor protein Patched (Ptch) localizes to the cilium. When cells receive Hedgehog ligands like Sonic Hedgehog (Shh), Ptch transports out of the cilium and another seven transmembrane containing receptor proteinSmoothened (Smo) transloates into the cilium from the cytoplasm. Smo localization to the cilium activates the Hedgehog signaling cascade which progresses with the processing of Gli transcription factors and expression of Hedgehog target genes (Ingham and Placzek 2006). In addition the Hedgehog signaling, other signaling pathways including Wnt signaling, receptor tyrosine kinase signaling and platelet derived growth factor alpha (PDGFalpha) signaling have also been shown to be regulated by the primary cilia (Schneider, Clement et al. 2005).

The primary cilia are made up of a nine doublet microtubules that extend out from the basal body, which are encapsulated by the ciliary membrane that is continuous with the plasma membrane. The basal body is derived from the mother centriole of the centrosome when mother centriole docks to the plasma membrane through its distal appendages upon stimulation of ciliogenesis. The basal body functions as the nine-fold symmetric template on which the axoneme can be built to adapt the same nine-fold symmetry and the axoneme functions as the scaffold that provides rigidity (Marshall 2008). The ciliary compartment, termed the transition zone (TZ), is located between the basal body and the axoneme and it functions as a gate to the cilium, regulating the transport of proteins into and out of the cilium. TZ is a highly structured assembly that

is composed of various proteins including CEP290, NPHP and MKS (Goncalves and Pelletier 2017).

2.2.3 CILIUM ASSEMBLY AND DISASSEMBLY

The primary cilium assembly events are coupled to cell cycle such that they assemble in quiescent cells and disassemble as cells enter cell cycle. In addition to quiescent cells, primary cilia are also present in differentiated cells. The cilium assembly and disassembly pathways are highly ordered set of events that are regulated by many proteins, most of which have been characterized to date through genetics, proteomics and transcriptomics screens.

The assembly of the primary cilium is induced when cells exit cell cycle through mitogen deprivation, serum starvation or differentiation signals. The critical events of ciliogenesis was defined initially by ultrastructural studies (Sorokin 1962) and later by live imaging experiments. The first step in ciliogenesis upon serum starvation is the formation of "ciliary vesicles" (CV) at close proximity to the distal appendages of the mother centriole. CV formation is regulated by the activity of Rab8 activator Rabin8 and the GTPase Exchange Factor (GEF) for Rab8 (Sanchez and Dynlacht 2016). Once CV form, they dock to the distal appendages to form a membranous cap around them and this cap is further elongated through the fusion of Rab8 positive vesicles (Lu, Insinna et al. 2015). Following formation of the membranous cap, the microtubule doublets start growing from the distal end of the mother centriole to extend out the ciliary axoneme. While the axoneme is elongating, concomitant vesicular trafficking enlarges the membranous cap such that the growing exoneme is encapsulated in a double membrane. Finally, the nascent cilium docks to the plasma membrane through fusion with the ciliary sheath.

The conversion of mother centriole to basal body during ciliogenesis involves activity of several functional modules. First, the centriole distal end capping proteins CP110 and CEP97 are removed. Evidence for the capping function of these proteins came from siRNA depletion experiments of these proteins that caused cilium formation in proliferating cells and overelongated centrioles (Spektor, Tsang et al. 2007). Removal of CP110 and its interaction partner CEP97 from the basal body cap is followed by

fusing of ciliary vesicle with the plasma membrane (Kobayashi, Kim et al. 2014). Specific cell types such as cytotoxic T lymphocytes (CTL) harness CP110 to mediate immunological synapse formation. In these cells, mother centrioles dock to the plasma membrane without removing the distal caps and this prevents the formation of cilium at the immunological synapses (Stinchcombe, Randzavola et al. 2015). In addition to removal of the distal cap, docking of the mother centriole to the plasma membrane requires distal appendages which are nine-fold symmetrical assemblies of proteins including CEP164, FBF1, CEP83 (Tanos, Yang et al. 2013) (Schmidt, Kuhns et al. 2012).

Cilia are composed of hundreds of proteins that were identified by a combination of approaches over the years. Given that cilia lack ribosomes for synthesis of these proteins, assembly and maintenance of cilium requires regulated transport of these proteins form the cytoplasm into the cilium. Ciliary protein import and export are regulated by various mechanism including the Intraflagellar transport (IFT) machinery, BBSome complex, vesicular trafficking, transition zone and centriolar satellites (Hsiao, Tuz et al. 2012, Chamling, Seo et al. 2014, Lechtreck 2015, Garcia-Gonzalo and Reiter 2017, Liu and Lechtreck 2018). IFT is the prominent and best characterized machinery for ciliary protein transport (Rosenbaum and Witman 2002). IFT is a highly conserved machinery that shares common ancestry with the coat protein complexes (COP1) (Jekely and Arendt 2006). IFT machinery is composed of retrograde moving IFT-B complex that mediates retrograde transport, IFT-A complex that mediates anterograde transport and adaptor BBSome complexes (Eguether, San Agustin et al. 2014). IFT trains walk along the ciliary axoneme through molecular motors and they transport ciliary building blocks and signaling molecules in and out of the cilium (Kozminski, Johnson et al. 1993). While kinesins function with the IFT complexes during the anterograde transport (Cole, Diener et al. 1998), dynein motors mediate the retrograde transport (Pazour, Dickert et al. 1999). Defects in IFT function causes the formation of morphologically abnormal cilia or complete failure in cilia formation (Scholey 2012).

Although IFT-mediated transport is defined as the predominant means of protein supply for the cilium, it is not the only system that mediates protein transport and its relative contribution to ciliary transport was not known. Recently, Luo W. et al tracked

the transport trajectories of cytoplasmic proteins into the cilium and showed that anterograde transport was predominantly mediated by the IFT machinery, retrograde transport exploited both IFT and passive diffusion. The relative contribution of IFT and passive diffusion to retrograde transport varies based on the transported protein.

Cilium disassembly is far less understood relative to ciliary assembly. One signal that induces cilium disassembly is the mitogen stimulation, which induces cells to enter the cell cycle. Mitogens trigger disassembly of the cilia in part through the activity of kinesins Kif2a and Kif24 (Kim, Lee et al. 2015, Miyamoto, Hosoba et al. 2015). Following the mitogen stimulation, PLK1 phosphorylates Kif2a. Phosphorylated Kif2a stimulates the microtubule depolymerizing activity of the kinase and axonemal microtubules are disassembled. Axonemal microtubule disassembly activities of Kif2a and PLK1 is followed by the activities of Nek2 and Kif24. Nek2 phosphorylates Kif24, inducing its microtubule depolymerizing activity. Activity of Kif24 following the disassembly of cilium prevents untimely assembly of cilium during the cell cycle (Sanchez and Dynlacht 2016).

Disassembly of cilium can be induced by factors other than mitogens; such as mechanical stress or heat shock. Elevating the hydrostatic pressure in the eye can cause the cilia of trabecular meshwork cells to shorten while exposure of chondrocytes to mechanical strain can reduce the number of ciliated cells and the cilium length in bovine species (McGlashan, Knight et al. 2010, Luo, Conwell et al. 2014). Elevated temperature exposure is known to cause 50% cilia loss in a population of ciliated NIH3T3 cells (Prodromou, Thompson et al. 2012). Ciliary disassembly can be achived through dismantling cilia by severing the ciliary axoneme. A putative breaking point along the cilium might allow for osmotic, mechanical or chemical factors to blow severing insults on the cilium (Blum 1971). Microtubule severing process mediated by Centrin is known to excise flagella in *C. reinhardtii* (Sanders and Salisbury 1989). Another way to disassemble cilium is through resorbtion where relocation of the building blocks of the cilium back into the cytoplasm occurs. Just like microtubules, cilia are also thought to exist in a steady state where balance between assembly and disassembly affect cilium length. In fact, blocking IFT prevents further incorporation of materials at the cilia and causes cilia to resorb (Marshall and Rosenbaum 2001).

2.3 CENTROSOME BIOGENESIS

Centrosome number in cells is maintained by a duplication and segregation mechanism linked to the cell At the G1/S transition, centriole duplication is initiated with the orthogonal formation of a nascent centriole termed “procentriole” next to the pre-existing parental centrioles. Once the procentriole forms, it elongates through S and G2 phases. Following the assembly of centrioles, they undergo maturate by accumulation of pericentriolar material around them. PCM start accumulating around them. Super resolution microscopy studies on vertebrate and fruit fly centrosomes revealed that PCM was exclusively forming around mother centrioles (Sonnen, Schermelleh et al. 2012). During mitosis, the two pair of centrioles are segregated to two daughter centrioles by the mitotic spinde and at the end of mitosis the linker between the two centrioles is dissolved in a process termed “centriole disengagement” to relicence the centrioles for duplication in the next cell cycle.

Centrioles are linked by two different types of linkers, which form and dissolve throughout the cell cycle. The first linker is the “S-M linker” that forms during S pahse and tethers newly formed procentrioles to the adjacent preexisting centrioles. This linker dissolves late in mitosis in a process called “centriole disengagement” and this dissolution is required for licencing the reduplication of centrioles in the next cell cycle. Key kinases that are required for centriole disengagement are PLK1 and Separase. The second type of linker is the “G1-G2 tether” that connects the proximal ends of the two parental centrioles and such connection is required for focusing the MTOC activity at the centrosome. G1-G2 tether assembles in G1 cells when the S-M linker dissolves during disengagement. The G1-G2 tether is composed of C-NAP1, Rootletin, CEP68, CDK5RAP2 and LRRC45 (Mayor, Stierhof et al. 2000, Yang, Adamian et al. 2006, Graser, Stierhof et al. 2007, He, Huang et al. 2013) Prior to mitosis, Hippo pathway components Salvador (hSav1) and mammalian sterile 20-like kinase 2 (Mst2) phosphorylate never in mitosis A-related kinase 2 (NEK2). In return, NEK2 phosphorylates C-NAP1 and rootletin, thereby, dissolving the centriole fibers and allowing the separation of duplicated centriole pairs to migrate away from one another and to form the spindle poles. Migration of the separated centrosomes is then maintained by Eg5-microtubule pathway (Mardin, Lange et al. 2010).

Laser ablation of centrioles form the centrosomes in mammalian cells results in the dispersal of the PCM and eventually to centrosome loss in human cells (Bobinnec, Khodjakov et al. 1998), indicating that centrioles are required for the maintenance of the centrosome. Following this study, Kirkham M. et al showed that amount of PCM recruited to the centrosome correlated with the centriole size, such that larger centrioles recruit more PCM in *C.elegans* (Kirkham, Muller-Reichert et al. 2003). In support of these findings, Basto R. et al discovered that gradual loss of centrioles in a centriole duplication incompetent *Drosophila* strain also led to the loss of PCM in the same cells (Basto, Lau et al. 2006). These studies underline the importance and necessity of centrioles in the stable assembly of the centrosome.

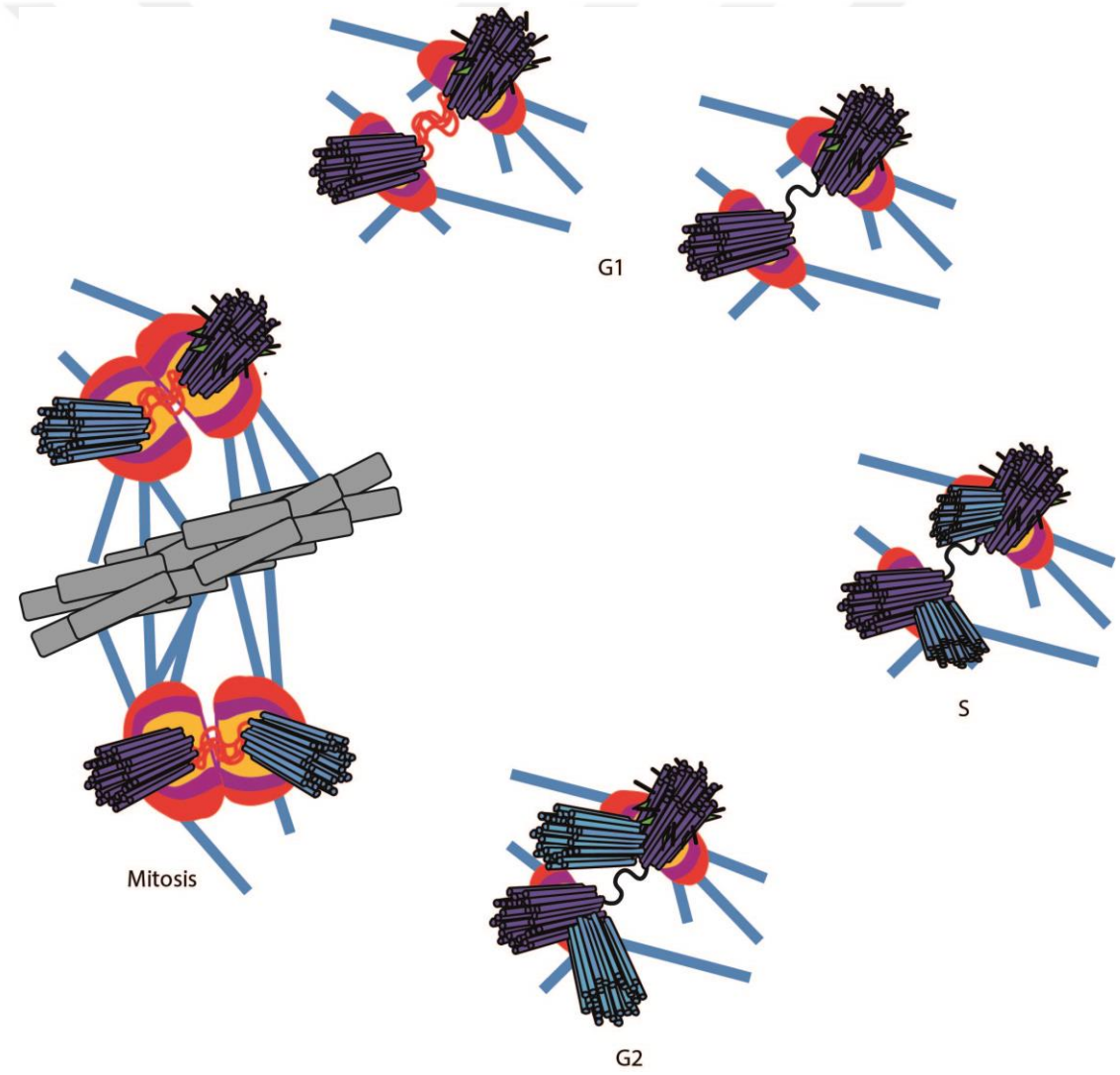


Figure 2.3-1. Centrosome Duplication and Cell Cycle Following the exit from mitosis, centrosome fibers (shown was red bundle of thread) are dissolved prior to procentriole assembly. This dissolution of the centrosome fibers seperates the two template centrioles,

priming them for the duplication round. Although the proteinaceous linker between the template centrioles is dissolved, another dynamic linker structure is assembled between disengaged centrioles (shown as black thread).Two nascent centrioles start forming adjacent to the walls of pre-existing centrioles. As the new centrioles emerge, they are also tethered to their templates by the same fibers that were dissolved prior to synthesis phase. Procentrioles elongate throughout S and G2 phases. Prior to mitosis, the dynamic linker between duplicating centriole pair is broken to allow for the migration of the centrosomes to the poles. In parallel to the centriole duplication, PCM also expands and recruits more tubulin binding complexes (notice the PCM size difference between G1 and mitotic centrosome) through the phosphorylation activity of PLK1. This expansion primes PCM to function as the spindle poles (Lee and Rhee 2011).

2.3.1 CENTRIOLE ASSEMBLY

Biogenesis of centrioles can occur through *de novo* assembly of hundred of centrioles in multiciliated cells of specialized epithelial cells or through canonical duplication of pre-existing centrioles in most animal cells (Anderson and Brenner 1971).

During canonical duplication, procentriole formation is initiated orthogonally at the proximal end of the pre-existing parental centrioles and this initiation requires disengagement of centrioles by dissolving the S-M linker in the previous cell cycle. When enough spatial room is available at the site of intiation, the protein kinase PLK4 is recruited to this site by centrosomal proteins CEP152 and CEP192. The recruitment of CEP152 to the centrosomer requires CEP63 (Brown, Marjanovic et al. 2013, Lukinavicius, Lavogina et al. 2013). Following its ring shaped localization at the proximal end of the pre-existing centrioles, PLK4 then dinamically re-localizes into a single dot. This PLK4 concentrate also co-localizes with the nascent centriole marker Sas-6, which forms the carthwheel to dictate the nine fold symmetrical organization of the centrioles (Kim, Park et al. 2013). In addition to SAS-6, localization of PLK4 at the site of centriole initiation is followed by the recruitment of the centriole duplication proteins CPAP, CEP135 and gamma-Tubulin.

Cytoplasmic homodimeric pool of Sas-6 is recruited to the centrosome through its coiled-coil domain (Brown, Marjanovic et al. 2013, Lukinavicius, Lavogina et al. 2013). Once in the centrosome, Sas-6 proteins start oligomerizing to form the nine fold symmetric cartwheel (Keller, Orpinell et al. 2014). In this symmetric structure, the N

terminus of the protein forms the inner core while the C terminus pokes out and forms the nine fold symmetric spokes (Kitagawa, Vakonakis et al. 2011). A report by Nakazawa Y. et al proposed that the formation of such cartwheel of nine-fold symmetry was key for the assembly of a nine fold symmetric centriole. In fact, Sas-6 homologue *bld12* mutant *Chlamydomonas* cells assembled centrioles of varying folds other than nine (Nakazawa, Hiraki et al. 2007), establishing the cartwheel as the key determinant of the centriole structure. This idea, however, was challenged by a recent work by Hilbert M. et al., who engineered Sas-6 proteins with cartwheel assemblies of various symmetries to find that the presence of abnormal cartwheel structures could still yield nine fold symmetric centrioles. This study revealed the existence of other regulatory elements in the process (Hilbert, Noga et al. 2016). In line with this finding, it was discovered that in the absence of the endogenous Sas-6, oligomerization incompetent Sas-6 truncations were able to assemble centrioles with nine fold symmetry and that oligomerization incompetent Sas-6 protein could be recruited and shaped into a nine fold symmetric cartwheel by the mother centriole (Wang, Acehan et al. 2015) and (Fong, Kim et al. 2014) (MERGE REFERENCES???). Together these studies all identified Sas-6 as a critical component of centriole and basal body biogenesis in different ogranisms (Leidel, Delattre et al. 2005, Hu, Liu et al. 2015).

Once the cartwheel assembly is established, protein CPAP, along with other proteins such as CEP120 and SPICE1 elongate the centriolar microtubules (Comartin, Gupta et al. 2013). Recent reports on CPAP and centriole elongation provide a better understanding of the protein's involvement in the process. Sharma A. et al showed that the PN2.3. domain of CPAP will induce microtubule growth at the centriole in a slow and controlled manner by binding to an exposed beta tubulin domain at the plus ends of microtubules (Sharma, Aher et al. 2016). Following this, Gopalakrishnan and Li groups discovered that two distinct mutations on amino acid residues 375 and 343 of CPAP elicit severe and mild reduction in CPAP-Tubulin interactions which eventually lead to shorter and abnormally longer centriole biogenesis, respectively (Zheng, Ramani et al. 2016). It is also known that CPAP overexpression causes abnormally long centrioles with CP110 protein capping at distal ends. Interestingly when overexpression of CPAP was coupled with depletion of CP110, cells assembled gigantic

centrioles reaching the lengths of 8 µms (Schmidt, Kleylein-Sohn et al. 2009). In fact it is known that while CP110 depletion causes early ciliation, CP110 overexpression prevents the centrioles from further assembly to form the longer cilium, indicating that CP110 might have a dose dependent ruler function for centrioles (Spektor, Tsang et al. 2007).

An electron microscopy based analysis of rhesus monkey oviducts revealed that while a small proportion of new centrioles was produced through pre-existing centriole templated assembly; a great majority (about 95%) of the new centrioles, however, was produced through de novo or "acentriolar" pathway. What was even more remarkable about this study was that in both de novo and centriole templated biogenesis of new centrioles, newly assembled centrioles were in close proximity to an amorphous or fibrous thick coat, pointing out to the existence of functionally homologous structures in two different pathways (Anderson and Brenner 1971). Multiciliated cells massively increase their centriole number through *de novo* centriole biogenesis to enable the simultaneous formation of hundreds of motile cilia (Lemullois, Boisvieux-Ulrich et al. 1988). De novo centriole biogenesis is triggered by the activities of CEP63 paralogue DEUP1, also known as CCDC67. An elegant study by Zhao H. et al showed that DEUP1 was assembling deuterosome complexes through the recruitment of components such as CEP152, PLK4 and Sas-6. The fact that both pre-existing centriole templated or de novo biogenesis of centrioles recruit almost same set of proteins indicates that establishment of de novo centriole biogenesis pathway might have occured through the duplication and differentiation of CEP63 gene where the paralogue might have enabled the harnessing of centriole biogenesis components differently for massive centriole production (Zhao, Zhu et al. 2013). Apart from its role in centriole biogenesis, DEUP1 is also known to be a tumor suppresor whose elevated levels causes increased apoptosis and induced G1 arrest (Yin, Xu et al. 2016).

2.3.2 PERICENTRIOLAR MATERIAL ASSEMBLY

Pericentriolar material (PCM) is a platform for protein complexes that regulate microtubule nucleation, stability and organization. Over the years, about a hundred proteins were identified as components of the PCM and only a subset of them were

shown to take part in the assembly and maintenance of the PCM. PCNT and CDK5RAP2 are two critical components that are required for pericentriolar material assembly. When they are overexpressedin cells, they induce the assembly of expanded PCM that is positive for other PCM components like CEP192, identifying functions to these proteins in the early stages of PCM assembly. In fact, super resolution imaging of the centrosome revealed that while PCNT localizes at the centrosome with its C terminus close to the centriole wall and with the N terminus stretching out across the width of the PCM, other components such as CEP152, NEDD1 and TUBG1 form distinct rings within the PCNT lineated space (Lawo, Hasegan et al. 2012). Such spatial organization supports the scaffolding function of PCNT during PCM assembly.

Once assembled into a compact unit, pericentriolar material expands further for entry into mitosis in a process termed centrosome maturation (Palazzo, Vogel et al. 2000). Phosphorylation of PCNT and Cnn by Polo Like Kinase 1 (PLK1) is required for accumulation of more PCM components such as gammaTubulin (Lee and Rhee 2011, Conduit, Feng et al. 2014). As the PCM matures and primes itself for mitosis, its ability to nucleate microtubules increases significantly (Piehl, Tulu et al. 2004). Aurora A kinase is another critical kinase that regulates the capacity of centrosomal microtubule nucleation, where Aurora A recruits XMAP215 to the centrosome to stabilize microtubule nucleation (Kinoshita, Noetzel et al. 2005). Proper and timely centrosome maturation is critical for the formation of bipolar mitotic spindles and cell cycle protession. In addition to the regulation of PCM assembly and maturation by kinases and scaffolding properties of other centrosomal proteins, pericentriolar material assembly is also fine tuned by the concentration of free tubulin in the cytoplasm and the GTP or GDP bound state of the protein (Gopalakrishnan, Chim et al. 2012).

Genetic screens in *C.elegans* have identified core components required to assemble a PCM, making in vitro assembly of nematode centrosome an attractive field. In fact, Hyman group has demonstrated that one of the core components, Spd-5 was capable of forming meshwork like scaffolds onto which other PCM proteins could be recruited in vitro (Woodruff, Wueseke et al. 2015). Remarkably, the same group was later on able to assemble purified Spd-5 proteins into a spherical condensate in the presence of crowding reagents such as polyethylene glycol (PEG). These condensates were

capable of selectively recruiting other downstream PCM components and microtubule associated proteins. Another remarkable fact on the nature of these condensates was that they had four fold higher concentration of tubulin than in the surrounding solution and they could emanate microtubule asters (Woodruff, Ferreira Gomes et al. 2017).

2.4 CENTROSOME FUNCTION AND DISEASE

The centrosome is the main microtubule-organizing center in animal cells, participating in a variety of cellular processes including chromosome segration by forming the mitotic bipolar spindle, maintaining cell shape and polarity and vesicular trafficking. Importantly, centrioles at the core of the centrosome also function as basal bodies for nucleating the formation of flagella and cilia, including the primary cilium, a nexus for developmetanlly important signlaing pathways like Wnt and Hedgehog signlaing. Given the key function of the centrosome/cilium-complex during cell division and signaling, defects in their structure and are associated with a variety of human diseases including cancer and ciliopathies. Ciliopathies are group of genetic disorders that are caused by mutations on genes encoding proteins that function during cilium assembly and function. Since almost all cells of the human body have cilia, ciliopathies manifest themselves as a combination of anomalies across the body such as polycystic kidney disease, retinal degeneration and microcephaly (Waters and Beales 2011).

2.4.1 Cancer

The association between centrosomal abnormalities and cancer was first observed in the late 1800's; indeed it was one of the first cell biological defects noted in cancer cells (Boveri 2008).The most obvious defect is that cancer cells often have supernumerary centrosomes, which is associated with genome instability due to chromosome attachment errors and increased invasiveness of cells through stimulation of Rac activity and actin polymerization at the cell cortex (Ganem, Godinho et al. 2009, Beroukhim, Mermel et al. 2010). Recent studies demonstrate that cells with extra centrosomes form multiple cilia, which disrupts ciliary signaling and epithelial organization, also a hallmark of many types of cancer. Remarkably, the mechanism by which cancer cells acquire an abnormal number of centrosomes is poorly understood, which requires dissecting the molecular mechanism of centrile duplication (Mahjoub and Stearns 2012).

Cancer cells evolved mechanism to prevent multipolar mitosis in order to increase their fitness that would be grossly perturbed genomic stability (Brinkley 2001). The assembly of supernumerary centrosomes into two individual poles is called “centrosome clustering”, which involves actin cytoskeleton and molecular motors (Kwon, Godinho et al. 2008). During the centrosome clustering process, cancer cells go through a transient multipolar spindle formation where kinetocore-microtubule attachment errors increase and can cause perturbations in the ratio of oncogenic and anti-oncogenic genes which can provide the chromosome instability environment for the malignancy to occur (Ganem, Godinho et al. 2009, Beroukhim, Mermel et al. 2010).

Although cancer cells exhibit centrosome amplification phenotype, whether centrosome amplification is the cause or the consequence of cancers was debatable until recently. While Vitre B. et al. showed that mice with supernumerary centrosomes don’t develop tumors although cells of the same recombinant mice strains showed increased mitotic errors such as µmuclei formation and aneuploidy (Vitre, Holland et al. 2015), Levine M.S. et al. found that centrosome amplification through overexpression of PLK4 was sufficient for spontaneous tumor growth (Levine, Bakker et al. 2017), confirming that extra centriole can cause tumor formation directly.

2.4.2 Microcephaly

Autosomal-recessive primary microcephaly (MCPH) is a human genetic disedase characterized by abnormal brain development where cortex size is dramatically reduced. Remarkably, ten out of twelve of the annotated mutations that are known to cause microcephaly are centrosome and/or spindle pole localizing proteins or proteins that regulate the localization of other proteins to the centrosome (Hussain, Baig et al. 2012). The remaining two affected proteins are CASC5 and ZNF335, which are kinetocore scaffold and nuclear proteins, respectively (Cheeseman, Hori et al. 2008, Yang, Baltus et al. 2012). In fact, centrosome has been implicated in a variety of cellular processes that affect brain development (Kuijpers and Hoogenraad 2011) and mice with supernumerary centrosomes have been shown to develop microcephaly as a result of the depletion in the neural progenitor pool in the brain (Marthiens, Rujano et al. 2013). Very interestingly, Zika virus infected human brain organoids that mimick microcephaly like phenotype also display centrosome structure aberrations that further support the organelle's implication in microcephaly development (Gabriel, Ramani et al. 2017). These lines of evidence identify centrosome-mediated functions as critical regulators of neurogenesis.

2.4.3 Retinal Degeneration

Retinal degeneration is the leading cause of blindness that are caused by loss or ireversable damage of the retinal photoreceptor cells or the improper function of the retinal epithelial cells. Retinal photoreceptor neurons are highly specilized photon responsive cells. These cells are compartmentalized into two distinct units where inner segment is responsible for protein and energy production and outer segment, either with the shape of a cone or a rod, is responsible for photon perception (Brockerhoff and Fadool 2011). Outer segment grows out as an extension of the cilium and its assembly is maintained by kinesin motors and intraflagellar transport protein 88 (IFT88) (Marszalek, Liu et al. 2000, Pazour, Baker et al. 2002).

Retinal degeneration occurs as these photoreceptors are lost or irreversibly damaged. Remarkablly, one third of retinal degeneration mutations have been mapped to genes that encode components of the centrosome/cilium complex (Liu, Zhou et al. 2002,

Hong, Pawlyk et al. 2003, Conkar, Culfa et al. 2017), corroborating the significance of centrosome/cilium-mediated cellular processes during retinal development and function.

2.5 EVOLUTION OF THE CENTROSOME

Centrosome, when literally translated, means the central body and the term itself has been used to annotate any organelle with the ability to maintain itself in the cell centre by emanating and organizing microtubules, to associate itself close to cell nucleus and with ability to double their number as the cells divide. Within the eukaryotic domain, centrosomes can be found widespread across different groups in different shapes. While higher fungi such as *Saccharomyces* and *Ichthyosporea* assemble spindle pole bodies which are organized as distinct layers of sheets, the majority of the eukaryotic lineages has a centrosome composed of centrioles. Although a morphological variation is clearly evident, both Dictyosteliids and higher fungi share centrosome components that suggest a common evolutionary origin (Kilmartin 2003, Mana-Capelli, Graf et al. 2009).

Flagellar machinery is widely conserved across eukaryotic domain and comperative ultrastructural analyses can help us orient ourselves in complex phylogenetic trees. Eukaryotic flagellates share a similar organization and architecture of the two basal bodies of the flagella, suggesting that all eukaryotes descended from a biflagellate organism. Comparative analyses of microtubule root architecture in Amorphea indicate that the common ancestor of Amorphea was also a flagellate organism (Leadbeater B.S.C et al. 2000). Phylogenetic analyses indicate that animal centrosomes might have evolved independently from Amoebozoa and that centrosome with highly similar characteristics across eukaryotic lineages could have arisen as a result of convergent evolution (Azimzadeh 2014).

3 MATERIALS AND METHODS

Cell culture and transfection

hTERT-immortalised retinal pigment epithelial (RPE-1, ATCC, CRL-4000) cells were grown in DMEM:F12 1:1 (Lonza) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermofisher Scientific). Human embryonic kidney (HEK293T, ATCC, CRL-3216) cells were grown in DMEM supplemented with 10% FBS. All cells were cultured at 37 °C and 5% CO2. All cell lines were authenticated by Multiplex Cell line Authentication (MCA) and were tested for mycoplasma contamination by MycoAlert Mycoplasma Detection Kit (Lonza). All cells were passaged when 80-90% confluency was reached. RPE1 cells were transfected with the plasmids using Lipofectamine LTX (Invitrogen). LTX and plus reagents were mixed with a 4:5 (uL) ratio in 250 uL Optimem (Gibco, Thermofisher Scientific) for a transfection of 2 ug plasmid DNA in six well plate. HEK293T cells were transfected with the plasmids using 1 µg/µl polyethylenimine, MW 25 kDa (PEI, Sigma-Aldrich, St. Louis, MO). For microtubule-depolymerization experiments, cells were treated with 10 µg/ml nocodazole (US Biological, Swampscott, MA) for 1 h at 37 °C.

Plasmids and Cloning

Full length cDNAs of CEP103 and CCDC66 were obtained from DNASU plasmid repository. Gateway recombination reactions using GFP-N1, GFP-C1 and BioID-C1 were used to generate CEP103-GFP, CEP103-MycBirA*, GFP-CCDC66. Briefly, Deletion constructs of CEP103 and CCDC66 were made by PCR amplification of the indicated regions using the primers listed in the appendix. PCR products were cloned into pDONR221. 3 uL from the PCR amplicon was mixed with 100 ng pDonr221 vector at 1 uL and 1 uL BP clonase. Gateway recombination reactions using GFP-C1 to produce C terminally GFP tagged deletion constructs or GFP-N1 to produce N terminally GFP tagged deletion constructs were used to generate the expression vectors. Briefly, 100 ng pDonr plasmid was mixed with 100 ng gateway compatible expression vector, 0.5 uL LR clonase enzyme and the volume was topped upto 5 uL using TE Buffer (10 mM Tris-HCl, pH: 8.0, 1 mM EDTA).

Antibodies

Antibodies used for immunofluorescence were rabbit anti-CEP103 (HPA023315; Merck), mouse anti-γ-tubulin (GTU-88; T5326; Merck) at 1:4000, rabbit antiGFP (rabbit anti-GFP at 1.5 µg/ml (as previously described in (Hatch et al., 2010), rabbit anti-Myc (sc-789; Santa Cruz Biotechnology) at 1:1000, mouse anti-Centrin3 (M01-3E6; Abnova) at 1:750, mouse anti α-tubulin (DM1A; ab40742; Abcam), mouse anti-Rootletin (sc-374056; Santa Cruz Biotechnology) at 1:500, mouse anti-Myc (9e10; M4439; Sigma-Aldrich) at 1:500 and mouse anti-polyglutamylated tubulin (GT335; AG-20B-0020-C100; Adipogen) at 1:2000. For immunostainigs, 10 fold diluted antibody aliquots were stored at 4°C. Antibodies used for western blotting were rabbit anti-CEP103 (HPA023344; Merck), rabbit anti-GFP at 0.15 µg/ml (as previously described in (Hatch et al., 2010)), mouse anti- beta actin (60008-1-Ig; Proteintech) at 1:10.000, rabbit anti-GAPDH (10494-1-AP, Proteintech) at 1:10.000, mouse anti-γ-tubulin (GTU-88; T5326; Merck) at 1:2000. While diluted primary antibodies were recollected and stored at -20°C for future use, diluted secondary antibodies were discarded following their first use.

Immunoprecipitation, BioID Pulldowns and Mass Spectrometry

For co-immunoprecipitation experiments, HEK293T cells were transiently transfected, incubated for 24 h, washed with PBS, and lysed in lysis buffer (50 mM HEPES, pH 7.4, 100 mM KCl, 1 mM EGTA, 1 mM MgCl2, 10% Glycerol, 0.3% NP40, protease inhibitor cocktail). Insoluble material was pelleted at 13.000 rpm for 15 minutes at 4°C. Supernatant was collected into another microcentrifuge tube and the pellet was discarded. 1 mg lysate was incubated with 10 uL GBP beads for 2 hours. Following the incubation, beads were washed in lysis buffer lacking the inhibitor cocktail for three times. After the last wash, beads were collected at the bottom by centrifuging at 1000 rpm for 1 minute at 4°C Pelleted beads were eluted in sample buffer (50 mM Tris, pH:

6.8, 2% SDS, 10% Glycerol, 1% beta-mercaptoethanol, 0.005% Bromophenol Blue) and run on SDS-PAGE gels.

For BioID pulldown experiments; HEK293T cells were transiently transfected with Myc-BirA* tagged full length and C terminal half (606-916) of CEP103 and MycBirA* tag alone. 24 hours post-transfection, cells were incubated in 10 uM biotin for 18 hours. Following the incubation with excess biotin, cells were lysed in BioID Buffer (50 mM Tris, pH: 7.4, 500 mM NaCl, 0.4% SDS, 5 mM EDTA, 1 mM DTT, 2% Triton X-100) containing 1mM DTT and inhibitor cocktail by tumbling for 30 minutes at room temperature. Lysate was sonicated at 30% amplitude twice for 20 seconds and at 40% amplitude twice for 20 seconds at 4°C. Equal volume of prechilled 50 mM Tris, pH: 7.4 was added into the lysate. Sonication was repeated using the same scheme. Lysate was spun down at 13.000 rpm for 15 minutes at 4°C. Supernatant was saved into another tube and pellet was discarded. 100 uL from the cleared lysate (supernatant) was mixed with sample buffer and boiled at 95°C to be stored as the initial lysate. Streptavidin beads were washed three times with 50 mM Tris, pH:7.4 at 4°C. For every mililiter of lysate was mixed with 30 uL previously washed streptavidin beads and left tumbling overnight at 4°C. Bead+lysate mixture was spun down at 1000 rpm for 3minutes to precipitate the beads. Supernatant was collected and saved in another tube in case efficient streptavidin-biotin binding did not occur. Beads were washed in Wash Buffer 1 (2% SDS in dH2O) twice and Wash buffer 2 (0.2 % deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, 50 mM HEPES, pH: 7.5) ,3 (10 mM Tris, pH: 8.1, 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA) and 4 (50 mM Tris, pH: 7.4, 50 mM NaCl) once by tumbling at room temperature (RT) for 8 minutes. Beads were spun down at 1000 rpm for 3 minutes at RT. Beads were resuspended in 100 uL 50 mM Ammonium Biocarbonate. $1/10^{th}$ of the pellet was mixed with sample buffer containing 2 mM biotin. Rest of the pellet was either further processed for mass spectrometry or snap-frozen and stored at -80°C.

Proteins bound to the streptavidin beads were reduced with 5 mM tris(2-carboxyethyl)phosphine) (TCEP) for 20 minutes and alkylated with 10 mM Iodoacetamide treatment in the dark for another 20 minutes. Proteins were digested with trypsin (Promega, Medison, WI, USA) at 37°C overnight. Digested samples were

acidified to a final concentration of 5% formic acid. Samples were spun down at 14.000 rpm for 30 minutes. Supernatant was transferred to a new microfuge tube without disturbing the bead pellet and then loaded into the mass spectrometry column.

Microtubule Sedimentation Assay

Hek293T cells were lysed in BRB80 buffer (80 mM PIPES, pH 6.8, 1 mM MgCl2 , 1 mM EGTA including protease inhibitors) and precleared by centrifugation at 15,000rpm for 15min. and 90,000 rpm for 5 min. at 4°C using a TLA-100 rotor. Supernatant was collected and mixed on ice with dithiothreitol (DTT) and GTP to 1 mM final concentration. Taxol was added to a final concentration of 25 µM in a step-wise manner by incubating for 1h at 30°C. Samples were loaded onto 40% glycerol BRB80 cushions and centrifuged at 55,000 rpm for 25 minutes using a TLS-55 or TLA 100 rotor. Supernatant and pellet fractions were separated by SDS–PAGE for analysis.

DNA Transformation into chemically competent *E.coli*

Chemically competent E.coli STBL3 strain was directly taken from -80°C and placed on ice. Bacteria were allowed to thaw at room temperature. Approximately 100 ng plasmid DNA was mixed with bacteria and cells were placed back on ice to be incubated for 10 minutes. DNA-bacteria mixture was then exposed to heat shock at 42°C for 30 seconds. Heat shocked bacteria were incubated on ice for 2 minutes. 1000 uL LB medium without antibiotics was mixed with cells and cells were incubated at 37°C for 1 hour to allow for the transcription of antibiotic resistance gene casettes. 100 uL of the total mixture was spread over LB agar plates containing the appropriate antibiotics required for colony selection. Plates were incubated overnight at 37°C. Next day, single colonies were selected to grow in the LB media with antibiotics for selection.

Immunofluorescence and microscopy

For immunofluorescence experiments, cells were grown on glass coverslips that were treated with ethanol for sterilization, washed with PBS twice and fixed in either 4% parafolmaldehyde at 37°C for 30 minutes or -20°C methanol for 10 minutes. Following fixation, cells were washed twice with PBS, blocked with PBS containing 3% BSA and 0.1% Triton-X and incubated with the indicated primary and secondary antibodies and dilution rates. Primary antibodies were incubated on coverslips either for 1 hour at room temperature or overnight at 4°C. Secondary antibodies were incubated on coverslips for 1 hour at room temperature. DNA was stained using DAPI at 1ug/mL in 2% BSA in dH2O. Coverslips were mounted using ProGold and images were acquired with the Leica SP8 confocal microscopy. Microscopy images were processed with Hyvolution tool under default parameters.

Cell extracts and Western Blotting

Whole cell extracts for Western blotting were prepared by washing cells in ice-cold PBS and lysing them in SDS sample buffer. Extracts were run on SDS-PAGE gels. Stacking gel was composed of 5% acrylamide-bisacrylamide (29:1), 126 mM Tris-HCl, pH: 6.8, 0.1 % SDS (sodium dodecyl sulphate), 0.1% TEMED (Tetramethylenediamine) and 0.1% APS (Ammonium persulfate). Composition of the separating gel was as the following; 12% acrylamide-bisacrylamide (29:1), 375 mM Tris-HCl, pH: 8.8, 0.2% APS, 0.2% SDS and 0.1% TEMED. Proteins in SDS-Gel was transferred to nitrocellulose membranes using wet transfer system. Briefly, PVDF membrane was soaked in methanol for 1 minute and placed into a sandwich with the SDS-gel placed right next to it. Transfer was performed for 2 hours at 4°C at 100 Volts. PVDF membrane was stained transiently with Ponceau-S solution. Following the staining, membrane was destained by incubating the membrane in TBS-T (Tris-Buffered Saline- Tween20). PVDF membrane was blocked in 5% non-fat dried milk powder solution for 1 hour at room temperature and incubated with the primary and secondary antibodies. Following antibody incubation, membrane was imaged using the LICOR imaging system.

Construction of the phylogenetic tree

Amino acid sequences of *Bos taurus, Danio rerio, Homo sapiens, Mus musculus* and *Rattus norvegicus* CEP103 protein were fed into NCBI's Protein BLAST and the phylogenic tree was constructed under default settings.

4 CEP103 is a novel centrosome protein that binds to microtubules

Centrosome number in cells is tightly regulated and in most cells, centrosome duplicates once per cell cycle, assuring that each daughter cell receives only one centrosome at the end of cell division. Centrosome number in cells is maintained by a duplication and segregation mechanism linked to the cell cycle. Centrosome duplication is initiated at the G1 to S transition of the cell cycle when one procentriole adjacent to each pre-existing centriole forms. Among the proteins that function in initiation of centriole duplication is the protein kinase PLK4, the cartwheel protein SAS-6 and the proximal PCM proteins CEP63 and CEP152 that recruit PLK4 to the site of initiation. Procentrioles elongate throughout S and G2 phases, which depends on several proteins including SAS-4/CPAP/CENPJ, POC5, CP110 and CEP97 (Watanabe, Honda et al. 2016). Once centrioles are elongated, the centriole paris are segregated to daughter cells by the mitotic spindle and the linker between the centriole pairs is dissolved by the separase activity to license centriole for another round of duplication in the next cell cycle, which is known as centriole disengagement (Tsou, Wang et al. 2009). Defects in the regulation of centriole duplication and segregation cycle causes centriole number abnormalities and the most common number abnormality is the supernumerary centrioles, which is a hallmark of many different cancer types. Supernumerary centrioles contribute to cancer by increasing aneuploidy and invasive behavior (Godinho and Pellman 2014). Importantly, a recent study showed that supernumerary centrioles induced by PLK4 overexpression in mouse causes cancer (Levine, Bakker et al. 2017).

Given the causal relationship between centriole number abnormalities and cancer, it is critical to dissect the molecular mechanism of centriole duplication throughout the cell cycle. Over the years, a number of different approaches including genetic, proteomics and transcriptomics screens identified many proteins that function during centriole duplication. To elucidate the molecular mechanism of centriole duplication, it is important to complete the parts list of centriole duplication proteins. To this end, we have focused on identification and characterization of novel centriole duplication

proteins through analysis of the BioID proximity interaction maps generated for known centriole duplication proteins (Firat-Karalar, Rauniyar et al. 2014).

Although commonly used techniques such as co-immunoprecipitation has been valuable in identifying proteins and protein-protein interactions important in centrosome duplication, these techniques are limited in identifying the insoluble, weak and transient interactions at the centrosome. Application of proximity labeling approaches overcame the challenges in identifying such interactions at the centrosome and revealed a more detailed and holistic picture on how these components cooperate to ensure proper centrosome duplication (Firat-Karalar, Rauniyar et al. 2014, Gupta, Coyaud et al. 2015). Biotin Identification (BioID) proximity labeling approach makes use of the fusions of protein of interest with the promiscuous biotin ligase BirA*. In cells expressing BirA*-fusions and treated with excess biotin, BirA* was shown to biotinylate proteins in the 20 nm proximity of the protein of interest (Roux, Kim et al. 2012). Application of the BioID approach to the proteins that function in centriole duplication including PLK4, CEP192, CEP63 and CEP152 identified both known and previously uncharacterized centriole duplication proteins. Studies that focused on the characterization of the novel hits from these screens revealed new proteins including CCDC14 and KIAA0753, which regulate centriole duplication through regulating CEP63 levels at the centrosome, which shows the power of the BioID approach in identifying new regulators of centriole duplication (Firat-Karalar, Rauniyar et al. 2014).

Among the the previously characterized proteins that are in the proximity interaction maps of centriole duplication proteins, CEP103 stands out by its specific and high confidence proximity relationship to CCDC67 (Firat-Karalar, Rauniyar et al. 2014). CCDC67, also known as DEUP1, functions in *de novo* duplication of centrioles in mouse multiciliated tracheal epithelial cells (Zhao, Zhu et al. 2013). CCDC67 was implicated as a potential tumor suppressor in gastric cancers and thyroid carcinomas (Park, Jang et al. 2012, Yin, Xu et al. 2016). Importantly, CCDC67 is paralogous to CEP63, which functions in canonical centriole duplication. CEP63 interacts with CEP152 and functions in recruitment of the protein kinase PLK4 to the site of procentriole

formation during iniation of centriole duplication (Sonnen, Gabryjonczyk et al. 2013). Depletion of CEP63 and CEP152 in mammalian cells cause centriole underduplication, where cells fail to efficiently duplicate the existing centrioles (Brown, Marjanovic et al. 2013). Given its proximity to the CEP63/CCDC67 duplication module, we focused on dissecting the function and regulation of CCDC67 as a new component of this duplicatoin module.

There are two lines of evidence that suggest a role for CEP103 in centrosome and/or cilium-mediated cellular processes. First, CEP103 was upregulated about fivefold during early stages of differentiation in mouse tracheal epithelial cell (MTEC) cultures based on a trancriptome screen (Hoh, Stowe et al. 2012). During differentiation of multiciliated tracheal cultures, hundred of centrioles are amplified through the de novo duplication pathway, which then dock to the apical surface to form hundreds of motile cilia. Assembly of hundreds of centrioles and cilia during MTEC differentiation requires upregulation of the genes that function in these processes. Therefore, the the transcriptomic analysis of the upregulated genes during MTEC differentiation provided a very powerful resource for identification of new components of centrosome and cilum biogenesis because. Second, in addition to identification of CEP103 as a proximity hit of the centriole duplication protein CCDC67 (Firat-Karalar, Rauniyar et al. 2014), CEP103 was later identified as a proximity partner of the centriolar satellite protein OFD1 in BioID experiments in HEK293T cells(Gupta, Coyaud et al. 2015). Centriolar satellites are ubiquitous and essential regulators of the mammalian centrosome/cilium complex and the underlying mechanism of this regulation is poorly understood. Importantly, they were recently shown to regulate centriole duplication through recruitment of centriole duplicatoin proteins, such as CEP63 to the centrosome (Firat-Karalar, Rauniyar et al. 2014, Kodani, Yu et al. 2015). Moreover, the centriolar satellite protein OFD1 functions during ciliogenesis (Lopes, Prosser et al. 2011). Therefore, dissecting the function and regulation of CEP103 as a putative centrosome and centriolar satellite component has the potential to reveal new mechanisms of centriole duplication and cilium formation in cells with primary cilium and cells with motile cilia.

In this study, we functionally and biochemically characterized CEP103 in mammalian cell lines. We showed that CEP103 localizes to the centrosome and in a subset of cells also to the centriolar satellites. Importantly, CEP103 also localizes to the interphase microtubule network and to the spindle microtubules and central spindle during mitosis. In vitro microtubule pelleting experiments revealed affinity of CEP103 to microtubules. Analysis of the CEP103 deletion mutants in localization and pelleting experiments identified the C-terminal half of the protein that contains a coiled coil domain to be required for its localization ot the microtubules and the centrosome. Overexpression of CEP103 disrupted the organization of centriolar satellites in cells. BioID proximity mapping of full-length CEP103 and its C-terminal half revealed extensive proximity interaction with centrosome and centriolar satellites proteins, including the duplication protein CCDC67, the centriolar linker protein CROCC2, the centriole protein FAM161A and the centriolar satellite scaffold PCM1. Using co-localization and co-immunoprecipiation experiments, we showed that CCDC67 interacts, directly or indirectly, with CCDC67, PCM1 and CEP63. Together, these findings identify CEP103 as a novel centrosome protein that localizes to the microtubules, interacts with duplication and satellites proteins and disrupts satellite organizatoin when overexpressed. Future studies are required to elucidate its function in centrile duplication and cilium formation.

4.1 Results

4.1.1 CEP103 is a centrosomal protein

CEP103 was previously identified as a proximity partner of the centriole duplication protein CCDC67 (Firat-Karalar, Rauniyar et al. 2014) and the ciliogenesis factor OFD1 (Gupta, Coyaud et al. 2015) in HEK293T cells in the BioID proximity labeling screens. Supporting its putative function in centrosome/cilium complex-mediated cellular processes, its expression was upregulated about fivefold during early stages of MTEC differentiation and when centrioles are massively amplified (Hoh, Stowe et al. 2012). Given that CEP103 was not previously characterized for its function and regulation, we first examined it for its domain architecture and evolutionary conservation. CEP103 contains 2 coiled-coil (CC) domains: CC1 (amino acids 25-487) and CC2 (amino acids

518-605) (Figure 1A). Orthologs of CEP103 are present in hemichordates and placozoans but absent in arthropods, nematodes and Chlamydomonas and such evolutionary conservation pattern in similar to CCDC67.

Based on its proximity interactions and evolutionary conservation, we hypothesized that CEP103 is a putative centrosome protein that functions in centrosome/cilium-related cellular processes. To test our hypothesis, we examined the localization of endogenous CEP103 and GFP-CEP103 in human retinal pigmental epithelial (RPE1) and HeLa cells using immunofluorescence experiments. A rabbit polyclonal antibody raised against the N terminus of CEP103 showed that CEP103 localizes to the centrosome in HeLa and RPE1 cells (Figure 1C). Interestingly, CEP103 also localizes to the centriolar satellites in a subpopulation of HeLa cells. Immunoblotting with the CEP103 antibody in human epithelial RPE1, epithelial HeLa, osteosarcoma U2OS cells and mouse epithelial IMCD3 cells cell extracts revealed that CEP103 is ubiquitously expreseed across different cell types and thus has a wide expression pattern (Figure 1D). Localization of proteins at the centrosome can be dependent and independent of microtubules. Upon depolymerization of microtubules using drugs like nocodazole, bona fide centrosome proteins still localize to the centrosome (Nakadai, Kishimoto et al. 1999, Suizu, Ryo et al. 2006).To test whether CEP103 is a bona fide centrosome protein or not, HeLa cells were treated with nocodazole and CEP103 levels at the centrosome was quantified in control and nocodazole-treated cells using quantitative immunofluorescence. While there was a small but significant (P value < 0.0001) decrease in centrosomal levels of CEP103, (28 %), CEP103 still localized to the centrosome relative to control cells, which identifies CEP103 as a bona fide centrosome protein (Figure 1F). In aggreement with this result, CEP103 also localizes to the centrosomes purified from HeLa cells using sucrose gradient centrifugation (Figure 1E).

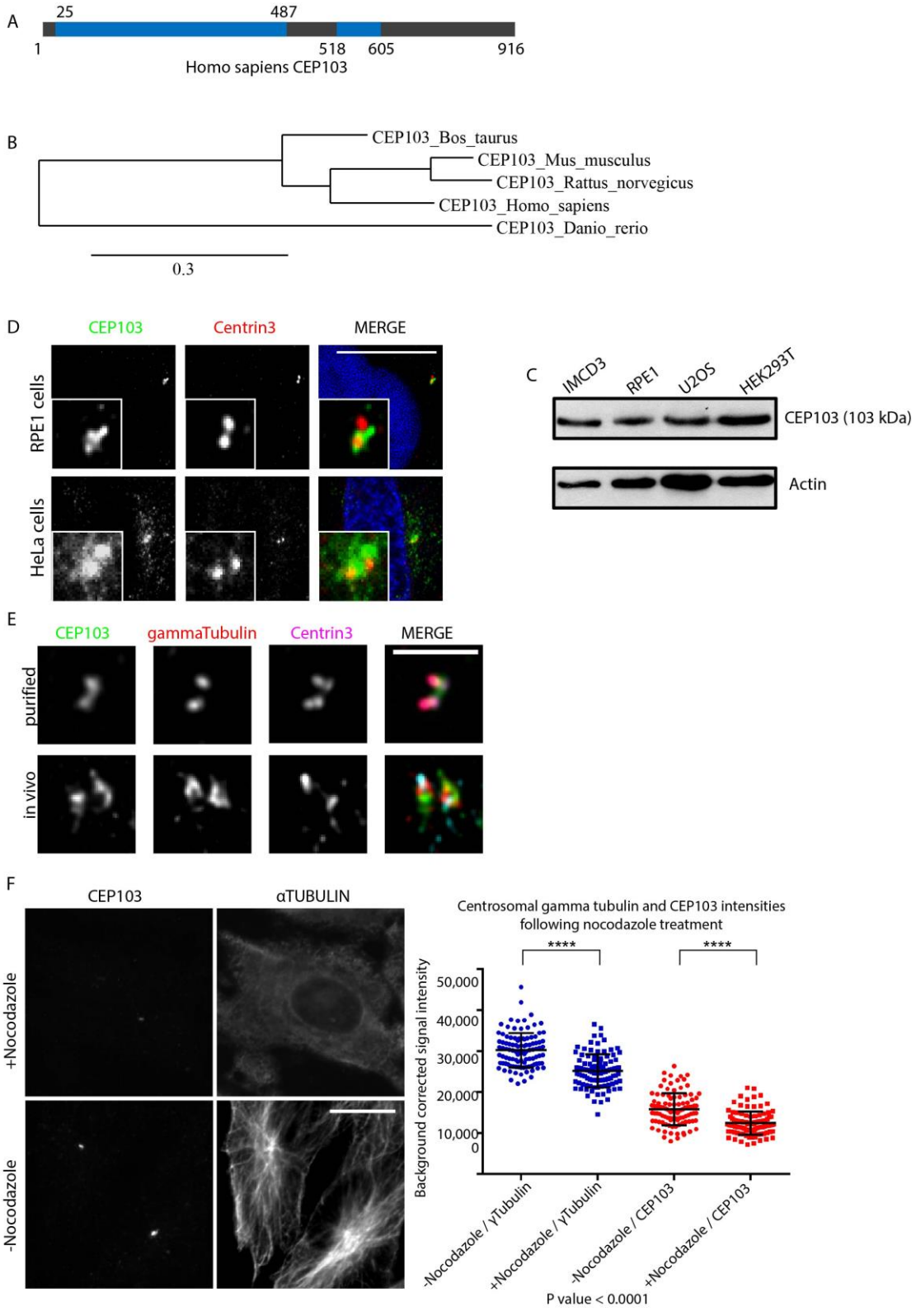


Figure 4.1-1. CEP103 is a centrosomal protein. A) Phylogenetic dendogram showing the relationship between CEP103 of different vertebrates. B) Schematic representation of human CEP103. CEP103 contains two major coiled-coil domains (depicted as blue boxes). C) CEP103 protein expression in different cell lines. Lysates from different human cell lines and a mouse cell line were run and blotted for CEP103. Beta Actin was used as loading control. D) CEP103 localizes to the centrosome. RPE1 cells were fixed and stained for CEP103 and centriolar

marker Centrin3. DNA stained with DAPI. Scale bar: 10 µm E) Localization of CEP103 at the centrosome. Cycling HeLa cells were fixed and stained for CEP103, gamma tubulin and Centrin3 (lower panel). Purified centrosomes from HeLa cells were spun down and embedded on coverslips, fixed and stained for CEP103, Centrin3 and gamma tubulin (upper panel). F) CEP103 centrosomal signal intensity following nocodazole treatment. RPE1 cells were treated with 5 ug/mL nocodazole for one hour. Cells were fixed in methanol and stained for CEP103 and gamma tubulin. Centrosomal signal was quantified in ImageJ. p value <0.0001.

4.1.2 CEP103 localizes to the proximal end of the centrioles

Centrosomes are spatially organized into different domains that are associated with different functions. For example, distal appendage proteins that function in ciliogenesis localize at the distal end of the mother centriole, centriole duplication proteins that function in the assembly of procentrioles localize to the proximal end of the centriole and PCM proteins localize in concentric rings of different sizes around the centriole (Lawo, Hasegan et al. 2012, Mennella, Keszthelyi et al. 2012). To determine the spatial localization of CEP103 at the centrosome, we applied high-resolution microscopy in cells stained for CEP103 and distal and proximal markers of the centriole. RPE1 cells were stained using antibodies against the centriole protein Centrin 3 with distal centriole localization, centriolar and ciliary microtubule marker polyglutamylated tubulin, centriole duplication protein CEP63 with proximal PCM localization, centriolar linker proteins C-NAP1 and Rootletin and visualized using the Hyvolution system. In serum starved ciliated RPE1 cells, CEP103 localizes proximal to polyglutamylated Tubulin and Centrin3 (Figure2B, upper panel). To better resolve the proximal site localization and to identify possible interactors of CEP103, we co-stained CEP103 along with centrosomal proteins that function in centriole duplication and centrosome cohesion. CEP103 localizes proximal to the centriole duplication protein GFP-CEP63 (Figure 2B, middle panel), but partially distal to the centriolar linker proteins Rootletin and C-Nap1 (Figure 2A and 2B). Interestingly, a small population of CEP103 proximal pool co-localized with C-NAP1, suggesting that CEP103 might function in centrosome cohesion. Together, the localization pattern of CEP103 is consistent with it being a component of the pericentriolar material and localization at the proximal end of the centrosome.

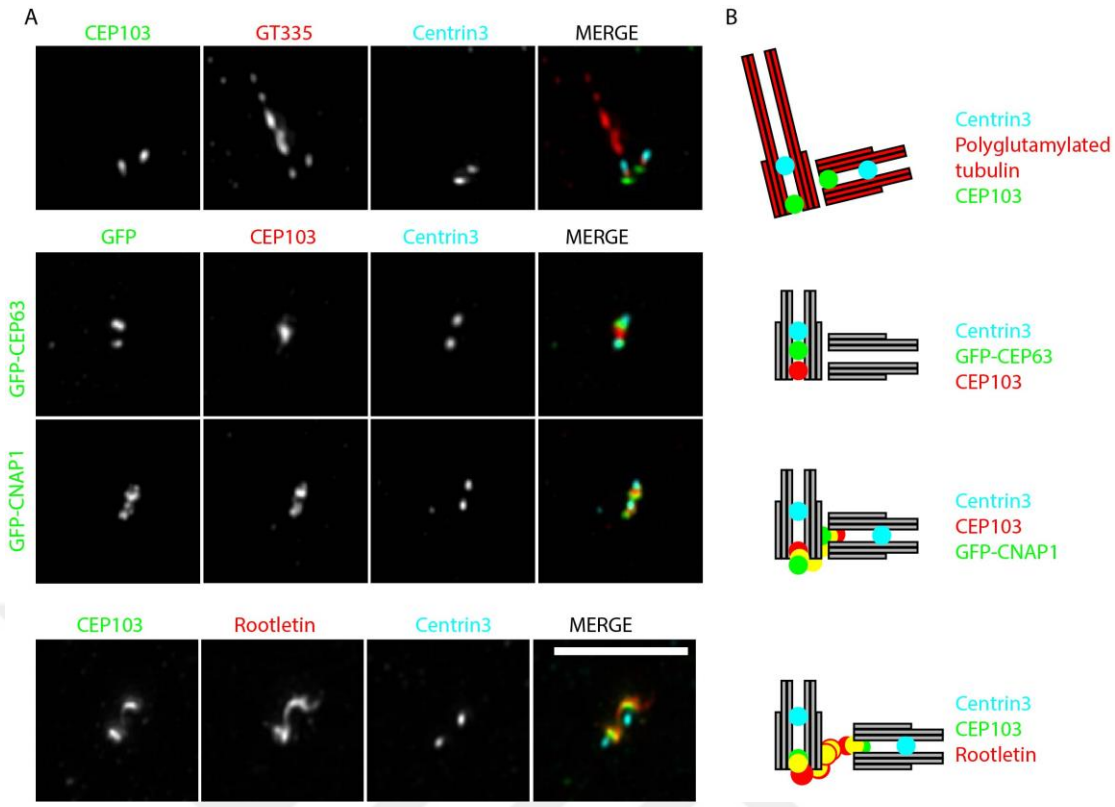


Figure 4.1-2. CEP103 is a proximal protein, partially colocalizing with centriole fiber proteins C-NAP1 and Rootletin. A) Immunofluorescence analysis of CEP103 localization in RPE1 cells using high resolution microscopy. RPE1 cells were fixed and stained for CEP103 and markers for polyglutamylated tubulin (GT335), Centrin3, Rootletin, C-NAP1 and Rootletin, GFP-CEP63, which stains the two centrioles, the cilium, distal end of the centrioles, centriole fibers and proximal ends of centrioles, respectively. Scale bar: 4 µm. B) Schematic representation of sub-centrosomal localization of CEP103 in RPE1 cells.

4.1.3 CEP103 localizes dynamically to the centrosome and the microtubules throughout the cell cycle

Centrosome undergoes dynamic changes during cell cycle. It forms the primary cilium in G1 phase, which disassembles before mitosis. Centrioles duplicate and elongate in S/G2 phases and centrosomes mature to form spindle poles during mitosis. To determine which cell cycle phases and events CEP103 is associated with, we determined its cellular localizaton throughout the cell cycle in RPE1 cells relative to the centriole distal end marker Centrin 3 and microtubules. CEP103 localizes to the proximal end of the centrosome throughout the cell cycle (Figure 2A). During centriole duplication in S/G2 phases and mitosis, it is enriched at the preexisting centrioles, in

aggreement with it being a component of the pericentriolar material, not the centrioles. Interestingly, CEP103 localizes to the spindle microtubules in metaphase and the microtubules of the central spindle in telophase and cytoskinesis, suggesting that CEP103 might be a microtubule binding protein. Supporting such affinity, CEP103 co-localizes with the radial interphase microtubule network in RPE1 cells where the its cytosolic pool is depleted by preextraction with detergent before methanol fixation (Figure 2B).

Given the association of CEP103 with interphase and mitotic microtubules in vivo, we next tested whether CEP103 interacts with microtubules using the in vitro microtubule plelleting assays using cell extracts from HEK293T cells. Briefly, cells were lysed and incubated in taxol to stabilize microtubules. Taxol-stabilized microtubules with their associated proteins were then spun on a glycerol cushion and the initial lysate, supernatant and pellet fractions were probbed for tubulin, CEP103, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as negative control and gamma-tubulin as positive control. GAPDH stayed in the supernatant and a fraction of gamma-tubulin co-pelleted with taxol-stabilized microtubules, which validates the fidelity of the pelleting experiments (Figure 3C). The co-localization experiments with alpha-tubulin and the in vitro microtubule pelleting experiments both suggest that CEP103 either directly or indirectly binds to microtubules in cells.

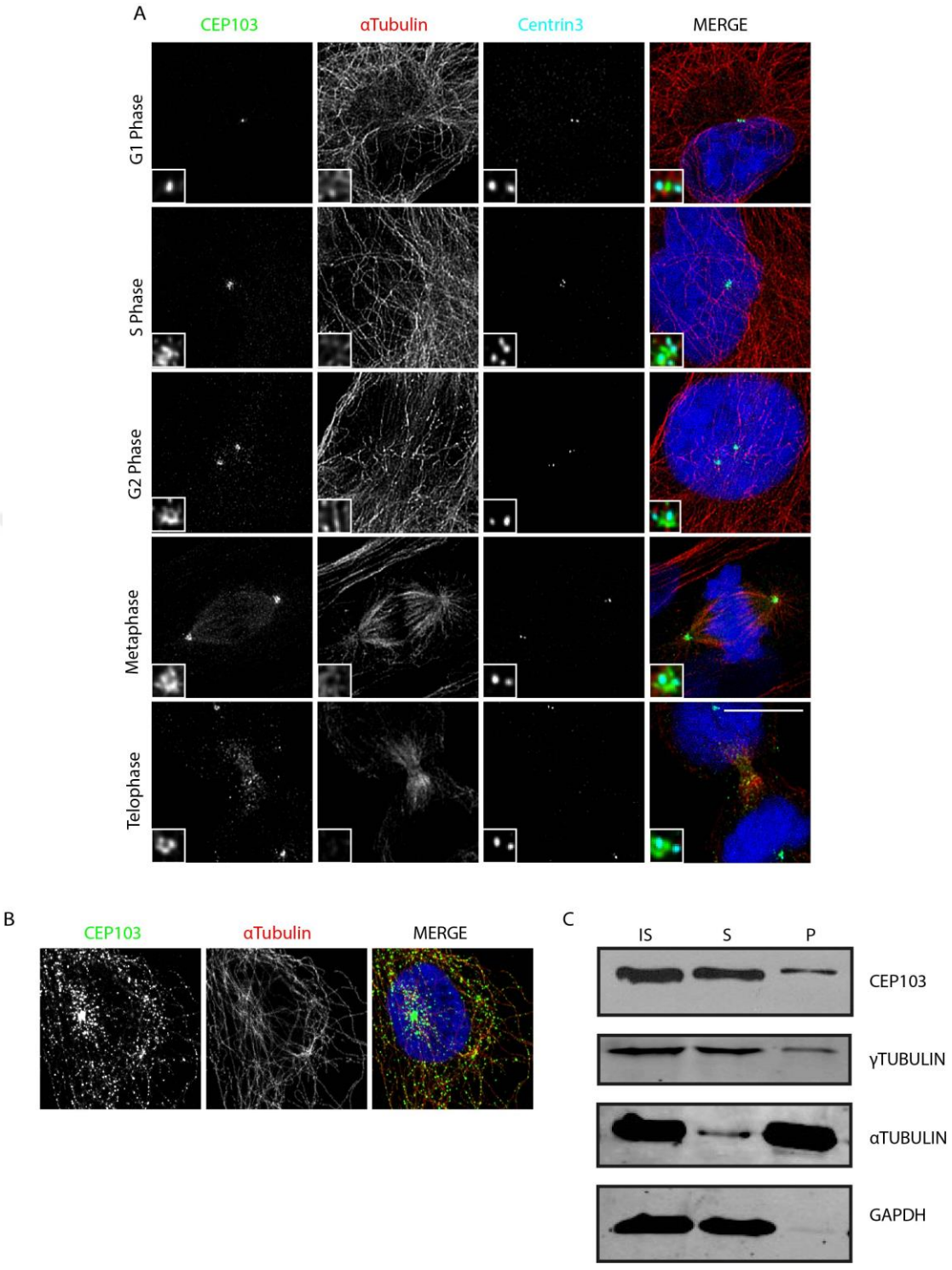


Figure 4.1-3. CEP103 dynamically localizes throughout the cell cycle and binds to microtubules A) Cell cycle localization of CEP103 in RPE1 cells. Cycling RPE1 cells were fixed and stained for CEP103, alpha tubulin and Centrin 3. DNA stained with DAPI. Scale bar: 10 µm. Inset: 4X B) CEP103 localizes to interphase microtubules. RPE1 cells were pre-extracted with 0.5% Tx-100 prior to methanol fixation and centrosomal signal of an interphase RPE1 cell was oversaturated to reveal the microtubule binding pool. DNA was stained with DAPI. C) CEP103 binds to microtubules *in vitro*. HEK293T cells were lysed and microtubules were stabilized using taxol. Stabilized microtubules were sedimented and samples from initial sample (IS),

supernatant (S) and pellet (P) were run on SDS-PAGE and blotted with antibodies against GAPDH, alpha tubulin, gamma tubulin and CEP103.

4.1.4 C terminus truncation mutant 606-916 is sufficient and required for CEP103's localization to the centrosome and the microtubules

To determine which region of CEP103 is required for its centrosome and microtubule localization, we generated a series of GFP-tagged CEP103 deletion mutants and determined their localization in transfected RPE1 cells stained for microtubules or centrioles. CEP103 is a 916 amino acid in length and contains 2 coiled-coil (CC) domains: CC1 (amino acids 25-487) and CC2 (amino acids 518-605) (Figure 1B). Full length GFP-CEP103 localizes to the centrosome in low-expressing cells. Interestingly, when overexpressed, full length CEP103 did not co-localize with microtubules, instead it formed large aggregates that do not co-localize with centriole marker centrin. Formation of aggregates upon overexpression of proteins is typical to centriolar satellite proteins, supporting the affinity of CEP103 to centriolar satellites as suggested based on immunofluorescence experiments in HeLa cells (Figure 1D). GFP-CEP103 (1-502) and GFP-CEP103 (1-605) spanning CC1 didn't localize to the centrosome or the microtubules (Figure 4A and 4B). While GFP-CEP103 (1-606) also formed aggregates in transfected cells, GFP-CEP103 (1-502) formed filamentous structures that did not co-localize with microtubules (Figure 4A and 4B). Importantly, GFP-CEP103 (606-916) localized to the centrosome and microtubules, while GFP-CEP103 (684-916) did not (Figure 4A and 4B). These data identifies the 606-916 amino acid region to be required for the affinity of CEP103 to the centrosome and microtubules. Given that this C-terminal fragment of CEP103, but not full-length, localizes to the centrosome and microtubules suggest this region is possible buried inside the full-length CEP103 such that its affinity to the centrosome and microtubules is inhibited.

To test whether CEP103 (606-916) has affinity to microtubules, we performed in vitro microtubule pelleting assays with extracts from HEK293T cells expressing GFP-CEP103 (606-916) or GFP as negative control. Consistent with the localization results, GFP-CEP103 (606-916), but not GFP alone, co-pelleted with taxol-stabilized microtubules (Figure 4C) and this results indicates the CEP103 (606-916) either directly or indirectly has affinity to microtubules.

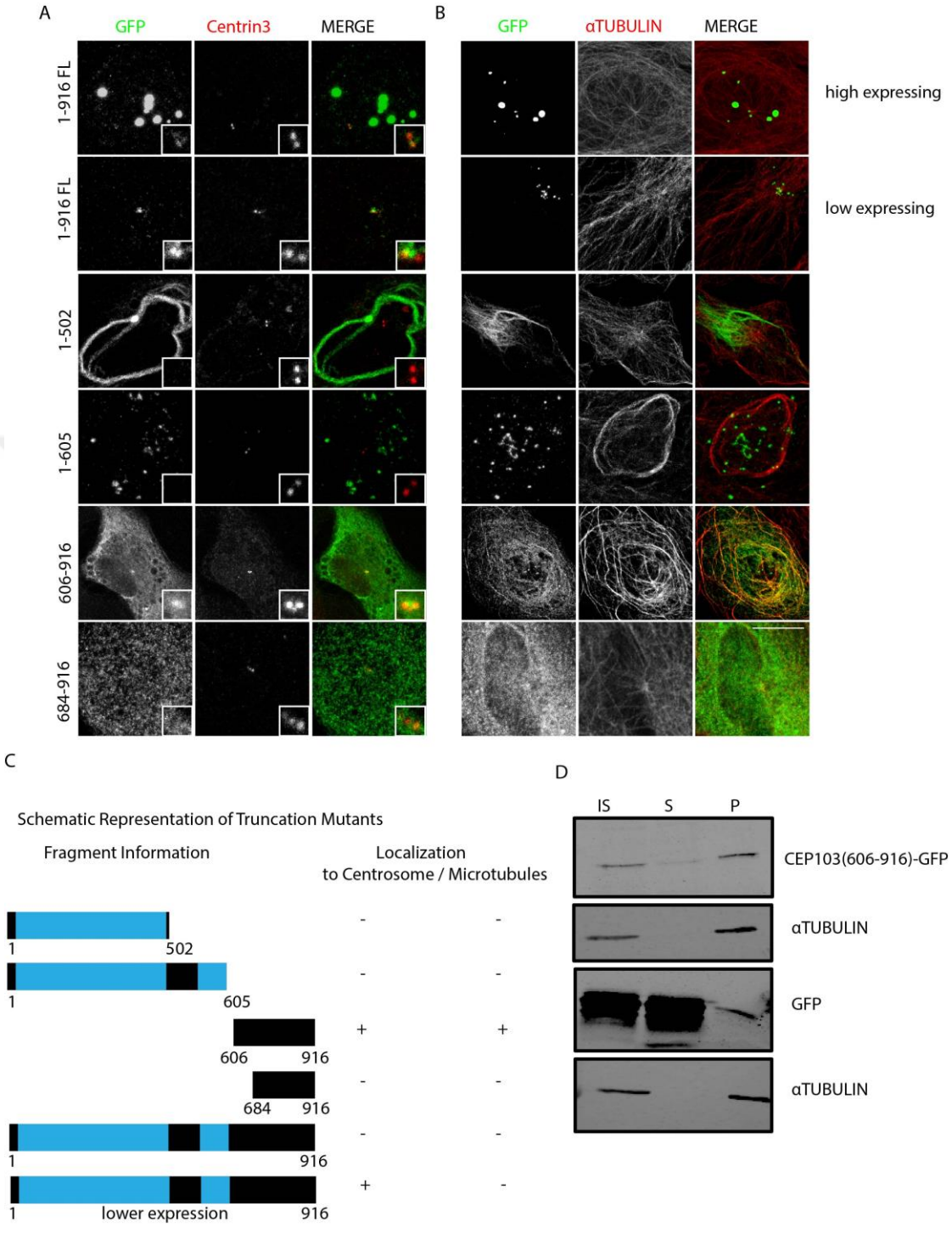


Figure 4.1-4. C terminus truncation mutant 606-916 is sufficient and required for CEP103's localization to the centrosome and the microtubules. Representative immunofluorescence images of RPE1 cells transfected with full-length and deletion mutants of CEP103, fixed and stained for A) GFP and Centrin 3 B) GFP and microtubules. Scale bar: 10 µm. C) Schmatic representation of full length CEP103 and its truncation mutants. Blue boxes represent the coiled-coil domains of the protein. Centrosome and/or microtubule localizing fragments were assigned as +, while the fragments that failed to show such localization were

assigned as -. D) GFP-CEP103 (606-916) binds to microtubules. HEK293T cells were transfected with either GFP alone or CEP103-GFP (606-916) constructs, *in vitro* microtubule pelleting assay was performed with the transfected cell lysates, IS, S and P were run on SDS-PAGE and blotted with GFP and alpha-tubulin. IS: Initial lysate, S: Supernatant fraction, P: Pellet fraction.

4.1.5 Filamentous structures formed by GFP-CEP103 truncations are not stable microtubules

GFP-fusions of full-length and deletion mutants of CEP103 induced formation of aggregates, filamentous structures and microtubule bundles in overexpressing cells. To determine whether the assembly and organization of these structures are dependent on an intact microtubule network and is so whether they are stable microtubules, GFP-fusions of full-length CEP103 (1-916), CEP103 (1-502) and CEP103 (503-916) were expressed in HeLa cells and colocalization of these proteins to microtubules was assessed before and after microtubule depolymerization. HeLa cells were transfected with the indicated constructs, treated with DMSO or 5 ug/mL nocodazole for 1 h, fixed and stained for GFP and microtubules. Aggregates formed by overexpression of the full length protein didn't show a morphological or spatial change following nocodazole treatment. (Figure 5, upper panel), suggesting that their assembly and localization does not depend on microtubules. GFP-CEP103 (1-502)-induced filamentous structures were still present following nocodazole treatment. Given that these structure also did not colocalize with microtubules, they might represent self-assembly of this fragment through its coiled coil domain. Finally, the localization of GFP-CEP103 (503-916) to interphase microtubules were disrupted after nocodazole treatment, indicating that nocodazole destabilizes the GFP-CEP103 (503-916)-bound microtubules. While this result confirms that GFP-CEP103 (503-916)-positive filaments are indeed microtubules, it also suggests that GFP-CEP103 (505-916) does not stabilize microtubules as stable microtubules, which resist microtubule deplymerization. These data indicate that microtubule-affinity of CEP103 is mediated through its (503-916) region and that its N-terminal (1-502) region plays an inhibitory role in its microtubule affinity through self-assembly into large aggregates.

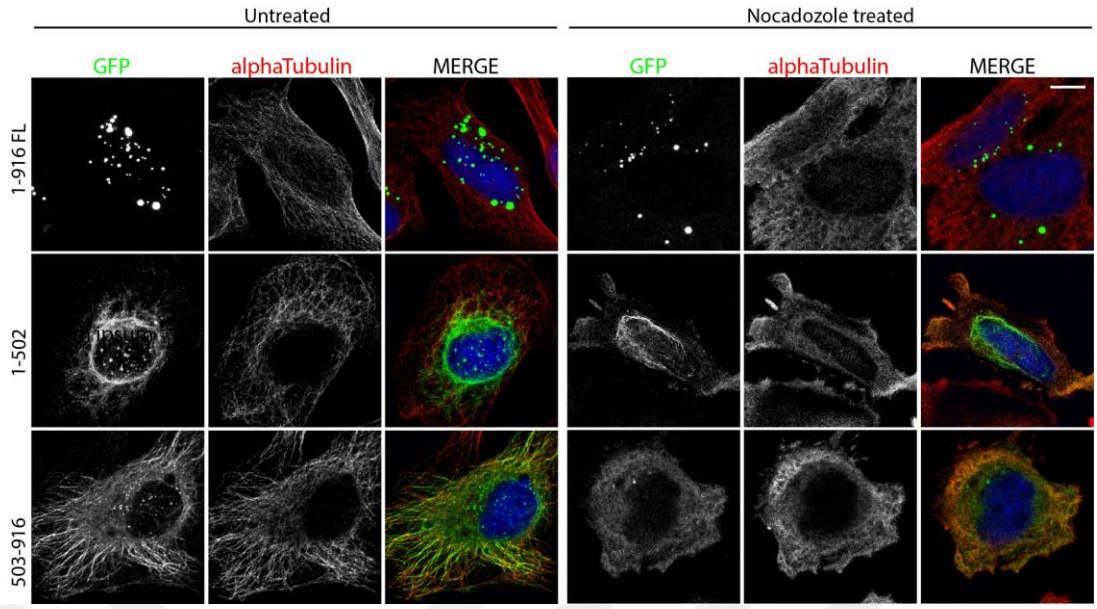


Figure 4.1-5. Filamentous structures formed by GFP-CEP103 truncations are not stable microtubules. HeLa cells were transfected with GFP-fusions of CEP103 (1-916), CEP103 (1-502) and CEP103 (503-916), treated with DMSO or 5 ug/mL nocodazole for 1h, fixed and stained for GFP and alpha-tubulin. DNA stained with DAPI. Scale bar: 10 µm.

4.1.6 CEP103 interacts with CEP63 and its paralog CCDC67

To gain insight into cellular functions of CEP103, we identified the proximity partners of CEP103 using BioID proximity labeling experiments. HeLa cells were transfected with myc-BirA*-fusions of full-length CEP103 (1-916) and its C-terminal half CEP103 (606-916), incubated with 50 um biotin for 18 h, fixed and stained for biotinylated proteins and centrosome marker gamma-tubulin. Both constructs localized to the centrosome and induced localized biotinylation at the centrosome after 18 h 50 uM biotin incubation (Figure 6A), confirming the fidelity of the BioID approach. Next, HEK293T cells were transfected with mycBirA* itself as control or myc-BirA* fusions of CEP103 (1-916) and CEP103 (606-916), incubated with biotin for 18 h, lysed in dentaturing lysis buffer and processed for pulldown of biotinylated proteins using streptavidin beads. The mass spectrometry analysis of the eluates identified the possible interactors of CEP103, which was filtered using myc-BirA* dataset as control.

Full-length CEP103 and its C-terminal truncation mutant had both overlapping and distinct proximity parners, which are listed Figure 6B. Several of these BioID hits stand

out by their function and localization at the centrosome/cilium complex. PCM1 is the scaffolding protein for centriolar satellites and is essential for satellite assembly (Dammermann and Merdes 2002). The proximity of both the full-length and the C-terminal fragment to PCM1 supports the affinity of CEP103 to centriolar satellites, consistent with the cellular localization data. DEUP1/CCDC67 is another centrosome protein with functions in *de novo* centriole biogenesis in multicilaited epithelial cells (Zhao, Zhu et al. 2013). Importantly, CEP103 was initially identified as a proximity partner for CCDC67 (Firat-Karalar, Rauniyar et al. 2014) and the reciprocal identification with our dataset further supports the interaction between these two proteins. Ciliary rootlet coiled coil rootletin family member-2 (CROCC2) is a component of the centrosome linker/it's a putative ciliary rootlet protein (C?????) and its identification is in aggreement with the partial localization of CEP103 a the centrosome fibers using high resolution microscopy an (Figure 3B). Finally, FAM161A is a microtubule associated protein, which localizes close to the centriole sall, functions in cilium formation and its mutations are linked to in retinitis pigmentose 28 (Zach, Grassmann et al. 2012).

To determine which proximity interactions reflect functional relationship in cells, we first assessed the co-localization of CEP103 with the selected candidates in immunofluorescence experiments. Aggregates that form by overexpression of centrosome and centriolar satellite proteins were shown to sequester proteins that have functional or physical relationship to them (Dammermann and Merdes 2002, Xie, Qin et al. 2016). Myc-CEP103-positive aggregates in transfected cells stained positive with GFP-CCDC67 and endogenous PCM1 (Figure 6C). We also transfected cells with GFP-FAM161A, which formed microtubule bundles when overexpressed and showed that endogenous CEP103 is recruited to these bundles (Figure 6D). Together, the co-localization experiments in cells support the proximity interaction of CEP103 to PCM1, FAM161A and CCDC67 and identify these proteins as putative proteins that function with CEP103 in centrosome/cilium-related cellular processes.

To determine which proximity hits reflected physical interaction with CEP103, we performed co-immunoprecipitation experiments in HEK293T cells with the candidates

we validated in co-localization experiments. Given that CCDC67 and CEP103 has reciprocal proximity interactions with each other and co-localize in cells, we first tested the interaction between these proteins by performing GFP pulldown experiments in HEK293T lysates expressing Myc-CEP103 and GFP-CCDC67. Myc-CEP103 co-precipitated with GFP-CCDC67, but not with GFP, indicating that Myc-CEP103 and GFP-CCDC67 interact when overexpressed in cells (Figure 6F). CCDC67 is paralogous to the centriole duplication protein CEP63 and its expression is specific to multiciliated cells where it regulated de novo centriole duplication through deuterosome formation (Zhao, Zhu et al. 2013). However, CEP63 is ubiquitiously expressed and functions in canonical centriole duplication (Lukinavicius, Lavogina et al. 2013, Zhao, Zhu et al. 2013). Given the evolutionary relationship between CCDC67 and CEP63 and their expression profile, we performed CEP63 pulldown experiments in HEK293T cell lysates expressing GFP-CEP63. Endogenous CEP103 co-precipitated with GFP-CEP63, but not with GFP (Figure 6E). Co-immunoprecipitation experiments indicate physical interaction of CEP103 to the centriole duplication proteins CCDC67 and CEP63. Importantly, these interactions suggest that CEP103 might function in canonical and de novo centriole duplication in different cell types.

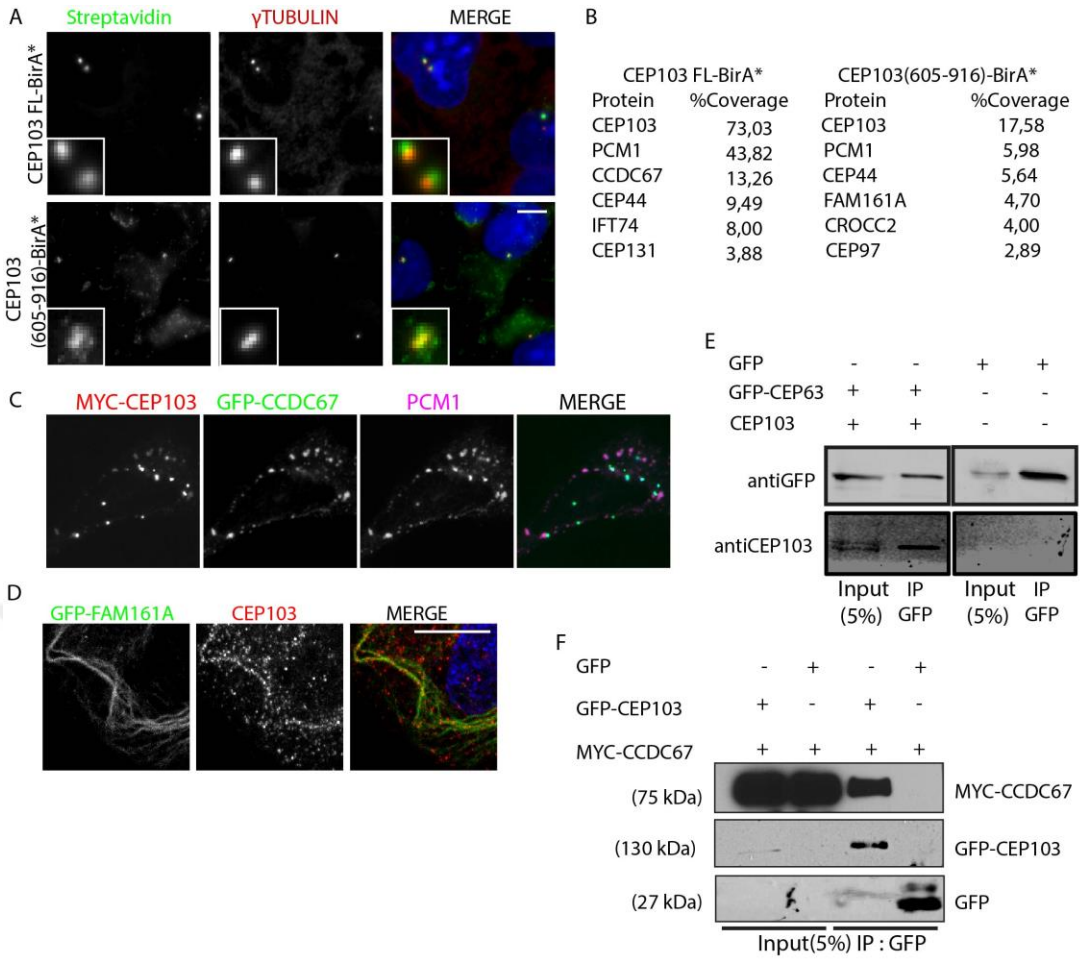


Figure 4.1-6. CEP103 interacts with CEP63 and its paralog CCDC67 A) Myc-BirA*-fusions of full-length CEP103 and its C-terminal mutant induces localized biotinylation at teh centrosome. RPE1 cells were transfected with Myc-BirA*-fusions of CEP103 (1-916) and CEP103 (606-916) constructs and treated with 50 uM biotin for 18h. Cells were fixed and stained fort the centrosome marker gamma-tublin and marker for biotinylated proteins with fluorescent streptavidin. B) Mass spectrometry analysis of proximity interactors of Myc-BirA*-fusions of CEP103 (1-916) and CEP103 (606-916). Proximity interactors are ranked in the order of percentage of coverage. C) MYC-CEP103-positive aggregates sequester GFP-CCDC67 and the centriolar satellite protein PCM1. HeLa cells were transfected with GFP tagged CCDC67 and MYC tagged CEP103. Transfected cells were fixed and stained for GFP, MYC and PCM1. D) PCM1 distinctively localizes at the periphery of GFP-CEP103-positive aggregates. RPE1 cells were transfected with GFP-CEP103 construct, fixed 24h post-transfection and stained for GFP and PCM1. Upper panel scale bar: 10 µm. Lower panel scale bar: 1 µm. E) GFP-FAM161A-induced microtubule bundles co-localizes with endogenous CEP103. HeLa cells were transfected with GFP-FAM161A construct, fixed and stained for GFP and CEP103. Scale bar: 10 µm. F) CEP103 and CEP63 physically interact in co-immunoprecipitation experiments. HEK293T cells were transfected with GFP or GFP-CEP63 and immunoprecipitation against the GFP tag was performed. 5% of the initial lysate and the

equal volume of pellet were run on SDS-PAGE gel. Western blots were incubated with anti GFP and anti CEP103 antibodies. G) GFP-CEP103 and Myc-CCDC67 physically interact in co-immunoprecipitation experiments. HEK293T cells were transfected with GFP-CEP103 and MYC-CCDC67 constructs. Immunoprecipitation against the GFP tag was performed. 5% of the initial lysate and the equal volume of pellet were run on SDS gel. Western blots were incubated with anti GFP and anti MYC antibodies.

4.1.7 Overexpression of CEP103 disrupts centriolar satellite organization

Overexpression of many centrosome and centriolar satellite proteins like CEP290 and CEP72 disrupts centriolar satellite organization by forming large aggregates that sequester PCM1. To test the phenotypic effects of CEP103 overexpression on centriolar satellite formation and organization, GFP-CEP103 was transfected to HeLa cells. GFP-CEP103 overexpression resulted in the formation of cytoplasmic aggregates that sequestered endogenous PCM1 and complete disappearance of the centriolar satellite pool of PCM1 (Figure 7). Interestingly PCM1 was sequestered to the periphery of these aggregates, suggesting that these structures might have spatial organization. The size of the aggregates induced by GFP-CEP103 correlated with its expression levels, such that cells expressing higher levels of the protein formed bigger aggregates (Figure 7). Together, these finding identify important roles for CEP103 in centriolar satellite assembly, dynamics and/or organization in cells.

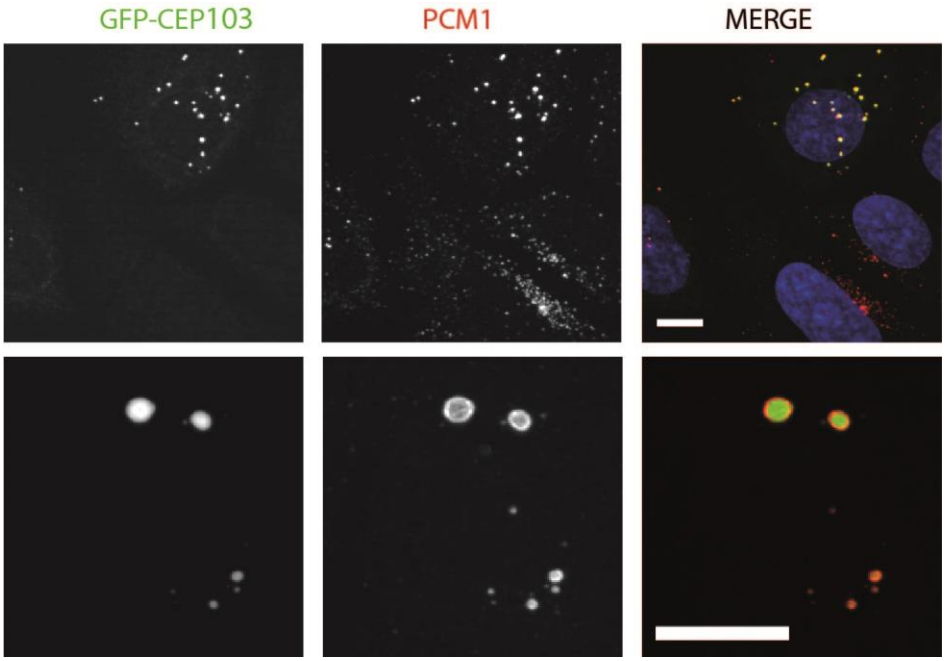


Figure 4.1-7. Overexpression of CEP103 disrupts centriolar satellite organization. HeLa cells were transfected with GFP-fusions of CEP103 construct, fixed and stained for GFP and the centriolar satellite marker PCM1. DNA is stained with DAPI. Scale bar is 10 µm.

4.2 Discussion

Just like DNA, centrosome duplication occurs once per cell cycle to ensure the correct segregation of both the genetic material and the centrosome itself (Nigg 2007). Deregulation of centrosome duplication causes centrosome number abnormalities that are conspicuous phenotypes of cancers (Kerketta, Ghosh et al. 2017). Extra centrosomes increase aneuploidy and invasive metastatic behavior in cancer cells (Tachibana, Gonzalez et al. 2005). Centrioles also nucleated the formation of the primary cilium, which is the signaling center for receiving and transmitting signaling pathways like Hedgehog signaling. Therefore, the function of centrioles in forming the cilium is essential during embryonic development (Klotz, Dabauvalle et al. 1990) and centrosome number aberrations are

Centrosome and cilium complex is involved in critical processes and is related to a broad spectrum of diseases in animals including ciliopathies and cancer. Complete understanding of the molecular mechanism of centrosome and cilium biogenesis is required to reveal the underlying defects of these diseases. To this end, we set out to complete the repertoire of proteins that function in these processes by functionally and biochemically characterizing the putative new components of these proteins detected in previous duplication proximity maps generated in our lab. Among the putative components, CEP103 stood out with its proximity relationship to duplication proteins and upregulation in MTEC transcriptome datasets.

The relationship of the previously uncharacterized protein CEP103 to the centrosome/cilium complex was first implicated in the transcriptomic analyses of mouse tracheal epithelial cells during their differentiation at air-liquid interphase (ALI) cultures. CEP103 gene was upregulated about five fold during MTEC differentiation, which involves a massive increase of in the number of centrioles and cilia to form hunders of cilia associated with the apical side of these cells (Hoh, Stowe et al. 2012). In aggrement with this finding, CEP103 was later identified as a proximity partner of

CCDC67 and OFD1 in proximity dependent biotin identification (BioID) experiments in HEK293T cells (Firat-Karalar, Rauniyar et al. 2014, Gupta, Coyaud et al. 2015).

We started off characterizing CEP103 by first identifying its subcellular localization in RPE1 and HeLa cells. Using an antibody raised against the N terminus of the protein, we discovered that CEP103 localized to the centrosome and in a subset of HeLa cells to the centriolar satellites. Centrosomal proteins have a microtubule dependent or independent localization at the centrosome (Nakadai, Kishimoto et al. 1999, Suizu, Ryo et al. 2006). To determine if localization of CEP103 to the centrosome is dependent on microtubules, we disturbed the microtubule network using microtubule depolymerizing drugs and showed that a significant pool of CEP103 still localized to the centrosome, identifying this protein as a bona fide component of the centrosome. Supporting this result, CEP103 was also detected in in centrosomes purified from HeLa cells.

As a microtubule based organelle, centrosome has a very complex structure composed of two centrioles and a surrounding pericentriolar material. Studies using high-resolution microscopy revealed centrosome as a highly ordered structure with functional and distinct sub-centrosomal domains (Lawo, Hasegan et al. 2012, Mennella, Keszthelyi et al. 2012) To associate CEP103 with known functional domains of the centrosome, we determined its localizatoin using high resolution microscopy relative to markers of the distal end of centrioles, proximal end of centrioles, centriole fibers, centriole barrel and PCM. This analysis identified CEP103 as a proximal component of the PCM, like the centriole duplication proteins CEP63 and CEP152 and this is in line with identification of CEP103 as a promixty partner of centriole duplication proteins. Interestinly, a sub-population of CEP103 co-localizes with the centriole linker Rootletin and C-Nap1, suggesting that CEP103 might function in centrosome cohesion.

Centrosome-mediated functions are dynamically regulated during cells cycle. Centrosomes organize the radial microtubule array of interphase cells. During S to G2 transition, procentrioles are duplication next to preexisting centrioles, which elongate throughout G2 phase and once the centrioles are fully elongated, they are segregated

by the mitotic spindle to the daughter cells. To gain insight into the function of CEP103 throught cell cycle, we determined its cellular localization in different cell cycle phases. In interphase cells, CEP103 localizes to the proximal end of the centrosome and the signal at the proximal end increases as cells enter mitosis. Impotantly, the signal of CEP103 at the centrosome during mitosis is enriched around the parental centrioels, supporting our characterization of CEP103 as a component of the PCM, but not the centriole. Strikinly, CEP103 also localizes to the spindle poles and microtubules in metaphase and central spindle microtubules in telophase, suggesting that it has affinity to microtubules. Consistent with in vivo localization data, CEP103 co-pelleted with microtubules in in vitro microtubule pelleting experiments. Future in vitro experiments with purified proteins are required to determine whether CEP103 binds to microtubules directly or not.

To define the regions required for centrosome and microtubule localization of CEP103, we analyzed a series of CEP103 deletion constructs in localization and pelleting experiments. The N-terminal half of the protein that harbors one or two coiled coil domains forms aggregates and filaments that are not microtubules anda are possibly self-assembled structures throught the dimerization of the coiled-coil domain. Aggregate formation as a result of overexpression is a commonly observed phenotype for centrosomal proteins because of their hydrophobic nature (Xie, Qin et al. 2016). The C-terminal half of the protein that lacks the coiled coil domains localizes to the centrosome and microtubules. When overexpresed, it induces formation of microtubule bundles. Overexpression of microtubule associated proteins can lead to bundling of microtubules into highly stable structures through intense acetylation of the filaments (Mori, Taniyama et al. 2015). Treatment of these cells with microtubule deplymerizing drugs disrupted the formation of these bundles, suggesting that CEP103 does not stabilize these microtubule structures. Interestingly, the full-length protein does not localize to the centrosome or microtubules, instead it induces formation of aggregaes like the N-terminal half. Together, this analysis indicates that the C-terminal half of the protein is required for its centrosome and microtubule localizatoin and the N-terminal half of the protein plays an inhibitory role in the localization of CEP103 to these structures, possibly through burying the binding regions in the fully-formed

protein. These results will be important in dissecting the structure-function relationship of CEP103 in future phenotypic characterization experiments.

To gain insight into the molecular mechanism and function of CEP103, we identified its proximity interactors using the BioID method. We performed the BioID experiments with the myc-BirA*-fusions of full-length CEP103 and its centrosome-localizing C-terminal half. Our mass spectrometry analyses of biotinylated proteins pulled down with streptavidin from lysates expressing BirA*-tagged CEP103 identified extensive interactions of these proteins with centrosome and centriolar satellite proteins, confirming our characterization of CEP103 as a novel centrosome protein and supporting its possible roles in centrosome-mediated processes. Interestingly, while de novo centriole biogenesis protein CCDC67 was among the proximity partners of CEP103; the paralog of CCDC67, CEP63, which function in canonical centriole duplication was not. To determine if the reciprocal proximity partnership between CEP103 and CCDC67 represented a physical interaction, we performed co-immunoprecipitation and showed that MYC-CCDC67 and GFP-CEP103 interacted. In aggreement with this result, CCDC67 and CEP103 co-localized in the aggregates induced by CEP103 overexpression in cells. Moreover, endogenous CEP103 co-precipitated with GFP-CEP63, suggesting that CEP103 might play roles in canonical centriole duplication in most cells and in de novo centriole duplication in multiciliated cells. The possible function of CEP103 in these processes will be studied in the future through siRNA depletion and CRISPR/Cas9 genome editing approaches in mammalian cell lines and MTEC cultures.

Cellular localization experiments with overexpressed CEP103 revealed a spatially distinct recruitment of the centriolar scaffolding protein PCM1 to the periphery of the CEP103-induced aggregates. In fugure experiments, we will attempt to purify CEP103 to establish in vitro assays for the assembly of these structures to test their ability to nucleate microtubules and form centrioles. Such experiments were successfull performed for the C.elegans Spd-5 (Woodruff, Wueseke et al. 2015, Woodruff, Ferreira Gomes et al. 2017), which identified PCM as a spherical condensate that concentrates tubulin and nucleate microtubules. Our work contributes to the

completion of the parts list for the centrosome and to date, we can say that the parts list of the centrosome for proteins is almost complete. The challenge forthe field is to understand how these parts come together to assemble the complex and highly ordered structrues, centrioles and cilia. Our characterization of CEP103 for its localizatin, overexpression phentoype and interaction revealed important results for elucidating the function of this protein. Future phenotypic experiments in cells with primary and motil cilia and in vitro reconstitution experiments are required to determine the role of CEP103 in health and in disease.

5 The retinal degeneration gene product CCDC66 has extensive interactions with centrosome and cilium proteins

5.1 INTRODUCTION

Primary cilium is the signaling center for developmentally important pathways including Hedgehog signaling. Defects in cilia formation and function are linked to human genetic syndromes, termed ciliopathies, which are characterized by a multitude of syndromes affecting retina, kidney and brain. Retinal degeneration is a prominent syndrome associated with many ciliopathies and is the leading cause of blindness. About one third of genes mutated in retinal degeneration are components of the centrosome/cilium complex, highlighting the functional significance of this structure in these specialized cells. Elucidating the molecular defects underlying retinal degeneration requires dissecting the function and regulation of the centrosome/cilium-complex components in the retina.

The human eye is a compartmentalized ovular structure that allows visible light through. At the back of the eye lays the retina. Retina is composed of different layers of cells. Photoreceptors are found deeper within this layer and are required for image acquisition. Two different types of photoreceptors; cone and rod shaped receptors are distributed over retina. Evident from their names, while rod shaped receptors have outer segment reminiscent of rods, cone shaped receptors have outer segments similar to a cone. Outer segments of these cells are highly specilized cilia and they have an increased membrane surface area. Rhodopsin molecules on these membranes are excited by the incoming visible light, converting light energy into chemical energy (Pulves D. et al. 2001). The light responsive cilium of photoreceptors is composed of three compartments: Outer segment, transition zone and basal body (Nemeth A.H. et al. 2009).

CCDC66 was first identified as a canine generalized progressive retinal atrophy (gPRA) gene (Dekomien, Vollrath et al. 2010). The disease manifests itself with night blindness

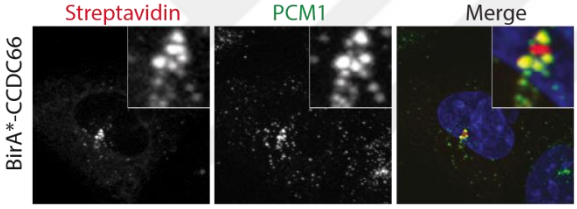
and loss of central and peripheral visual fields. These manifestations occur as a result of degeneration of the retina. Screening of the Shapendoes breed of dogs affected by the disease showed that the CCDC66 gene was mutated in all individuals carrying the disease. Mutational analysis revealed a single base pair insertion at the N terminus of the protein, leading to an early stop codon. CCDC66 prominently localized to the inner segments of the photoreceptors (Petersen-Jones 2005, Dekomien, Vollrath et al. 2010). Following this work, Gerding et al generated CCDC66 null mouse models using gene trapping and CCDC66-/- mice exhibited retinal degeneration thatmanifested itself as early as 13 days of age of growing offspring (Gerding, Schreiber et al. 2011). These lines of evidence suggest an important function for CCDC66 in retinal development and function, which we set out to characterize in ciliated mammalian cell lines.

We elucidated the function and regulation of CCDC66 in mammalian centrosome/cilium-complex-mediated cellular processes based on three lines of evidence. First, as detailed above, CCDC66 is one of the genes that is mutated in retinal degeneration, a prominent syndrome of ciliopathies. Second, a comprehensive RNA-sequencing on purified tubulin polymers of *Xenopus* extracts identified CCDC66 mRNA to be associated with microtubules, suggesting that CCDC66 might have microtubule-mediated function. Third, BioID pulldowns of various centrosome proteins including CEP72 and >>>> identified proximity CCDC66 as a proximity interactor. (Gupta, Coyaud et al. 2015, Conkar, Culfa et al. 2017).

Work in our laboratory identified CCDC66 as a component of the centrosome, primary cilium, centriolar satellites and microtubules using in vitro and in vivo experiments. Interestingly, CCDC66 has a very dynamic localization patter throughout cell cycle. It localizes to the centrosome and satelliets in interphase cells, relocalizes to spindle poles and microtubules in metaphase and centrosome and central spindle in telophase and to the primary cilium upon serum starvation. To gain insight into the cellular functions of CCDC66 and its molecular mechanism, we identified the proximity interactors of CCDC66 is asynchronous cells, which revealed extensive interactions of

CCDC66 with centrosome, centriolar satellite and microtubule associated proteins (Figure 1A) (Conkar, Culfa et al. 2017). During my thesis, I performed co-immunoprecipitation experiment to determine which of the proximity interactions are reflected as physical interactions and showed that CCDC66 interacts with the centriolar satellite protein PCM1 and the transition zone protein CEP290. Once I identified the interactos of CCDC66, I performed co-immunoprecipitation experiments with the CCDC66 truncation mutants to define the region that mediates the interaction of CCDC66 with centriolar satellites. Together, my findings revealed that CCDC66 function during ciliogenesis is mediated through its interaction with PCM1 and CEP290, which was previously shown to function during primary cilium formation (Drivas and Bennett 2014).

A



B

BirA*-CCDC66

Protein	NSAF
CCDC66	20
PCM1	1.31
HSPA1A	1.10
TUBA1B	0.88
ALMS1	0.65
AZI1	0.56
TUBA1A	0.52
HSPA1L	0.50
HSPA8	0.45
CEP152	0.45
DDX3X	0.42
EIF5	0.39
OFD1	0.33
MAP4	0.29
CEP290	0.26
MPHOSPH9	0.23
HDLBP	0.23
TJP1	0.21
CKAP5	0.20
CCDC138	0.20
MRE11A	0.20

Şekil 5.1-1 Adapted from Conkar et al. Localization of and protein landscape around BirA* tagged CCDC66. A) RPE1 cells were transiently transfected with Myc-BirA* tagged CCDC66 constructs and incubated in 1 mM biotin for 18h. Cells were fixed and stained with fluorescent streptavidin and anti PCM1 antibodies to label the biotinylated proteins and the centriolar satellites. B) Mass spectrometry analysis of proximity interactors of Myc-BirA*-CCDC66. Interactors are listed according to their NSAF values, with the highest scores placed

on top. Proteins in black were previously known to localize to the centrosome. Proteins in bold were previously identified as CCDC66 interactors. Proteins in gray were not associated with centrosome and cilium before.

5.2 Results

5.2.1 CCDC66 interacts with centriolar satellite proteins PCM1 and CEP290

CCDC66 has extensive proximity interactions with centrosome, centriolar satellite and microtubule-associated proteins (Figure 1A) (Conkar, Culfa et al. 2017). Several proteins among these proximity interactors stand out by their high proximity to CCDC66 and their functional and structural importance during centrosome and cilium biogenesis. One of these proteins is PCM1 (pericentriolar material 1), which is the molecular marker for centriolar satellites (Balczon, Bao et al. 1994, Dammermann and Merdes 2002). Centriolar satellites are an array of 70-100 nm electron dense granules that localize around the centrosome in vertebrate cells. Although centriolar satellites can be ubiquitously found across vertebrate species; their localization, molecular composition and numbers varies in different cell types and tissues (Kubo and Tsukita 2003). PCM1 is essential for the assembly of centriolar satellites and is used as a marker to label and identify these structures (Dammermann and Merdes 2002, Wang, Lee et al. 2016). Recent evidence implicates centriolar satellites in critical functions not only for the assembly and function of centrosome/cilium-complex but also for other cellular processes such as autophagy and neurogenesis (Firat-Karalar, Rauniyar et al. 2014, Tollenaere, Mailand et al. 2015). Mutations in satellites are linked to schizophrenia, ciliopathies, primary microcephaly and leukemia (Kim, Krishnaswami et al. 2008, Datta, McQuillin et al. 2010). The function of PCM1 in satellite assembly and centrosome/cilium-mediated processes makes this protein a potentially interesting candidate for elucidating CCDC66 functions in cells.

Another interesting proximity protein for CCDC66 is CEP290, which is the most mutated gene in across a wide spectrum of ciliopathies including Joubert syndrome and Leber Congenital Amaurosis (LCA) (Nemeth A.H. et al. 2009). Importantly, mutations in CEP290 cause retinal degeneration in humans and mouse models of

CEP290 also has retinal degeneration phenotypes (Chang, Khanna et al. 2006). CEP290 localizes to the centriolar satellites and the transition zone in ciliated cells, where it functions as the gatekeeper for regulating proteins trafficking to the cilium (Craige, Tsao et al. 2010). The causal relationship of CEP290 to retinal degeneration and its localization to the centrosome and centriolar satellites in cells makes this protein a promising interacting partner for CCDC66.

To determine whether CCDC66 interacts with PCM1 and CEP290, we performed immunoprecipitation experiments in HEK293T cells overexpressing Myc- and GFP-tagged candidate proteins. While Myc-CCDC66 interacted with GFP-PCM1, we didn't detect interaction between Myc-CCDC66 and GFP-CEP290 (Figure 1A). Interestingly, when cells were transfected with Myc-CCDC66, GFP-CEP290 and tomato-PCM1 and GFP pulldown was performed, both Myc-CCDC66 and td-PCM1 pelleted with GFP-CEP290, indicating that these there proteins forms a complex and the interaction between CCDC66 and CEP290 is mediated by the scaffolding protein PCM1 (Figure 1B). In both cases, the negative control GFP did not pulldown any of the candidates, validating the specificity of the immunoprecipitation data (Figure 1A and 1B)

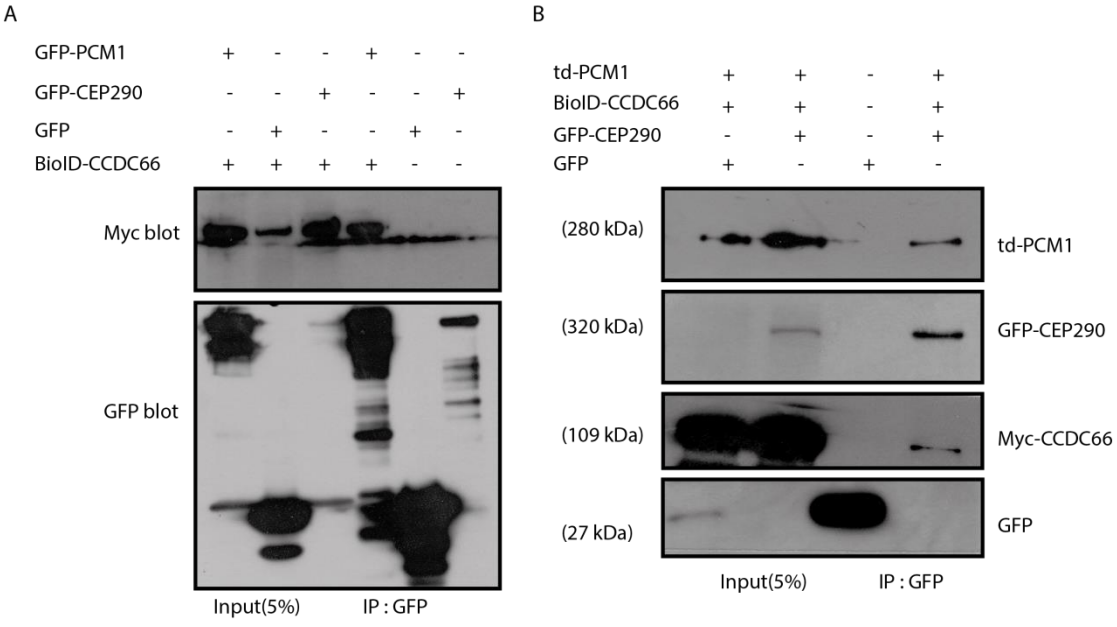


Figure 5.2-1. CCDC66 interacts with centriolar satellite proteins PCM1 and CEP290 . A) Co-immunoprecipitation between Myc-CCDC66 and GFP-PCM1 or GFP-CEP290 following co-expression in cells. Cell lysates were incubated with GBP (GFP Binding Protein) beads and

immunocomplex was precipitated. Co-precipitated (IP) samples were probbed with anti-GFP and anti-Myc antibodies. B) Co-immunoprecipitation between Myc-CCDC66, tomato(td)-PCM1 and GFP-CEP290. Cell lysates were incubated with GBP beads and immunocomplex was precipitated. IP samples were probbed with anti-GFP, anti-Myc and anti-PCM1 antibodies.

5.2.2 PCM1 doesn't interact with the retinal degeneration mutant Myc-CCDC66 RD

Having discovered a physical interaction between CCDC66 and the centriolar satellite scaffold PCM1, we moved onto characterizing regions required for mediating its interaction with PCM1. To this end, we co-transfected HEK293T cells with full length GFP-PCM1 and Myc-fusions of full-length CCDC66 and its truncation mutants and performed GFP pulldowns. Among the truncation mutants is CCDC66-RD (amino acids 1-217) that mimics the truncated proteins described in dogs with a point mutation in CCDC66 and this point mutation causes a framseshift that introduces a premature stop codon at amino acid 207 (Dekomien, Vollrath et al. 2010). While Myc-fusions of CCDC66 (1-949), (1-844) (483-844) (322-864) (322-478) (1-564) (685-949) (483-864) pelleted with GFP-PCM1, Myc-fusions of CCDC66 (845-949), (1-121) (1-321) did not pellet with it. Importantly, Myc-CCDC66 RD didn't interact with PCM1, suggesting that the interaction of CCDC66 with centriolar satellites is important for photoreceptor development and function and that this interaction is disrupted in retinal degeneration, possibly explaining the underlying diseases mechanism (Figure 2).

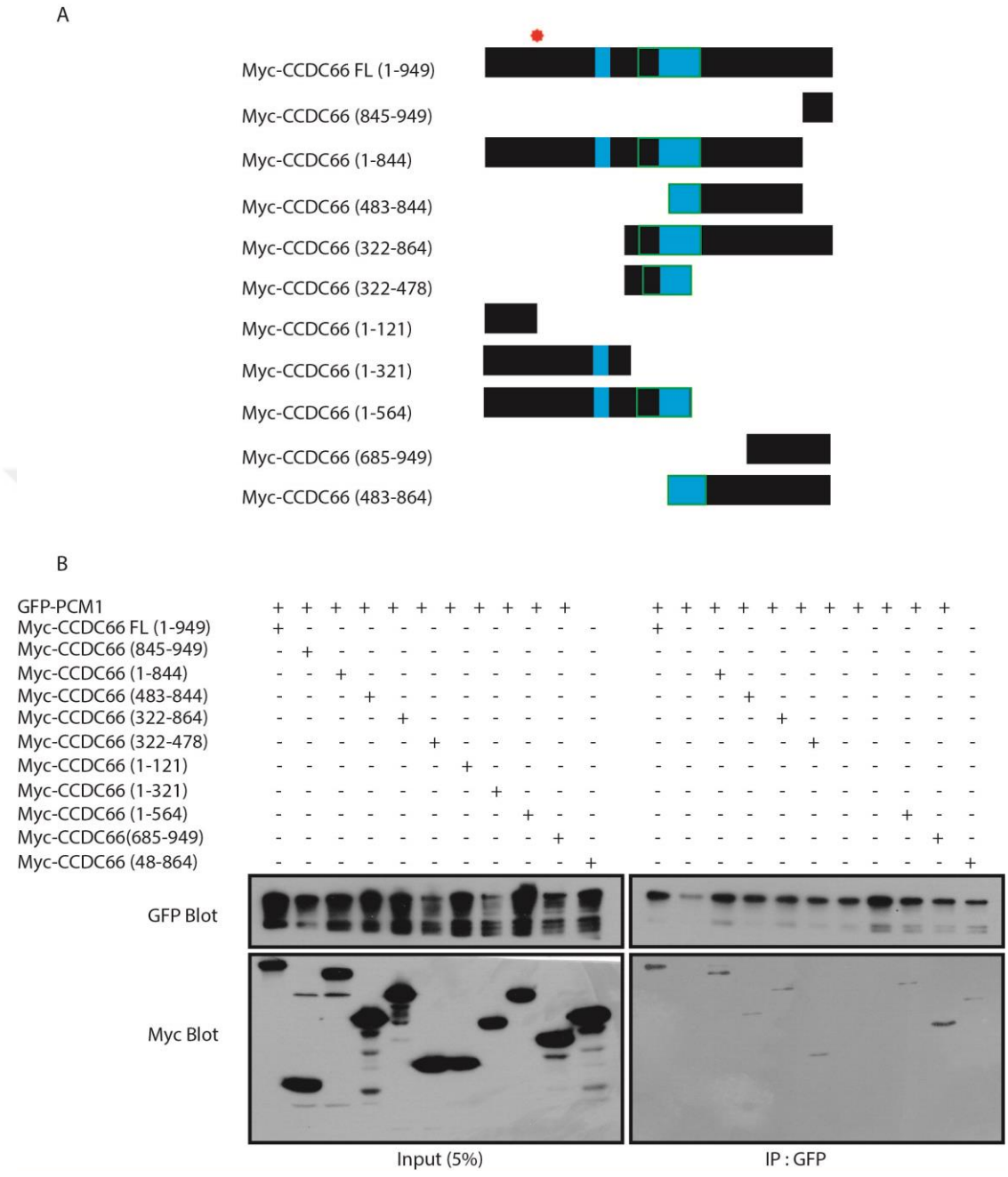


Figure 5.2-2. Truncation mutant mimicking the protein product of mutated CCDC66 gene in retinal degeneration doesn't interact with PCM1. A) Schematic representation of CCDC66 full length and truncation mutants including the retinal degeneration mutant RD. Red Asterisk indicates the amino acid region corresponding to the mutated nucleotide sequence causing retinal degeneration. Blue boxes represent the coiled-coil domains. Green box represents the conserved CCDC66 domain of the protein. B) Co-immunoprecipitation between Myc-CCDC66 fragments and GFP-PCM1 following co-expression in cells. Cell lysates were incubated with GBP (GFP Binding Protein) beads and immunocomplex was precipitated. Co-precipitated (IP) samples were probbed with anti-GFP and anti-Myc antibodies.

5.3 Discussion

In this chapter of the thesis, we elucidated the molecular mechanism of CCDC66 functions by determining its interacting partners and identifying its regions that mediate these interactions. CCDC66 was previously discovered as a canine generalized progressive retinal atrophy (gPRA) gene in dogs and CCDC66 knockout mice developed retinal degeneration (Petersen-Jones 2005, Gerding, Schreiber et al. 2011). Retinal degeneration is complex genetically heterogenous disease characterized by the the progressive loss of light sensitive receptors in the retina and it is a common feature of retinal dystrophies. Majority of the reported retinal degeneration mutations are linked to single genes and a small subset of these mutations are digenic or multiallelic (Nemeth A.H. et al. 2009). Importantly, almost one third of the retinal degeneration mutations map to the genes encoding centrosome and cilium proteins, which highlights the importance of the proper centrosome/cilium-complex in retinal development and function. The photoreceptor cells of the retina bear specialized sensory cilia that transmit visual transduction cascade. Moreover, the retinal epithelial cells are important for the development and function of photoreceptors and their dysfunction also causes retinal degeneration (Jiang, Esteve-Rudd et al. 2015). In order to elucidate the underliying molecular defects of retinal degeneration, it is essential to dissect the mechanisms that regulate the assembly and function of the centrosome/cilium complex in retinal cells.

Work in our laboratory identified the protein landscape proximate to CCDC66 through application of BioID-based proximity dependent biotinylation to cells expressing Myc-BirA* fusion of CCDC66. This analysis revealed extensive interaction of CCDC66 with the components of the centrosome/cilium complex and microtubule associated proteins (Figure 1). To gain insight into function and molecular mechanism of action for CCDC66, we first tested the physical interaction of CCDC66 with a subset of its proximity partners. Using co-immunoprecipitation experiments in cells expressing fusions of CCDC66, CEP290 and PCM1, we showed that CCDC66 interacts with CEP290 and PCM1, suggesting that CCDC66, CEP290 and PCM1 forms a functional complex.

Surprisingly, we failed to detect the same physical interaction between CCDC66 and CEP290 when they are overexpressed together. This could be because the centrilar satellite protein PCM1 mediates the interaction between CCDC66 and CEP290.

Identification of physical interaction of CCDC66 to CEP290 and PCM1 is critical for providing insight into the cellular functions of CCDC66 as well as the underlying molecular mechanism of these functions, because both PCM1 and CEP290 are well characterized for their cellular functions. PCM1 is required for the assembly of centriolar satellites and is used as a marker to label and identify these structures. Centriolar satellites are regulators of centrosome/cilium-mediated cellular processes (Balczon, Bao et al. 1994, Dammermann and Merdes 2002, Kubo and Tsukita 2003). They were recently shown to be essential for cilium formation in retinal epithelial pigmental cells (Wang, Lee et al. 2016). CEP290 is a component of centriolar satellites and the transition zone in ciliated cells and functions as a gatekeeper for regulating ciliary content. Just like CCDC66, CEP290 mutations are also known to be associated with retinal generation phenotype (Kim, Krishnaswami et al. 2008) and CEP290 mutant mice also develop retinal degeneration phenotype due to the perturbed interaction between CEP290 and Retinitis Pigmentosa GTPase Regulator (RPGR) (Chang, Khanna et al. 2006). Together, these lines of evidence suggested possible functions for CCDC66 during cilium formation and ciliary functions. Work in our laboratory tested these functions using siRNA depletion experiments and identified important regulatory functions for CCDC66 during cilium formation, centriolar satellite organization and ciliary trafficking (Conkar, Culfa et al. 2017).

Once we identified CCDC66 as a component of centriolar satellites through its physical interaction with PCM1, we next determined the regions that mediate this interaction. To this end, we performed co-immunoprecipiation experiments to test the interaction of PCM1 with a series of CCDC66 truncation mutants. Among these truncation mutants is the CCDC66 retinal degeneration (RD) mutant, which is composed of the first 207 amino acids of the protein, mimicking the truncated protein product in dogs with retinal degeneration (Dekomien, Vollrath et al. 2010). Interestingly, while the majority

of the fragments did interact with full length GFP-PCM1, the retinal degeneration mutant failed to interact with PCM1. This result suggests that the retinal degeneration phenotype caused by CCDC66 mutant protein could be associated with its inability to interact with the centriolar satellites. Following our work on phenotypic characterization of CCDC66 for cilum-related processes, an RNA-sequencing experiment discovered that CCDC66 circular RNA (circRNA) was overexpressed in colon cancer and polyps. Using siRNA depletion, Hsiao et al also show that decreased expression of CCDC66 circRNA leads to reduced migration and increased cell death independent of apoptosis (Hsiao, Lin et al. 2017). The functions of CCDC66 as a component of centrosome/cilium-complex and microtubules can also in part explain the reported cancer phenoytpes.

This chapter of the thesis sought to understand the relationship between retinal degeneration gene CCDC66, centriolar satellite protein PCM1 and another centrosome/cilium complex protein CEP290. Challenging CCDC66 as a human retinal dystrophy gene, a recent study identified a frameshift deletion in a patient suffering from retinal degeneration. Khan et al performed targeted next generation sequencing (NGS) of 44 retinal dystrophy genes on patients suffering from retinal dystrophy and identified a CCDC66 mutation in affected individuals. Their segregation and linkage analyses, however excluded CCDC66 variant because the gene didn't follow the inheritance of the phenotype (Khan, Budde et al. 2018). Although CCDC66 mutation did not follow the phenotype, we know that retinal dystrophies show high genetic heterogeneity and mutations on the same gene can result in different phenotypes depending on the type of cell it exerts its effects (Nemeth A.H. et al. 2009). Given that CCDC66 knockout mouse also develops retiunal degeneration and our work identified critical function of CCDC66 in cililum-mediated cellular processes, we are confident in the link between CCDC66 and retinal degeneration and we plan to perform future experiments in further dissecting the molecular mechanism of CCDC66 in cilium formation and ciliary trafficking.

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APPENDIX: List of primers

Fragment (aa)	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
1-916 full length	**GGGGACAACTTTGTACAAAAAAGTTGAT** ATGCTGCCACTGGGCTCAGAG	**GGGGACAACTTTGTACAAGAAAGTTGT** TAACTACAACATTATGGACTGA
1-502	**GGGGACAACTTTGTACAAAAAAGTTGAT** ATGCTGCCACTGGGCTCAGAG	**GGGGACAACTTTGTACAAGAAAGTTGT** CTGCCTGAGAAGCATCTGTGC
503-916	**GGGGACAACTTTGTACAAAAAAGTTGAT** GGCACAGATGCTTCTCAGGCAG	**GGGGACAACTTTGTACAAGAAAGTTGT** TAACTACAACATTATGGACTGA
606-916	**GGGGACAACTTTGTACAAAAAAGTTGAT** CCTTTAAAGATGTCCTCACCTCATG	**GGGGACAACTTTGTACAAGAAAGTTGT** TAACTACAACATTATGGACTGA
1-605	**GGGGACAACTTTGTACAAAAAAGTTGAT** ATGCTGCCACTGGGCTCAGAG	**GGGGACAACTTTGTACAAGAAAGTTGT** GGATCCAACACATCTTCTAGATGC
684-916	**GGGGACAACTTTGTACAAAAAAGTTGAT** GAACCCCTGGAGCCGGCCGCTC	**GGGGACAACTTTGTACAAGAAAGTTGT** TAACTACAACATTATGGACTGA

Fragment (amino acid)	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
1-949	**GGGGACAACTTTGTACAAAAAAGTTGAT** ATGAACTTGGGAGATGGTTTAAAGCTTG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAAAATGAAGAACCAAAACTCTCTTC
1-322	**GGGGACAACTTTGTACAAAAAAGTTGAT** ATGAACTTGGGAGATGGTTTAAAGCTTG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAACAAACACTGCTGACATCAGCCAGC
1-478	**GGGGACAACTTTGTACAAAAAAGTTGAT** ATGAACTTGGGAGATGGTTTAAAGCTTG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTATCTAGAAGTGTCATGTCCCTTTTG
483-949	**GGGGACAACTTTGTACAAAAAAGTTGAT** GGTGTTGATACAATACAAATGG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAAAATGAAGAACCAAAACTCTCTTC
322-949	**GGGGACAACTTTGTACAAAAAAGTTGAT** ACACCTACAACCGGAAGCCAGGTTG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAAAATGAAGAACCAAAACTCTCTTC
1-121	**GGGGACAACTTTGTACAAAAAAGTTGAT** ACACCTACAACCGGAAGCCAGGTTG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAAGTTTTTTTGAATAATCCCATAATG
685-949	**GGGGACAACTTTGTACAAAAAAGTTGAT** AGAATAGAGAAACAAACAAAACAC	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAAAATGAAGAACCAAAACTCTCTTC
322-478	**GGGGACAACTTTGTACAAAAAAGTTGAT** ACACCTACAACCGGAAGCCAGGTTG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTATCTAGAAGTGTCATGTCCCTTTTG
483-844	**GGGGACAACTTTGTACAAAAAAGTTGAT** GGTGTTGATACAATACAAATGG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAGGATGATTCAGAAATCCCAGATGAT

1-844	**GGGGACAACTTTGTACAAAAAAGTTGAT** ATGAACTTGGGAGATGGTTTAAAGCTTG	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAGGATGATTCAGAAATCCCAGATGAT
843-949	**GGGGACAACTTTGTACAAAAAAGTTGAT** CATTTTATTCCGTATGTTCGAACA	**GGGGACAACTTTGTACAAGAAAGTTGT** TTAAAATGAAGAACCAAAACTCTCTTC

gateway sequence / annealing sequence

VITAE

Efraim Culfa was born in Elazig in 1992. He received his bachelor degree from the Deparment of Molecular Biology and Genetics of Istanbul University in 2015. During his bachelor studies, he worked in an electron microscopy centre at Ulm University and later on in Max Delbrück Centre for Molecular Medicine in Germany, where he studied the interaction between a GAP (GTPase Activating Protein) and Roundabout receptor 1 (Robo1). Following his graduation, he started Masters at Koc University where he focused on studying the regulation of centrosome/cilium complex biogenesis. There are many wild animals Efraim wants to see in real life; especially whales and elephants and leopards and great horned owls.