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CEP78, a Novel Centrosomal Protein

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CEP78, a Novel Centrosomal Protein

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Abstract

Background: The centrosome is a tiny organelle well-known for its role in establishing the bipolar spindle during cell division. Defects in centrosome function often give rise to human diseases including cancer and kidney cyst formation. We are interested in studying the function of one novel centrosomal protein named CEP78, identified in a proteomic screen for novel centrosomal components. **Methods and results:** Treatment of cells with nocodazole, a microtubule-depolymerizing agent that specifically depolymerizes cytoplasmic microtubules but not the stabilized centrosome microtubules, showed that CEP78 is a stable centrosomal component. Colocalization of this protein with other centrosomal markers such as CEP164, SAS6, Centrin, Polyglutamylated tubulin and POC5 at different phases of the cell cycle indicated that CEP78 specifically localizes to the distal end of the mother and daughter centrioles. There are 2 CEP78 dots during the interphase and as the cells go through mitosis, procentrioles mature, and the number of CEP78 dots increases to 4 dots per cell and by the end of telophase each daughter cell has 2 CEP78 dots. Characterization of CEP78 functional domains showed that Leucine-rich repeats are necessary for centrosomal localization of the protein. In addition, we found that overexpression of CEP78 did not change the number of centrioles and centrosomes but decreased the number and intensity of CEP170 dots (sub-distal appendage protein) without a decrease in the expression level of this protein. Further studies showed that there is no interaction between these 2 proteins. Finally,

overexpression of CEP78 protects microtubules from depolymerization in the presence of nocodazole, suggesting its ability to bind microtubules. **Conclusion:** Our findings suggest that CEP78 is targeted to the distal end of mature centrioles via its lecuine-rich repeats, where it could be involved in centriolar maturation or regulation of sub-distal appendage assembly and/or remodeling, a structure known to nucleate and anchor microtubules. Understanding the function of CEP78 will shed light on the role of the centrosome in cell cycle.

Resumé

Contexte: Le centrosome est un petit organite bien connu pour son rôle dans l'établissement du fuseau bipolaire pendant la division cellulaire. Les déficiences de la fonction du centrosome donnent souvent lieu à des maladies humaines, y compris le cancer et la formation de kystes rénaux. Nous sommes intéressés à étudier la fonction d'une nouvelle protéine centrosomale nommée CEP78, identifiée dans un criblage protéomique pour de nouveaux composants centrosomaux. **Méthodes et résultats :** Le traitement des cellules avec le nocodazole, un agent qui dépolymérise spécifiquement les microtubules cytoplasmiques mais pas les microtubules stabilisés du centrosome, a montré que CEP78 est un composant centrosomal stable. La colocalisation de cette protéine avec d'autres marqueurs centrosomaux tels que CEP164, SAS6, Centrine, tubuline polyglutamylée et POC5, à différentes phases du cycle cellulaire a indiqué que CEP78 est précisément à l'extrémité distale des centrioles, mères et filles. Il existe deux points CEP78 au cours de l'interphase et les cellules passent par la mitose, procentrioles maturent, et le nombre de points de CEP78 augmente à 4 par cellule et, à la fin de la télophase chaque cellule fille possède 2 points CEP78. La caractérisation des domaines fonctionnels de CEP78 a montré que des répétitions riches en leucine sont nécessaires pour la localisation centrosomale de la protéine. En outre, nous avons constaté que la surexpression de CEP78 ne change pas le nombre de mères/procentrioles mais diminue le nombre et l'intensité des points de CEP170 (protéine d'appendice sous-distal) sans

diminution du niveau d'expression de cette protéine. D'autres études ont montré qu'il n'y a pas d'interaction entre ces deux protéines. Enfin, la surexpression de CEP78 protège des microtubules contre la dépolymérisation en présence de nocodazole, ce qui suggère qu'il possède la capacité de lier les microtubules. **Conclusion :** Nos résultats suggèrent que CEP78 est destiné à l'extrémité distale des centrioles matures par ses répétitions riche en lecuine, où il pourrait être impliqué dans la maturation ou la régulation de l'assemblage ou de la rénovation de l'appendice sous-distal centriolaire, une structure connue dans la nucléation des microtubules et d'ancrage. Comprendre la fonction de Cep78 contribuera à éclaircir le rôle du centrosome dans le cycle cellulaire.

Mots clés: CEP78, Centrosome, CEP170, Microtubules

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List of Abbreviations

γ-TuRCs: γ-Tubulin Ring Complexes

ARJP: Autosomal Recessive Juvenile Parkinsonism

BBS: Bardet-Biedle Syndrome

CDKs: Cyclin-Dependent Kinases

CIN: Chromosomal INstability

CLS: Centrosome Localization Signal

CPAP: Centrosome P4.1 Associated Protein

DMP: DiMethyl Pimelimidate

HAP1: Huntington-Associated Protein 1

IFT: IntraFlagellar Transport

LB: Luria broth

LRRs: Leucine-Rich Repeats

M: Mitosis

MAPs: Microtubule-Associated Proteins

MCPH: MicroCePHalies

MTOCs: Microtubule Organizing Centers

NuMA: Nuclear Mitotic Apparatus

PBS: Phosphate Buffered Saline

PCD: Primary Cilia Dyskinesia

PCM: PeriCentriolar Material

PKD: Polycystic Kidney Disease

PLK1: Polo-Like Kinase 1

RPM: Revolutions Per Minute

STIL: SCL-Interrupting Locus

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Finally, I want to dedicate my work to my family who have always supported and encouraged me in my life.

Chapter 1 Introduction

Cell cycle deregulation is a common feature of several cancers. This cycle is controlled by different pathways and organelles. Due to its microtubule nucleation ability and the role it plays in mitotic spindle assembly, centrosome is a key player in this regulation. Therefore, understanding the biology of centrosomes is key to better understanding of cell cycle.

1.1 Centrosome structure

The centrosome is a non-membranous organelle in the periphery of the nucleus during interphase (Fukasawa 2005, Schatten 2008). It consists of 2 centrioles (mother and daughter) embedded in an electron dense pericentriolar material (PCM) (Figure 1). The PCM contains proteins that regulate centrosome functions and is also involved in microtubule nucleation and anchoring (Dammermann, Muller-Reichert et al. 2004, Korzeniewski, Hohenfellner et al. 2013). The centrioles are microtubule-based cylinders that are arranged orthogonally and are characterized by a 9-fold radial symmetry. The distal and proximal ends of centrioles have different functions. While the distal end of mother centriole is involved in ciliogenesis, its proximal end is the site of centriole duplication and that is where procentrioles, the centrioles in early

stage of development, start to form. Centrioles have polarity in terms of structure and composition and due to generational difference; the two centrioles are structurally and functionally different (Ou, Zhang et al. 2004, Kitagawa, Vakonakis et al. 2011, Bornens 2012). Structurally, the mother centriole carries two sets of appendages at the distal and sub-distal end. Two main distal appendage proteins include CEP164 and Odf2 and some important sub-distal appendage proteins are CEP170, Cenexin, Ninein, EB1 and ε-tubulin. Studies on sub-distal appendages indicate that these proteins are acquired during G1 and unlike distal appendages, disappear at the onset of mitosis (Guarguaglini, Duncan et al. 2005). Distal appendages are important for docking of the basal body to the cell membrane whereas sub-distal appendages participate in anchoring microtubules, endosome recycling and forming basal body, a structure at the base of cilia which promotes microtubule nucleation (Dammermann, Muller-Reichert et al. 2004, Tsang and Dynlacht 2013). Mother and daughter centrioles are also different functionally. Although both centrioles can nucleate microtubules and accumulate PCM, microtubule anchoring is only done by mother centriole through its sub-distal appendages (Bornens 2012).
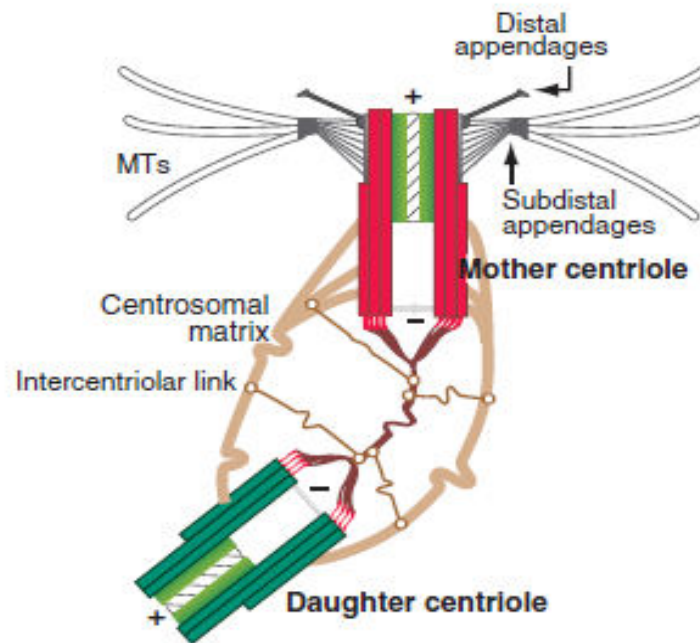
Figure 1. The centrosome of human cells. Photo adapted from Bornens (Bornens 2012).

So far 500 proteins have been identified as centrosomal by mass spectroscopy. Some of these proteins such as γ-tubulin are permanently associated with centrosome and remain in centrosome even after treatment with microtubule depolymerizing agents such as cold, nocodazole, colchicine derivatives. These proteins do not need microtubules for their centrosomal localization. However, some other centrosomal proteins such as Nuclear Mitotic Apparatus protein (NuMA) are cell-cycle-specific and temporarily associated with centrosome (Schatten 2008).

Centriole biogenesis happens through two pathways,canonical and *de novo*. In the first and most common pathway, canonical, procentrioles form in association with the old centrioles whereas the *de novo* pathway is activated in the absence of centrioles and occurs in multiciliated cells. The second pathway is also thought to take place primarily at deuterosomes (Brito, Gouveia et al. 2012).

During centriole assembly, a disc of fibrous material forms first adjacent to the proximal end of the parental centriole. Next, a set of 9-fold symmetric spokes connected to a central axis form the cart wheel within this material. As the assembly of centriolar microtubules begins at the tips of the spokes, the structure elongates to form the mature centriole. Although in most dividing cells, mother and daughter centrioles template the formation of only one centriole per cell cycle, in ciliated tissues, up to 8 centrioles can form simultaneously around the parent centriole. During differentiation, multiciliated cells assemble multiple basal

bodies around structures of unknown composition called deuterosomes (Dammermann, Muller-Reichert et al. 2004, Azimzadeh and Marshall 2010, Korzeniewski, Hohenfellner et al. 2013).

1.2 Centrosomal proteins and their functions

The centrosome is involved in cell shape, cell division, and transport of vesicles, cell polarity and motility through microtubule organization. Centrosomal proteins can be divided into 2 categories:

1. Structural proteins such as γ-tubulin, α-tublin, β-tublin, centrin, pericentrin, Ninein, C-Nap1, centriolin, CP110, cenexin, ODF2, CEP170 and PCM1

2. Regulatory molecules such as Cdc2, Cdk1, PLK1, Nek2 and Dynactin

Due to the importance of the structural proteins, some of them are further discussed here.

Gamma-tubulin: One well-studied structural centrosomal protein is Gamma-tubulin which is localized in PCM. This protein is conserved in eukaryotes and is a component of tubulin ring complex (γ-TuRC). γ-TuRC plays a role in microtubule nucleation by covering the minus ends of microtubules. This helps facilitate

the growth of protofilaments, the microtubule subunits (Schatten 2008).

Pericentrin: Another well-known centrosomal protein is pericentrin which forms a complex with γ- tubulin and needs the motor protein dynein for its centrosome localization. This protein acts as a scaffold for anchoring numerous proteins and is essential for centrosome and spindle organization (Schatten 2008, Delaval and Doxsey 2010).

Centrins: These proteins are conserved Ca2+ binding centrosomal proteins that are associated with centrioles and are important for centriole duplication (Schatten 2008).

NuMA (Nuclear Mitotic Apparatus protein): NuMA is a regulatory centrosomal protein involved in the organization of mitotic apparatus during mitosis. It has microtubule binding capacity and converges spindle microtubule ends to poles. It also acts as nuclear matrix protein during interphase (Zeng 2000, Schatten 2008)

CEP170: This is a sub-distal appendage protein which gets phosphorylated by Polo-like kinase 1 (PLK1). It associates with spindle apparatus during mitosis. This protein has several microtubule binding domains and possibly plays a role in microtubule organization (Guarguaglini, Duncan et al. 2005).

Ninein: This is another sub-distal appendage protein which acts as a docking site for γ-tubulin complex. It also participates in the anchorage of microtubule minus-ends (Moss, Bellett et al. 2007).

C-Nap1: C-Nap1 is important for centriolar cohesion and is regulated through phosphorylation by NEK2. It is involved in establishing link between the pair of basal bodies/centrioles through the protein rootletin which is a physical linker between the centrioles and binds to C-Nap1 (Yang, Adamian et al. 2006).

CP110: This protein is a substrate of Cdk2 and is involved in centriole duplication. It also acts as a cap for the distal end of centrioles and in this way controls their length. Another important function of this protein is the negative modulation of cilia assembly through cooperation with CEP97 (Schmidt, Kleylein-Sohn et al. 2009, Tsang and Dynlacht 2013).

Centriolin: This centriolar protein localizes to the mother centriole and induces the assembly of primary cilia (Hinchcliffe 2003).

CEP164: This component of distal appendage is indispensable for primary cilia formation and localizes to the mother centriole (Graser, Stierhof et al. 2007).

SAS6: SAS6 is one of the several proteins involved in the early stage of procentriole assembly and is essential for the nine-fold symmetry of the centriole (Nakazawa, Hiraki et al. 2007).

POC5: This protein localizes to the distal end of the centrioles and is important for centriole elongation and hence full maturation of procentrioles (Azimzadeh, Hergert et al. 2009).

The various functions of centrosomal proteins underline the importance of the centrosome in cellular function and the role it plays in regulation of several proteins.

1.3 Cell Cycle

1.3.1 The cycle

Cell cycle is a crucial cellular event which takes place in order to divide and duplicate cells. It consists of 2 distinct stages: interphase (G1, S and G2) and mitosis (prophase, metaphase, anaphase and telophase). The landmark of interphase is DNA replication which occurs during the S phase. G1 and G2 are the gap phases of interphase that prepare the cell for DNA synthesis and mitosis (Schafer 1998). Also, we must remember that sometimes cells enter a resting phase called G0 which means no proliferation and no DNA replication. Following DNA synthesis in interphase, mitosis (M) begins during which the replicated chromosomes get segregated into two cells. The 4 phases of mitosis are prophase, metaphase, anaphase and telophase (Vermeulen, Van Bockstaele et al. 2003). During prophase, chromatin becomes condensed to form chromosomes and the nucleolus disappears. In early prometaphase, the nuclear membrane dissolves and kinetochores are formed around centromeres where microtubules attach to move the chromosomes. During metaphase, spindle fibers align the chromosomes ensuring that only one copy of each chromosome is received by each new nucleus. During anaphase, the paired chromosomes separate and move to opposite sides of the cell. Finally, during telophase new membrane surrounds the

chromatids at the opposite poles and chromosomes go back to their chromatin form. Following mitosis, the spindle fibers disperse and the cytokinesis begins. During this stage, actin contracts around the cell center and divides the cell into two new daughter cells. Cell division is controlled and regulated by different pathways and cell organelles including centrosomes (Morgan 2007). In the next section, these regulations will be further discussed.

1.3.2 Control of cell cycle

The control of cell cycle is vital for cell survival. The main players in the regulation of cell division are cyclin-dependent kinases (CDKs) which act by phosphorylating their target proteins. CDKs have a stable expression level throughout the cell cycle and are activated by cyclins required for different stages of cell cycle. During G1, CDK2 already activated by cyclin E, phosphorylates Histone H1. This helps regulate the progression from G1 to S and is important for chromosome condensation and DNA replication. Next, cyclin A participates in both G2 and G2/M transition through the activation of CDK2 and CDK1. Furthermore, CDK4/CDK6/cyclin D phosphorylates Rb, which in turn release E2F, allowing E2F to activate transcription. Same is true for CDK2/ cyclin E. Finally, mitosis is regulated by CDK2 and cyclin B (Vermeulen, Van Bockstaele et al. 2003). In order for the cell cycle to progress

properly, there are several checkpoints which work through regulating the CDK activity. When there is a defect in DNA synthesis or chromosome segregation, the checkpoints become active and arrest the cell cycle for the repair to be done (Malumbres and Barbacid 2009).

It seems that the events in the cell cycle are tightly coordinated with the centrosome cycle.

1.3.3 Centrosome and cell cycle

The centrosome has several functions during cell division. During interphase, it serves by nucleating microtubules, organizing cytoplasmic organelles and forming primary cilia. During mitosis, the centrosome plays an important role in bipolar spindle assembly and this is controlled by a checkpoint monitoring microtubule defects and their attachments to kinetochores (Schwartz and Shah 2005).

For these functions, the centrosome cooperates with CDKs and cyclins. For instance it modulates G1 progression and entry into S phase through cyclins A/E. It has been shown that cyclin E has a centrosome localization signal (CLS) motif which is necessary to target cyclin E to the centrosome and controls the S phase initiation. Also, cyclin A binding to the centrosome might control the entry into S phase. Centrosome might control the interphase

through other pathways as well. For instance, studies have shown that removing the core centrosomal components such as centriolin, a mother centriole protein, delay cytokinesis and induces G1 arrest. Another example of such studies indicates that the overexpression of AKAP450, a PCM protein, induces cytokinesis defect and G1 arrest through p53 or p38. Finally, G2/M transition could be arrested by disruption of the interaction between γ-Tubulin ring complexes (γ-TuRCs) and pericentrin which anchors this complex at centrosomes. These findings imply the significance of centrosomes in the regulation of interphase events during the cell cycle (Matsumoto and Maller 2004, Doxsey, McCollum et al. 2005, Sluder 2005, Loffler, Lukas et al. 2006).

Centrosome can also regulate mitosis. During prophase of mitosis, the activation of cyclinB/CDK1 occurs in centrosome. Also, the activation of cyclinB/CDK2 by cdc25 is centrosomal dependent. First, cdc25 gets phosphorylated by Aurora-A which localizes to centrosome during mitosis and then the activated cdc25 removes the inhibitory phosphate residues from CDK2 to control mitotic progression. Furthermore, the centrosome participates in DNA damage repair. This is done through negatively regulating cdc25 by Chk1 which accumulates at centrosomes in response to the DNA damage caused by ultraviolet radiation or Hydroxyurea treatment (Doxsey, McCollum et al. 2005, Sluder 2005, Loffler, Lukas et al. 2006).

1.4 Centrosome Cycle

Centrosomes need to be duplicated and segregated in synchrony with chromosomes. There are four phases in centrosome cycle: centriole disengagement, centriole duplication, centriole maturation and centriole separation. In summary, at the end of mitosis, the two centrioles of each centrosome disengage but remain in close proximity. During S phase, each centriole nucleates a procentriole along its wall, and in G2 phase, the centriole pairs accumulate more PCM required for microtubule nucleation and anchoring to mature into two centrosomes required for mitosis (Figure 2).

a. Centriole disengagement

This phase starts in prophase and ends at the end of telophase. During centriole disengagement, the tight orthogonal positioning of the two centrioles in each centrosome pair is released and they move to a near parallel position. This stage is mainly controlled by PLK1 and Separase. First, PLK1 promotes the removal of Cohesin from centrosomes. Next, Separase cleaves Cohesin at the centriole to complete this process. Centriole disengagement is important for centriole duplication and for limiting it to once per cell cycle (Azimzadeh, Hergert et al. 2009, Bettencourt-Dias, Hildebrandt et al. 2011, Nigg and Stearns 2011, Korzeniewski, Hohenfellner et al. 2013).

b. Centriole duplication

Since each daughter cell inherits one centrosome upon cytokinesis, it is essential that the centrosome duplicates before mitosis so that it can establish bipolarity and correct mitotic spindles. Centriole duplication starts early G1 and continues till G2. During this phase, PLK4 is first recruited to the wall of the mother and daughter centrioles by CEP152. The recruited PLK4 then phosphorylates E3-ubiquitin ligase which in turn stabilizes its substrate SAS-6. Finally, SAS-6 plus SCL-interrupting locus protein (STIL) and CEP135 form a cartwheel that helps define the centriole nine-fold symmetry of procentrioles (Azimzadeh, Hergert et al. 2009, Bettencourt-Dias, Hildebrandt et al. 2011, Nigg and Stearns 2011, Korzeniewski, Hohenfellner et al. 2013).

c. Centriole elongation and maturation

The new formed procentrioles elongate during S and G2 phase. SAS-4 promotes this process and CP110 acts as a cap for the distal end of centrioles to limit microtubule extension. The proteins POC5, OFD1, CEP120 and SPICE1 help this process as well. Following the elongation, the daughter centriole acquires distal and sub-distal appendage components such as Ninein, CEP170 and ODF2 and becomes fully mature. This phase is called maturation and is important for microtubule anchoring and ciliogenesis. Mature centrioles also accumulate more PCM proteins such as CEP152 and CEP192 which are involved in the recruitment of centriole duplication factors. CEP215 is also the

PCM protein essential for PCM assembly in the maturation process. Another important event of this stage is the significant increase of microtubule nucleation activity in centrosomes due to the proteins Aurora A and PLK1. For this, PLK1 first recruits Aurora A to the centrosome which co-localizes with γ-tubulin and then this protein in turn recruits the proteins necessary for microtubule stabilization, such as NDEL1 (Azimzadeh, Hergert et al. 2009, Bettencourt-Dias, Hildebrandt et al. 2011, Nigg and Stearns 2011, Korzeniewski, Hohenfellner et al. 2013).

d. Centriole separation

During most of the cell cycle, the mother and daughter centrioles are connected to each other by Rootletin and C-Nap1, the components of the centrosomal linker. This link needs to be broken at the G2/M transition so the two new centrosomes can separate and move to the opposite sides of the cell and form the bipolar mitotic spindles. The proteins participating in this process are NEK2A, MST1/2, PLK1 and Eg5. First, MST1/2 kinases phosphorylates the protein kinase NEK2A which in turn phosphorylates C-Nap1 and rootletin to promote centrosome separation. Eg5 compliments this process by compensating for NEK2A activity if reduced and its recruitment to centrosome is done by PLK1 phosphorylation (Azimzadeh, Hergert et al. 2009, Bettencourt-Dias, Hildebrandt et al. 2011, Nigg and Stearns 2011, Korzeniewski, Hohenfellner et al. 2013).
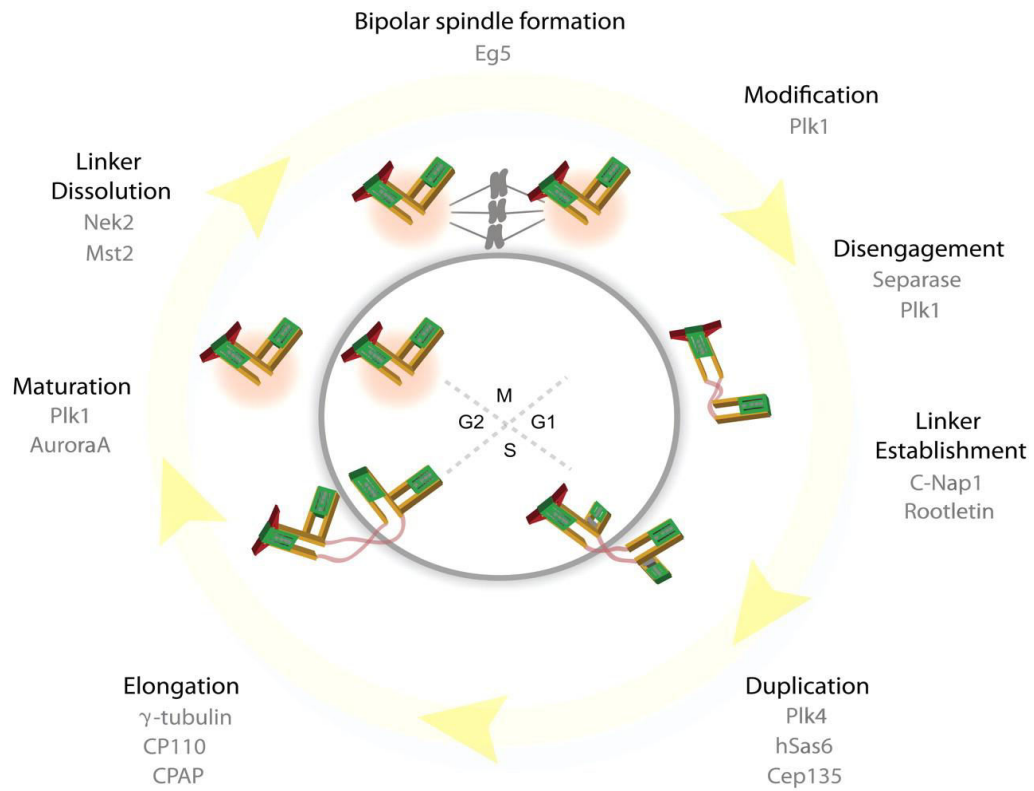
Figure 2. The centrosome cycle. Photo adapted from Mardin et al (Mardin and Schiebel 2012).

1.5 Cilia

Although eukaryotic cilia are conserved, they come in different sizes and functional roles (Quarmby and Parker 2005) . These structures are centriole-derived protrusions on the cell surface that contain microtubules and consist of axoneme and basal body. Axoneme is the microtubule structure of cilium and grows from ciliary basal body. Basal body which is at the base of eukaryotic cilia, is the same as mother centriole and participates in axoneme assembly (Bettencourt-Dias and Glover 2007). Similar to centriolar microtubules, axoneme microtubules are made of αβ tubulin heterodimers and are surrounded by ciliary membrane which is different from the cell membrane. There are 2 types of Cilia: 1. Primary or non-motile cilia which consist of 9 doublet microtubules and lack molecular motors. These cilia are usually one per cell and are specialized sensory structures. 2. Motile cilia which consist of 9 doublet microtubules surrounding a central pair of singlet microtubules and may be several hundred per cell (figure 3). These cilia need the motor protein dynein for their motility (Satir and Christensen 2007).

Cilia grow at their distal tips and motor proteins transport ciliary precursors for assembly and maintenance (Quarmby and Parker 2005). Signaling molecules, receptors and tubulins are transported to primary cilia by intraflagellar transport (IFT) and motor proteins such as dynein and kinesin-2 (Tsang, Bossard et al. 2008). Since cilia do not have protein synthesis machinery, they

depend on IFTs for their assembly. IFTs perform in 2 directions due to the protein complexes, IFT-A and IFT-B. IFT-A is involved in both anterograde and retrograde transport of molecules, whereas IFT-B is only involved in transport from cell body to cilia and directs anterograde transport (Tsang and Dynlacht 2013).

Most ciliated cells are in G0 of the cell cycle. For these cells to enter the mitosis stage, first the cilia need to be resorbed and when mitosis is complete, the cilia will be reassembled (Quarmby and Parker 2005). There are 3 distinct stages in cilia assembly. First, a Golgi-derived vesicle containing membrane proteins destined to the ciliary compartment binds the distal end of the mother centriole and the axoneme starts to form. This vesicle accumulates the essential structures inside the centriole to form the basal body. Next, vesicles create a sheath around the axoneme in which the microtubules are assembled. Finally, the axoneme reaches the cell surface and its membrane fuses to the plasma membrane to form the ciliary necklace (Pedersen, Veland et al. 2008).

THE CILIUM DISSECTED

Cilia consist of a ring of microtubule scaffolding called the **axoneme**, covered by cell membrane

Molecules are transported via intraflagellar transport (IFT) using **motor proteins** that travel up or down the microtubules

The axoneme is anchored in the cell by a **basal body**

IFT raft

Ciliary membrane

A cross-section of an axoneme reveals either a **9+0** or a **9+2** arrangement of microtubules

Ciliary membrane

Doublet microtubules

9+0
(primary cilium)

Singlet microtubules

Dynein arms

Radial spokes

9+2
(motile cilium)

In motile cilia, tubules work with the dynein arms and protein spokes that link them to produce movement
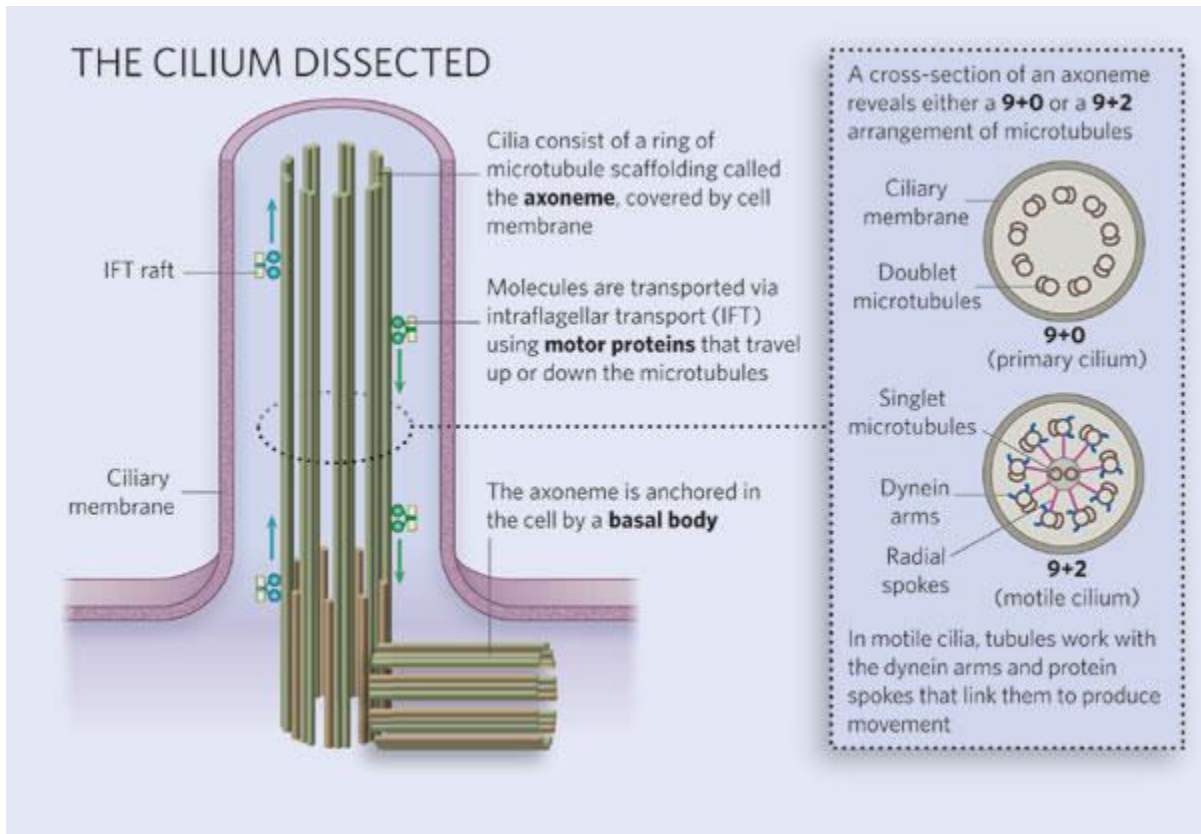
Figure 3. Diagram of ciliary structure. Photo adapted from Ainsworth (Ainsworth 2007).

1.6 Cytoskeleton

The centrosome is involved in cytoskeleton regulation by its active participation in the assembly of microtubules, a cytoskeleton component that plays important roles in transport of proteins and organelles, cell polarity and mitotic spindles (Luders and Stearns 2007). The cytoskeleton plays an important role in 3 cellular functions. First, it organizes the cell content and components. Second, it helps connect the cells with the external environment physically and biochemically. Finally, it is implicated in cellular movement (Fletcher and Mullins 2010).

Three main polymers of cytoskeleton are actin filaments, microtubules and intermediate filaments. The polymerization and depolymerization of actin filaments and microtubules lead to changes in cell shape and with the help of motor proteins, cellular components are organized. The differences between the 3 cytoskeletal subunits go back to their mechanical stiffness, dynamics of their assembly, their polarity and molecular motors associated with them (Fletcher and Mullins 2010).

Microtubules are the stiffest subunit and have a very complicated assembly dynamic. Their stiffness is beneficial in the interphase stage of cell cycle by assembling the radial array of microtubules that help the intracellular traffic. During mitosis, microtubules form mitotic spindles which enable chromosome alignment through dynamic instability of microtubules (Fletcher and Mullins 2010).

Although actin filaments are less rigid than microtubules by themselves, high concentration of the crosslinkers binding them make stiff isotropic, bundled and branched networks. These networks are involved in chemotaxis, cell-cell communication and phagocytosis. Unlike microtubules, actin filaments elongate steadily in the presence of nucleotide-bound monomers and their assembly is in response to the local activity of signaling systems. The intermediate filaments are the least stiff subunit and are not polarized. They interact with both microtubules and actin filaments through plectins and are usually assembled in response of mechanical stress (Fletcher and Mullins 2010).

Microtubules are tubular polymers composed of α and β tubulins that associate to form protofilaments with the β-tubulin subunit on the plus end of microtubules and α-tubulin subunit on the minus end. A third member of tubulin family, γ-tubulin is important for microtubule nucleation and assembly. Microtubule assembly needs GTP hydrolysis so the GDP-tubulin is stabilized at the plus end by a short cap (Luders and Stearns 2007, Wade 2009). The α and β monomers are 55 kDa and both consist of 450 amino acid residues. Tubulin is subject to several post-translational modifications like acetylation, detyronization and polyglutamylation. These modifications determine the stability of microtubules (Wade 2009).

When tubulin concentrations are low, the microtubule nucleation process is kinetically limiting. Therefore, nucleation takes place in specific structures called microtubule organizing centers (MTOCs) such as centrosome (Wiese and Zheng 2006). During interphase,

microtubules get nucleated in MTOCs rich in γ-TuRC whereas during mitosis they nucleate on centrosomes which are located at spindle poles and the astral microtubules are formed dynamically (Wade 2009).

A large number of proteins interact with microtubules and are referred to as microtubule-associated proteins (MAPs). Two classical types of MAPs isolated from brain are the high-molecular-weight MAPs (200-300 kDa) and the lower molecular weight tau proteins which is 55 kDa. The main role of these proteins is microtubule stabilization against dynamic instability (Wade 2009). The motor proteins, kinesin and dynein, are important microtubule partners during cell division in eukaryotes. Kinesins have 2 conserved regions which are responsible for ATP-binding and microtubule-bindng. Conventional kinesins move toward plus end of microtubules at 1µm/s in vitro. Dyneins also use ATP energy to move but they move towards the minus end of microtubules. Dyneins can move laterally and reverse direction as well. They have 1-3 heavy chains plus several intermediate and light chains. Their important function is in orientation of mitotic spindle and in nuclear migration (Wade 2009).

1.7 Diseases

There are 2 types of centrosome abnormalities: 1. structural defect and 2. numerical aberrations. The structural defects are largely due to changes in the expression levels of different centrosomal proteins or altered posttranslational modifications that would lead to an enlarged centrosome or reduction in MT nucleation. Also, a reduction of centrosome size reduces spindle length. Structural defects are common in tumors. As for numerical aberrations, overduplication of centrosome is a good example of these kinds of defects and is widely found in tumors. Both these aberrations could cause diseases (Greenan, Brangwynne et al. 2010, Bettencourt-Dias, Hildebrandt et al. 2011). Some common ones are discussed below.

1. *Aneuploidy*

Centrosomal deregulation usually leads in chromosomal instability (CIN) and aneuploidy. Aneuploidy is the result of chromosome missegregation and is caused by abnormal mitotic spindle assembly. This is mostly a numerical defect (Kumar, Rajendran et al. 2013).

2. Cancer

Important evidence of the role of centrosomal defects in tumorigenesis came from the fact that p53 knock down resulted in centrosome amplification in mouse fibroblasts and skin tumors. Centrosome abnormalities are often observed in breast, prostate, lung, colon and brain cancers. There are several pathways leading to centrosome overduplication. First, the overexpression of PLK4 or mutation in oncogenes or tumor suppressors will cause centriole over-duplication. Another pathway is through cell division failure and cell-cell fusion which causes tetraploidisation (Nigg 2006, Bettencourt-Dias, Hildebrandt et al. 2011).

Centrosomal amplifications and defects usually occur very early in tumorigenesis and are associated with initiation of chromosomal changes. These defects get more severe with tumor progression. In a study on cervical carcinoma, centrosomal amplification increased 20% in epithelia of grade 1 tumors, 5o% in grade 2 tumors and finally in grade 3 tumors, this increase was 70%. In tumor cell lines, centrosome overduplication is mainly caused by the reduced activity of p53 and the overexpression of its inactivating protein, Mdm2, which allows polyploid cells to proliferate rather than undergoing apoptosis (Saunders 2005).

Some studies have suggested the link between DNA damage and centrosome numerical abberations. For instance, DNA damage could lead to centrosomal splitting in *Drosophila* and mammalian cells. Furthermore, the overexpression of ATM/ATR could result in

this amplification. The consequences could be cell cycle arrest or errors in mitosis (Saunders, 2005).

3. *Brain development*

The most common phenotypes in this category are neural migration disorders such as lissencephaly, disorders of brain growth such as microcephalic osteodysplastic primordial dwarfism and primary microcephalies (MCPH) in which the size of brain is significantly reduced. The genes affected by primary microcephalies are either involved in centriole duplication or centrosome maturation. Centrosome P4.1 associated protein (CPAP) and CEP152 are MCPH proteins essential in both of these processes. Also, MCPH mutations could lead in a reduction of the whole body including the size of the brain (Bettencourt-Dias, Hildebrandt et al. 2011).

4. *Ciliopathies*

Defects in motile cilia cause pathologies referred to as primary cilia dyskinesia (PCD). Patients with PCD show body asymmetry which is an indication of the importance of ciliary motility in directional flow in early embryos and initiation of normal left-right developmental program. Mutations sometimes happen in the primary cilia and cause defects in its structure or function which would lead in diseases such as polycystic kidney disease (PKD),

nephronophthisis, retinitis pigmentosa, Bardet-Biedle (BBS) and Joubert and Meckel syndrome. Although cilia structure might not be altered in these disorders, its sensory function might have defects and therefore affects multiple organs such as kidney, retina, brain, bones and liver (Bettencourt-Dias, Hildebrandt et al. 2011).

6. *Defects in intracellular transport*

Because of its microtubule organizing ability, centrosome plays a crucial role in intracellular transport and spatial organization of cellular organelles. Huntington disease is one of the neurodegenerative disorders that is a consequence of defects in microtubule-dependent vesicular transport. This disease is characterized by loss of cognitive function and motor defects. Huntington-associated protein (HAP1) binds to dynactin and pericentriolar material 1 protein (PCM1) which is involved in centrosome and basal body function. Studies in fibroblast cultures of patients with Huntington disease exhibit aberrant centrosome numbers, a reduction in mitotic index, an increase in aneuploidy and finally persistence of midbody (Badano, Teslovich et al. 2005).

Since centrosome deregulation is the cause of several diseases, it is important to study novel centrosomal proteins and their functions as potential therapeutic targets.

1.8 CEP78

CEP78 is a novel centrosomal protein first identified in 2003 through proteomic characterization of human centrosome. In this study, a mass-spectrometry analysis of human centrosomes in interphase was performed and 23 new components were discovered. CEP78 was one of them (Andersen, Wilkinson et al. 2003). The *CEP78* gene is located on chromosome 9q21. Human CEP78 protein has several isoforms, the biggest one a 78 kDa protein consisting of 722 amino acids. This protein has orthologs in mouse, chicken, lizard, tropical clawed frog, zebrafish and fruit fly. As for the structure of this protein, it consists of 4-6 Leucin-rich Repeats (LRRs) and one coiled-coil domain. Very few papers have discussed possible CEP78 functions. In one such studies, the possible role of CEP78 in centriole anchoring and ciliogenesis was discussed (Azimzadeh, Wong et al. 2012). Also, in a study carried out on the effect of standard treatments on immune responses in prostate cancer patients, CEP78 was one of the proteins recognized for its treatment associated autoantigen reactivity (Nesslinger, Sahota et al. 2007). In a study carried out in 2012, CEP78 expression upregulated 5 fold by noise stress in rat cochleae (Han, Hong et al. 2012). Another study on the genes altered by ethanol treatment during neurodevelopment showed that CEP78 expression decreased on E 14/16 and P 4/7 (Kleiber, Mantha et al. 2013). Finally, a study in 2013 claimed the interaction between CEP78 and PLK4, CP110 and CEP97 (Baffet, Martin et al. 2013). Since all these three proteins are involved in

centriole duplication, it is necessary to look at the possible role of CEP78 in this process. The objective of my project was to further study CEP78 localization, function and interactions.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1   Chemicals

β-glycerophosphate,   β-mercaptoethanol,   AEBSF,   Ampicillin, Aprotinin,   CaCl2,   DAPI,   DMP,   DTT,   EDTA,   Ethanolamine, Glutathione, Glycerol, Glycine, HCl, Hepes, IPTG, KCl, Leupeptin, Methanol, MgCl2, Tris, Na Borate, NaCl, Nocodazole, NP-40, Paraformaldehyde, PBS, SDS, Triton

2.1.2   Solutions, Buffers and media

2.1.2.1 Coomassie

(50% Methanol; 10% Acetic Acid; 0.2% Coomassie Blue; dH2O)

2.1.2.2 ELB+ Buffer

(1M Hepes pH 7; 5M NaCl; 0.5M EDTA pH 8; 10% NP-40; 1mM DTT; 0.5mM AEBSF; Leupeptin 2μg/ml; Aprotinin 2μg/ml; 10mM NaF; 50mM β-glycerophosphate; dH2O)

2.1.2.3 Glutathione elution buffer

(100mM Tris pH 7.9; 120mM NaCl; 20mM Glutathione; 1mM DTT; 0.2mM AEBSF, dH2O)

2.1.2.4 4X Lower Gel Buffer

(1.5mM Tris-HCl pH 8.8; 0.4% SDS; dH2O)

2.1.2.5 0.1 HEMGN

(100mM KCl; 25mM Hepes pH 7.6; 0.2mM EDTA pH 8; 12.5mM MgCl2; 10% Glycerol; 0.1% NP-40; 1mM DTT; 0.2mM AEBSF; Leupeptin 2 µg/ml ; Aprotinin 2 µg/ml, dH2O)

2.1.2.6 Maniatis 5x SDS Page Running Buffer

(25mM Tris; 250mM glycine; 0.1% SDS; dH2O)

2.1.2.7 Stripping buffer

(20% SDS, 14.4M β-mercaptoethanol; 1M Tris; dH2O)

2.1.2.8 4X Upper Gel Buffer

(0.5M Tris-HCl pH 6.8; 0.4% SDS; dH2O)

2.1.2.9 Western Transfer Buffer

(50mM Tris; 380mM Glycine; 0.1% SDS; 20% Methanol; dH2O)

2.2   Methods

2.2.1   Bacterial methods

2.2.1.1   Purification of GST- tagged CEP78 and GST proteins

The bacteria *E.coli* DH5α strain containing truncated C (amino acids 590-722) and N (amino acids 1-146) terminal CEP78 plasmids were inoculated from glycerol stock in Luria broth (LB) media containing 100 μg/ml Ampicillin and grew overnight. The protein expression was induced by adding 1M IPTG to the cultures and incubating them at 20°C for 16 hours. Next, the bacteria were pelleted at 4000 rpm. After the pellets were washed with 1X PBS (Phosphate Buffered Saline), they were resuspended in 0.1 HEMGN buffer. Then, the bacterial suspensions were sonicated 3 times with 15 second bursts at the microtip limit. The lysates were centrifuged at 10000 rpm(Revolutions Per Minute) and the supernatants were transferred to Eppendorf tubes. Next, the 50% slurry Glutathione agarose beads were prepared. To do this, the beads were first resuspended in 0.1 HEMGN buffer and rocked for 1 hour at room temperature. Next, they were equilibrated in 0.1 HEMGN buffer and finally resuspended in 0.1 HEMGN buffer to make 50% slurry. The beads then were added to the extracts and the samples got incubated at 4°C for 1 hour. After the incubation, the samples were spun at 3000 rpm and the supernatants were

aspirated. The beads were washed with 0.1M HEMGN and then eluted with 1ml glutathione elution buffer for 20 minutes and spinned at 3000 rpm. Finally, the eluates were dialyzed against 0.1M HEMGN at 4°C overnight. The dialyzed proteins were stored at -80°C or run on a 10% SDS-PAGE gel and coomassie stained.

2.2.1.2   CEP78 Antibody purification

2.2.1.2.1 Making Columns

At first, the concentration of dialyzed GST and GST-CEP78 proteins was measured by running them on SDS-PAGE, doing a Coomassie staining and comparing the intensity of their bands to the ones of different BSA concentrations. Next, the GST-agarose beads were added to the proteins and incubated at 4°C for 2 hours. After the binding, the samples were spinned down at 1000 rpm and washed with 1X PBS. Next, the beads were washed and resuspended in 0.2M Na Borate pH=9. For crosslinking, solid DMP(Dimethyl pimelimidate) was added to beads and they were incubated at room temperature for 30 minutes. Next, the samples were spun at 1000 rpm and the beads were washed, resuspended in 0.2M Ethanolamine pH=8 and incubated at room temperature. After 2 hours, the samples were spun at 1000 rpm and the beads were washed with 1X PBS and 0.1 Glycine pH=2.5. Following another

round of washing with 1X PBS, the beads were transferred to the columns.

2.2.1.2.2 Purifying antibodies

First, rabbits were immunized against CEP78 truncated proteins and their serums were collected by Cocalico Biologicals company. Then the serums were loaded on the GST column and incubated at room temperature. After an hour, the flowthrough was collected from the GST column and added to the GST-CEP78 column and incubated in room temperature. One hour later, the beads were washed with 1X PBS and the antibody elution was carried out with fractions of 0.1M Glycine pH=2.5. The eluates were then collected in the Eppendorf tubes already containing 1M Tris HCl pH=8.

2.2.1.3 Transformation of competent cells

First, the bacteria *E.coli* DH5α strain competent cells were thawed. Next, 10 ng DNA was added to the competent cells and they were incubated on ice. Then, the samples were heat shocked first at 42°C for 45 seconds and then back on ice for 5 minutes. Later, LB media was added to the cells and the samples

were incubated at 37°C. After an hour, the cells were spinned down at 9000 rpm and the supernatant was aspirated. Finally, the pellet was resuspended in LB media and plated on LB plates containing appropriate antibiotic which were incubated at 37°C overnight.

2.2.2   Cellular methods

2.2.2.1 Immunofluorescence Assay

First, the cells were washed with 1X PBS. Next, they were fixed with 100% iced Methanol or 4% Paraformaldehyde and washed with 1X PBS. Following permeabilization with PBS-1% Triton, cells were washed with 1% PBS and blocked with PBS-3% BSA-0.1% Triton. Then they were incubated with the primary antibody. After one hour incubation, the cells were washed with PBS-0.1% Triton. Next, they were incubated in dark with the secondary antibody-fluorochrome-labeled. One hour later, the cells were washed with PBS-0.1% Triton and incubated with DAPI in dark for 7 minutes. Then, they were washed with 1X PBS and H2O. Once dry, the coverslips were mounted on slides using mounting media. Finally, they were sealed with nail polish.

2.2.2.2 Western Blotting

First, the cells were harvested by spinning at 1000 rpm for conical tubes or at 3000 rpm for microcentrifuge tubes. The supernatant was aspirated and the pellet was washed with 1X PBS. Next, the sample was lysed with ELB+ buffer and its protein concentration was measured via Biorad protein assay and Spectrophotometry. Then, the sample and loading dye were loaded on 10% gel and run at 150V. Once the running step was complete, the transfer to Nitrocellulose membrane was carried out at 60V for one hour. Next, the membrane was blocked in 3% milk and incubated with the primary antibody. After 1 hour incubation at room temperature, the membrane was washed with H2O and incubated with the secondary antibody at room temperature. One hour later, the membrane was washed with H2O. Finally, ECL was added on the membrane and developing was carried out.

2.2.2.3 Knock Down with siRNA

First, the cells were plated in 6 well plates so that at the time of transfection, they were 40%-50% confluent. For transfection, the siIMPORTER reagent from Millipore company was diluted with

serum-free medium in one microcentrifuge tube and in another tube siRNA oligo, siRNA diluent and serum-free medium were mixed so that the final concentration of the oligo was 100 nM. Next, the content of both tubes were mixed and incubated at room temperature for 5 minutes. Finally, the mixture was added to the cells and the cells were incubated at 37°C.

2.2.2.4 Plasmid Transfection with Transit reagent

First, the cells were plated in 6 well plates so that at the time of transfection, they were 60%-70% confluent. For transfection, the Transit reagent was first diluted with serum-free medium and next 2µg plasmid was added to it. The mixture was then incubated at room temperature for 20 minute. Finally, the mixture was added to the cells and the cells were incubated at 37°C.

2.2.2.5 Plasmid transfection with CaCl2

First, the cells were plated so that at the time of transfection, they were 60%-70% confluent. For transfection, 20µg DNA, 2.5M $CaCl_2$, 1ml 2X HEPES and H_2O were mixed and incubated at room

temperature for 20 minutes. Finally, the mixture was added to the cells and the cells were incubated at 37°C.

2.2.2.6 Immunoprecipitation

First, the cells were lysed in ELB+ buffer for 30 minutes. Next, the lysate was spinned down at 14000 rpm and the supernatant was transferred into new Eppendorf tubes. Then, 2µg antibody was added to the supernatant and the sample was incubated at 4°C for 1 hour. Following the incubation, 50% slurry protein A/G beads were added to the sample and once again the beads were incubated at 4°C for one hour. Next, the sample was spinned down at 3000 rpm in the cold and washed 3 times with ELB+ buffer. Finally, loading dye was added to the sample for western blotting.

2.2.2.7 Centrosomal localization study

First, the cells were treated with 10µM Nocodazole and incubated at 37°C. After an hour, the cells were fixed and stained with α-tubulin.

2.2.2.8 Microtubule Assay

First, the cells were treated with 10µM Nocodazole and incubated at 4°C. After an hour, Nocodazole was aspirated from plates, the media was replaced and the cells were incubated at 37°C for 2, 5 and 20 minutes. Finally, the cells were fixed and stained with α-tubulin antibody.

Chapter 3 Results

CEP 78 is a novel protein first identified through a mass-spectrometry analysis of human centrosome(Andersen, Wilkinson et al. 2003). It consists of 722 amino acids and structurally has 4-6 Leucine-rich repeats and a coiled coil domain. The objective of this project was to study the localization and function of this protein. For the localization study, experiments were planned to look at the cell cycle pattern of this protein and its centrosomal localization. As for the functions of CEP78, its possible role in different stages of centrosome cycle and its effect on some other centrosomal proteins were studied.

3.1 CEP78 is an intrinsic component of Centrosome.

It had already been shown that CEP78 is a centrosomal protein. In order to study whether CEP78 is a permanent component of centrosome or it requires microtubules for its centrosomal localization, retinal pigment epithelial (hTERT-RPE or RPE) cells were treated with nocodazole which is a microtubule depolymerizing agent. RPE cells were used because they are

normal diploid, have normal centrosome number and morphology, and undergo normal cell division. These traits makes them good candidates for studying the effect of a new centrosomal protein on other centrosomal components. γ-tubulin and α-tubulin were used as control proteins. In fact, what we expected was that the net pattern of alpha-tubulin disappeared due to microtubule depolymerization whereas the genuine centrosomal proteins would remain. Following an immunofluorescence assay and staining the cells with α-tubulin, γ-tubulin (a permanent centrosomal protein) and CEP78 antibodies, it was observed that the net pattern of α-tubulin disappeared whereas γ-tubulin and CEP78 proteins remained at the centrosome. These results indicated that CEP78 is indeed a stable centrosomal component and does not require microtubules for its centrosomal localization [Figure 4].
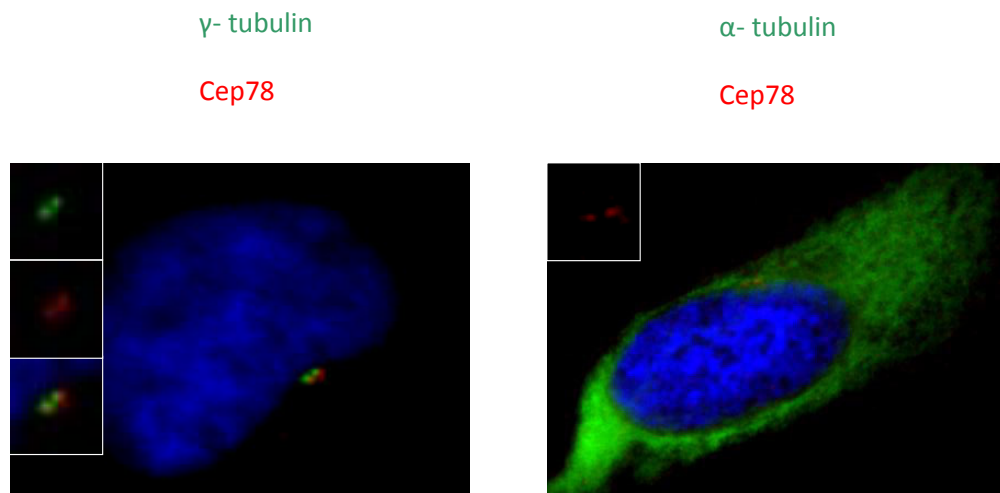
γ- tubulin

Cep78

α- tubulin

Cep78

Figure 4: CEP78 is an intrinsic component of centrosomes and does not require microtubule for its centrosomal localization. RPE cells are treated with nocodazole for 1 hour, fixed and stained with α-tubulin,γ-tubulin and CEP78.

3.2 CEP78 is a centriolar protein present at the distal end.

To see whether CEP78 is present at the distal or proximal end of centrioles, co-localization of this protein with other centrosomal proteins was studied in RPE cells by immunofluorescence assay and fluorescence microscopy. Several proximal (C-Nap1, Polyglutamylated Tubulin) and distal (CEP170, POC5) proteins were studied at this step. The results indicated that CEP78 did not colocalize with the proximal proteins C-Nap1 and Polyglutamylated Tubulin. However, the distal proteins CEP170, Centrin and POC5 showed a close co-localization with CEP78. In conclusion, CEP78 localizes to distal end of centrioles [Figure 5].
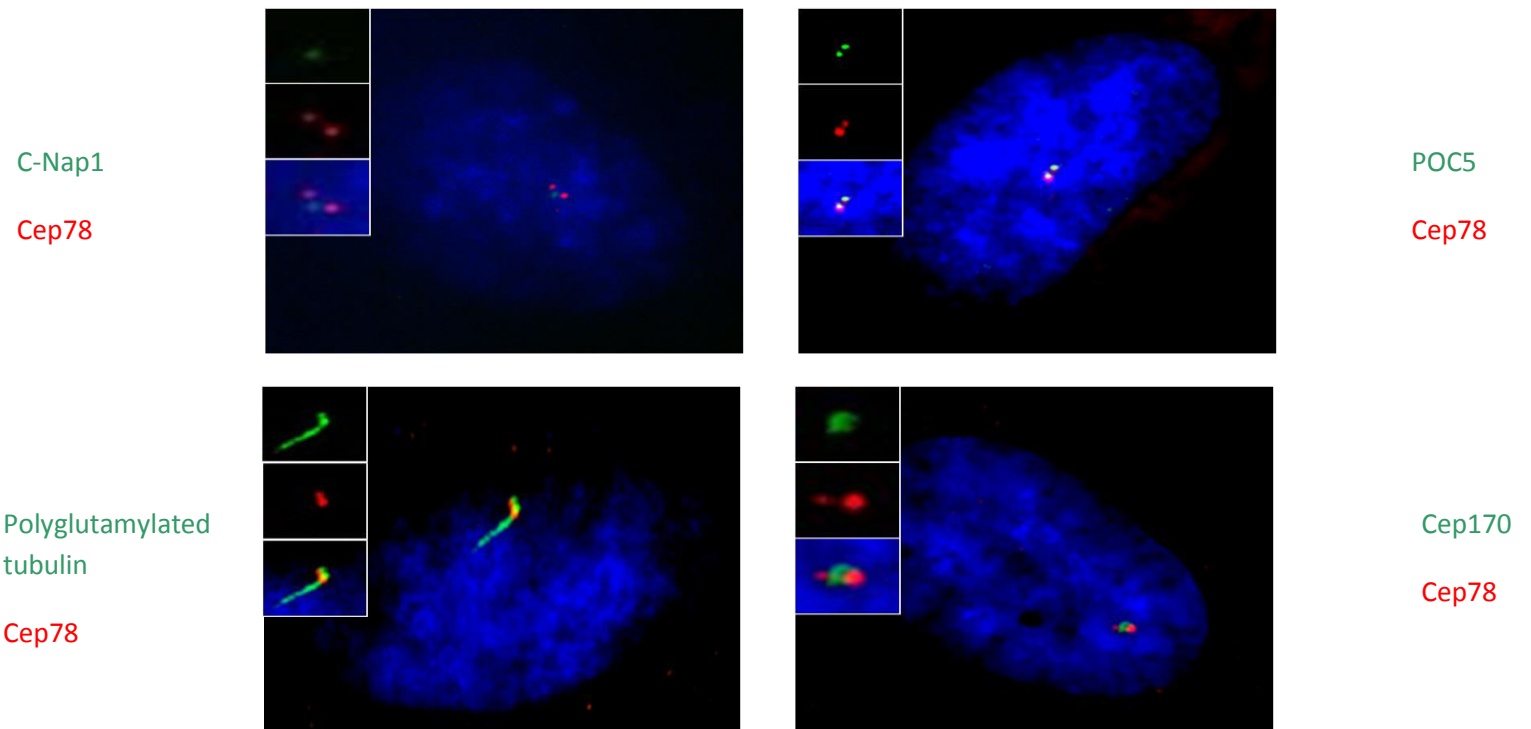
C-Nap1

Cep78

POC5

Cep78

Polyglutamylated
tubulin

Cep78

Cep170

Cep78

Figure 5: CEP78 localizes to the distal end of centrioles. RPE cells are fixed and stained with CEP170, Polyglutamylated tubulin, C-Nap1, POC5 and CEP78.

3.3 CEP78 localizes to mature centrioles.

In order to study the cell cycle pattern of CEP78, co-localization with centrin (centriolar marker on mother, daughter and procentrioles), was carried out in RPE cells at different stages of cell cycle by immunofluorescence assay and fluorescence microscopy. Different stages of the cell cycle were identified based on centrin and DAPI staining in asynchonzied cells and Polyglutamylated tubulin for G0 cells. Centrin is a centriolar marker which appears as 2 dots during G1 (mother and daughter centrioles) and 4 dots during S, G2 and mitosis (mother, daughter and procentrioles). During the G0, G1, S and early G2 phases of interphase, there were 2 CEP78 dots in the cells with the intensity of one dot stronger than the other one. My previous colocalization study with CEP170 (a sub-distal appendage protein on mother centriole) had indicated that the stronger dot belongs to the mother centriole. Measuring the intensity of these dots using the software Velocity showed that the mother centriole dot was 1.9 times stronger than the daughter centriole dot. In order to calculate the above number, first a number of images were taken by the microscope camera and each image was analyzed separately by the Velocity software to measure the intensity of the CEP78 dots on mother and daughter centrioles. Finally, an average was taken of the intensity differences. During late G2 phase of interphase, the intensity of the mother and daughter

centrioles became quite equal implicating that daughter has matured into a mother. Also, 2 new weak CEP78 dots started to appear on the procentrioles evolving to the daughter centrioles. As the cells went through mitosis, the intensity of the new dots increased gradually. In prophase the CEP78 dots have a quite diffused staining but by the end of telophase, 2 obvious CEP78 dots could be observed in each daughter cell. The co-localization study with Centrin indicated that CEP78 dots only localized to the mother and daughter centrioles but not procentrioles and that CEP78 is stronger on the mother compared to the daughter centriole[Figure 6].
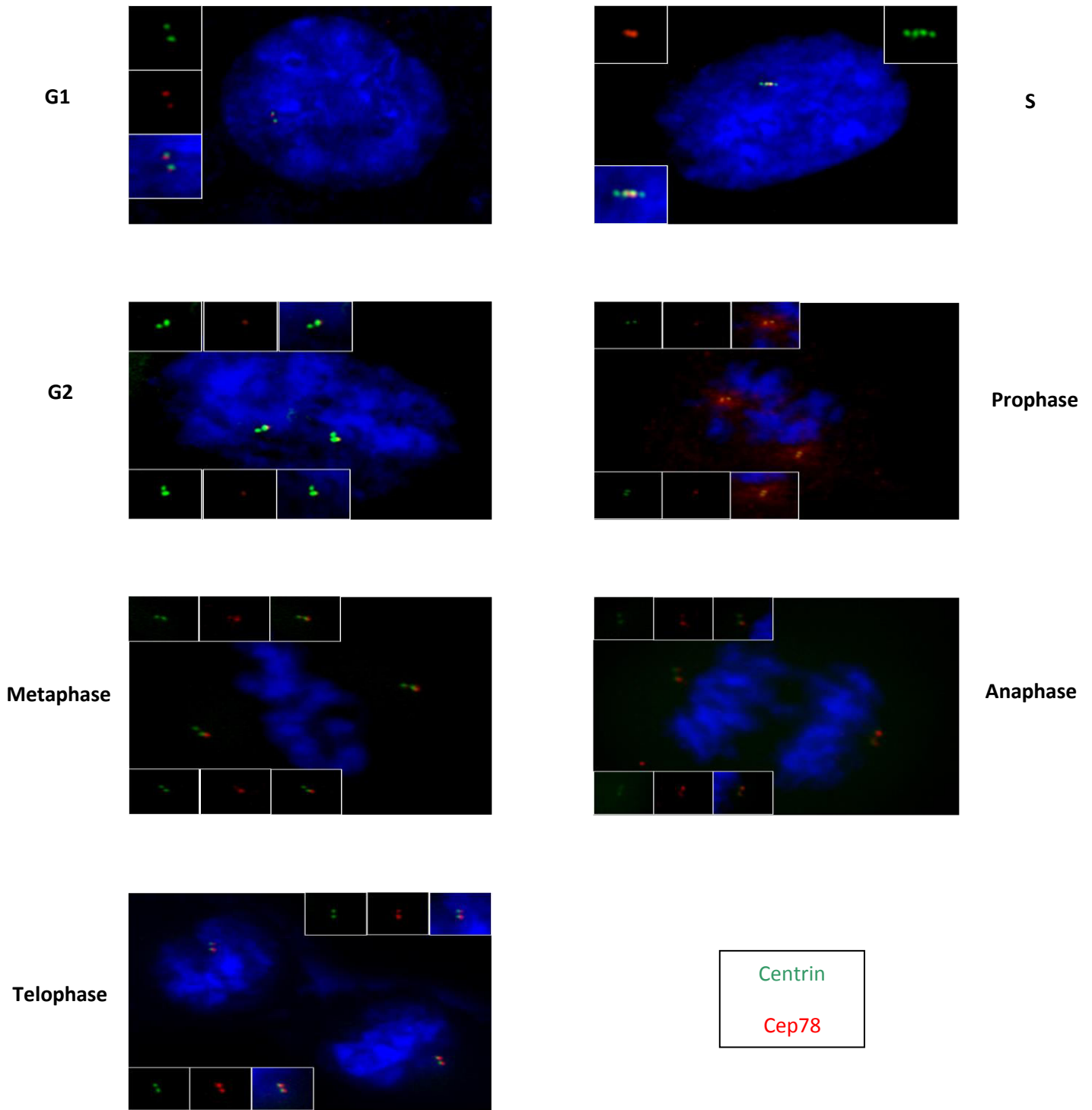
G1

S

G2

Prophase

Metaphase

Anaphase

Telophase

Centrin

Cep78

Figure 6: CEP78 localization pattern at different stages of cell cycle. RPE cells are fixed and stained with Centrin and CEP78.

3.4 Leucin rich repeats are responsible for centrosomal localization of CEP78.

The CEP78 protein consists of 4-6 Leucin rich repeats (LRRs), amino acids 147-308, and one coiled coil domain, amino acids 450-497. To address the importance of these domains, several CEP78 fragments with deletion in one or some of these domains were expressed in RPE cells and their expression pattern was studied by immunofluorescence assay and fluorescence microscopy. The results showed three distinct phenotypes: centrosomal localization, microtubule binding and aggregate formation [Table 1]. All these three patterns were observed after overexpression of full length CEP78 as well. While the fragments with deletion in any LRRs could not localize to centrosome (Δ147-174, Δ226-254, Δ255-282 and Δ283-308), the fragments that contained all the LRRs localized to centrosome clearly (Δ450-497, 1-445). About 70% of the cells expressing full length CEP78 (1-722) also showed centrosomal localization [Figure 7]. Furthermore, some of the fragments including the fragment 221-445 as well as 40% of the cells expressing full length CEP78 showed microtubule binding pattern. In fact, 70% of the cells expressing fragment 221-445, showed the net pattern of microtubules. This fragment contains the three middle LRRs. Although the microtubule binding pattern is observed, further studies are required to confirm this binding. Finally, the

expression of some of the fragments including 1-220 resulted in aggregate formation. This pattern was only observed in 15% of the cells expressing the full length CEP78 [Figure 8].The protein aggregates could be indicative of a malfunction of the normal process of protein turnover or a problem in the recruitment of the protein to centrosome. These results show that LRRs are crucial for centrosomal localization and probably MT binding of CEP78. They can function by interacting with other proteins that help recruit CEP78 to centrosome or microtubules.

Construct	Centrosomal localization	Microtubule binding
Full Length Cep78	74%	42%
Δ (47-174)	2%	0%
Δ (226-254)	0%	0%
Δ (255-282)	0%	0%
Δ (283-308)	0%	0%
Δ (450-497)	83%	10%
1-220	0%	0%
221-722	0%	33%
1-445	42%	32%
221-445	7%	70%
446-722	0%	0%

TABLE 1: Percentage of RPE cells transfected with CEP78 fragments showing centrosomal localization or microtubule binding pattern

Δ (147-174)

Δ (226-254)

Δ (255-282)

Δ (283-308)

Δ (450-497)

1-445

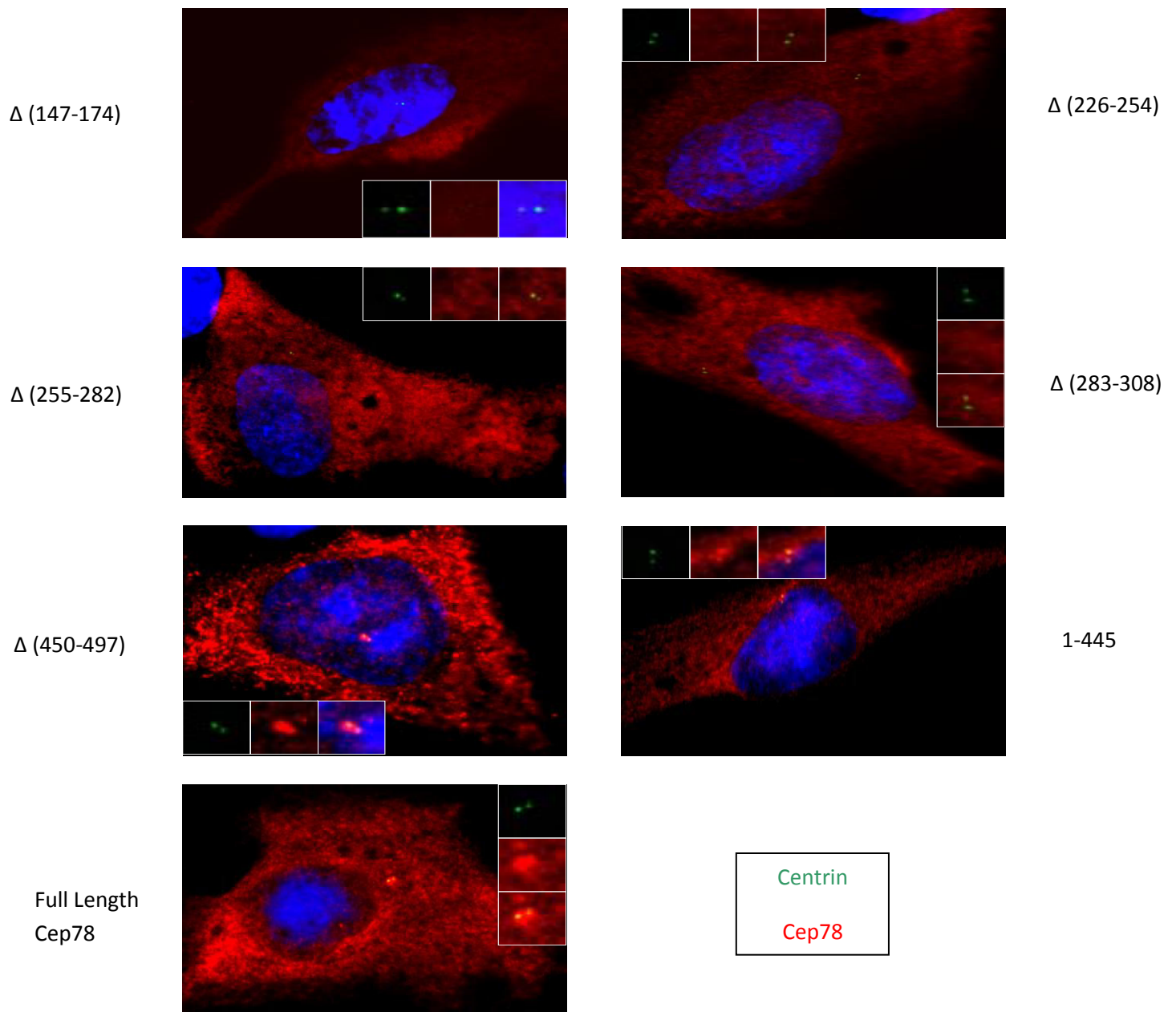Full Length
Cep78

Centrin

Cep78

Figure 7: LRRs are necessary for centrosomal localization. RPE cells are transfected with flag tagged CEP78 fragments, fixed and stained with flag and Centrin.
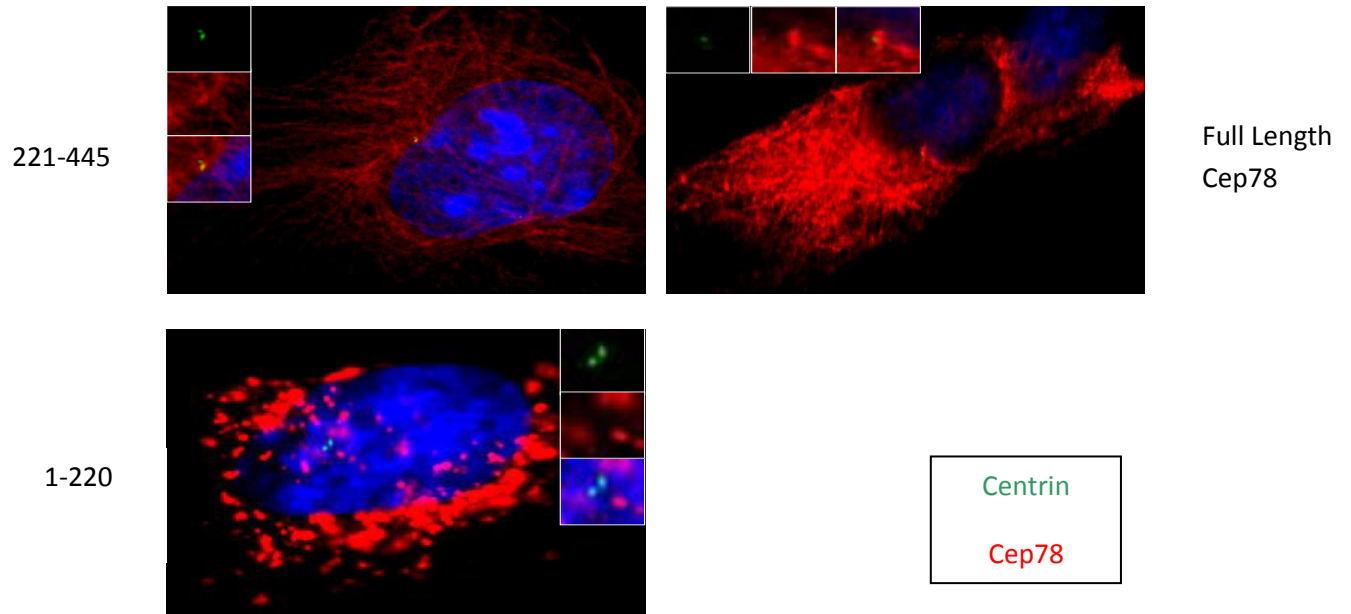
221-445

Full Length
Cep78

1-220

Centrin

Cep78

Figure 8: Some fragments show a) microtubule binding b) aggregate formation. RPE cells are transfected with flag tagged CEP78 fragments, fixed and stained with flag and Centrin.

3.5 CEP78 overexpression does not result in centriole duplication or accumulation.

Since CEP78 is a centriolar protein, I speculated that modulation of its protein levels could affect the number of centrioles within a cell. In order to study the possible role of CEP78 in centriole duplication or accumulation, the full length protein was expressed in RPE cells and its effect on CEP164 (mother centriole marker), SAS-6 (procentriole marker) and centrin (marker of mother, daughter and procentrioles) was studied. Since most cells were in G1 phase and only had one mother centriole, most control cells had only one CEP164 dot. Similar to control, most transfected cells had 1 CEP164 dot and there was no increase/decrease in the number of mother centrioles. Studying SAS-6 showed no significant difference between the control and transfected cells either and most cells had zero (no procentriole in G1 phase) or 2 (2 procentrioles from G2) SAS-6 dots based on the stage of cell cycle. This meant that there was no change in the number of procentrioles. Also, looking at centrin confirmed the results of CEP164 and SAS-6 since the number of centrioles did not change. So these experiments indicate that CEP78 overexpression does not result in centriole duplication or accumulation [Figure 9].
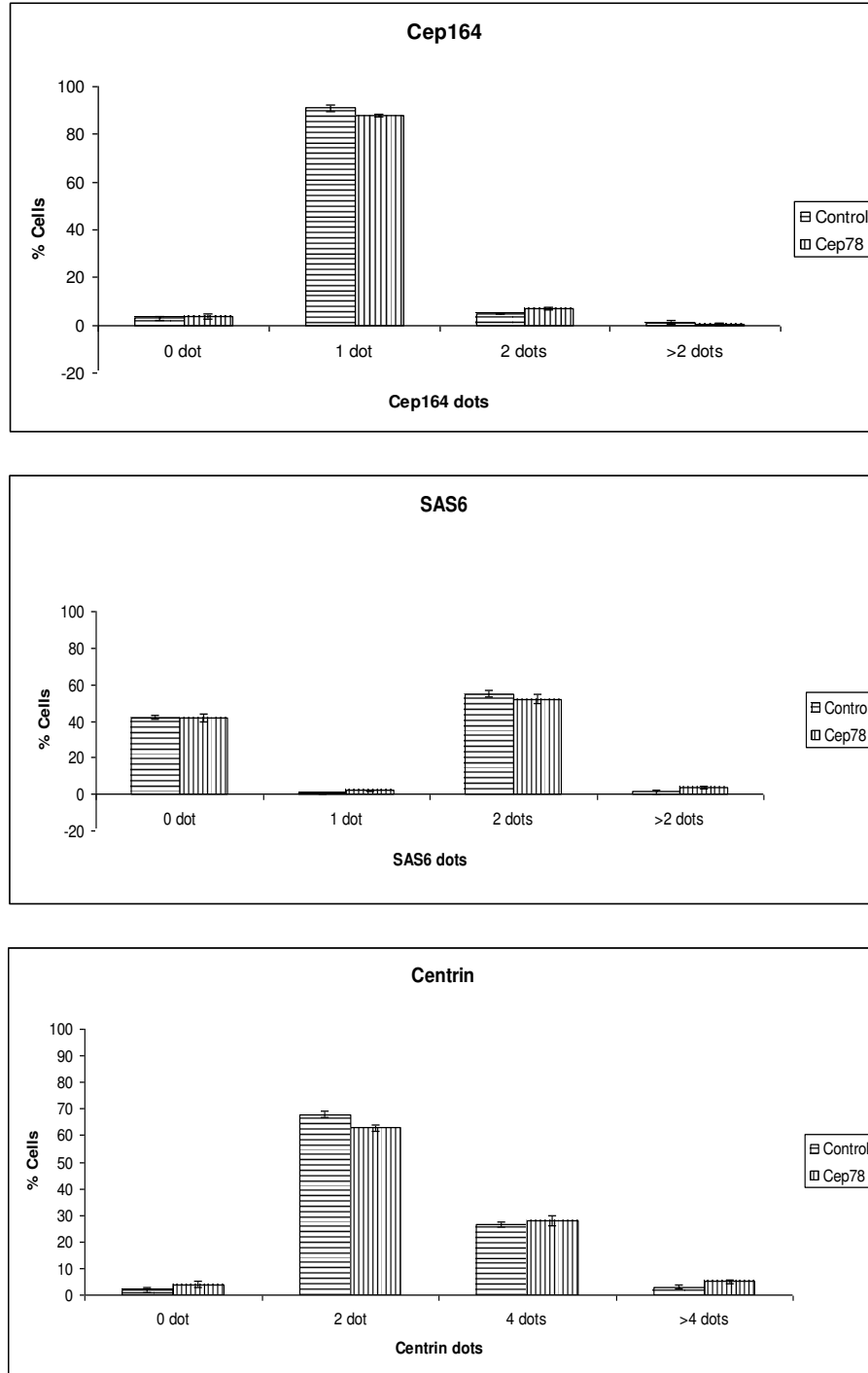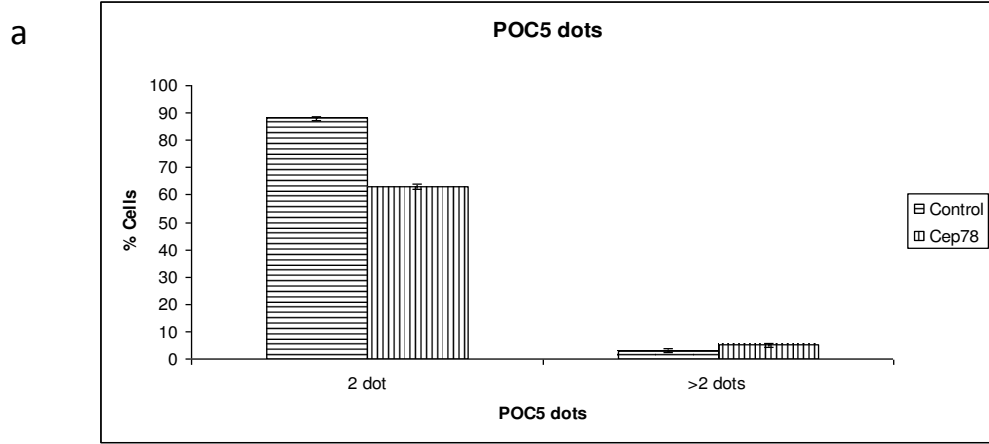
Figure 9: CEP78 overexpression does not cause centriole overduplication or accumulation. RPE cells are transfected with flag tagged full length CEP78 and CAIP (control), fixed and stained with flag, CEP164, SAS6 and Centrin.

3.6 CEP78 overexpression does not affect or bind POC5 and PLK1, proteins involved in centriole maturation.

As mentioned before, two new endogenous CEP78 dots start to appear at late G2 and also the intensity of mother and daughter dots becomes equal at this point. Since this phase coincides with centriole maturation in centrosome cycle, there is the possibility that CEP78 is involved in this process. For this purpose, the full length CEP78 was expressed in RPE cells and its effect on POC5 was studied by immunofluorescence assay and fluorescence microscopy. POC5 is a protein involved in centriole elongation and has a cell cycle pattern similar to CEP78, that is there are 2 POC5 dots during G1 and S phase and late G2, 2 new weak POC5 dots start to appear that become strong gradually. The results indicated that similar to the control, the transfected cells had mostly 2 dots and there was no significant difference in the number of POC5 dots [Figure 10a]. Also since POC5 is a distal centriolar protein, its interaction with CEP78 was studied by expressing full length flag-CEP78 and flag (control) in 293 cells and doing a flag immunoprecipitation to pull down CEP78 protein and its interacting proteins.This was followed by Western blotting of POC5. There was no interaction between the 2 proteins [Figure 10b]. The interaction between CEP78 and PLK1, another protein involved in centriole maturation, was studied with the same method discussed for POC5 as well. No interaction was observed

between CEP78 and PLK1 either [Figure 10b]. Therefore, CEP78 does not interact with POC5 or PLK1, proteins involved in centriole maturation and its overexpression does not have an effect on the number of POC5 dots. It would be interesting in the future to study the effect of overexpressing POC5 on the number of CEP78 dots and also the effect of CEP78 overexpression on centriole elongation.

Figure 10: a) CEP78 overexpression does not affect POC5 dots. RPE cells are transfected with flag tagged full length CEP78 and CAIP (control), fixed and stained with flag and POC5. b) CEP78 does not interact with POC5 or PLK1. 293 cells are transfected with flag tagged full length CEP78 and Flag (control), immunoprecipitated for flag and western blot was carried out for POC5. IN represents input and IP represents immunoprecipitation.
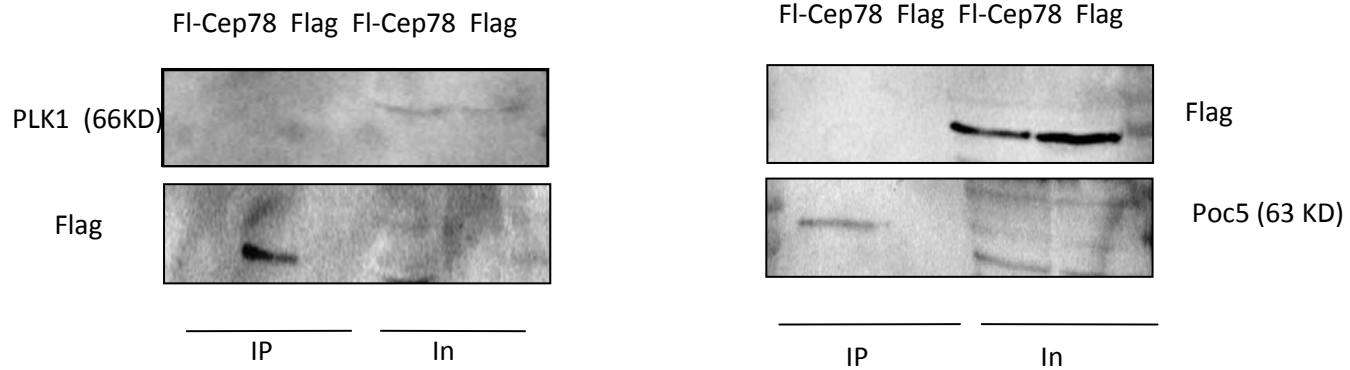
3.7 CEP78 overexpression does not have a significant effect on other centrosomal proteins.

In order to study the effect of CEP78 overexpression on other centrosomal proteins and the PCM integrity, the full length protein was expressed in RPE cells and the cells were stained for different centrosomal markers including γ-tubulin and Pericentrin by immunofluorescence assay.   No significant difference was observed in the number of any of the above centrosomal proteins. These results indicate that CEP78 overexpression does no affect PCM integrity and other centrosomal components [Figure 11].
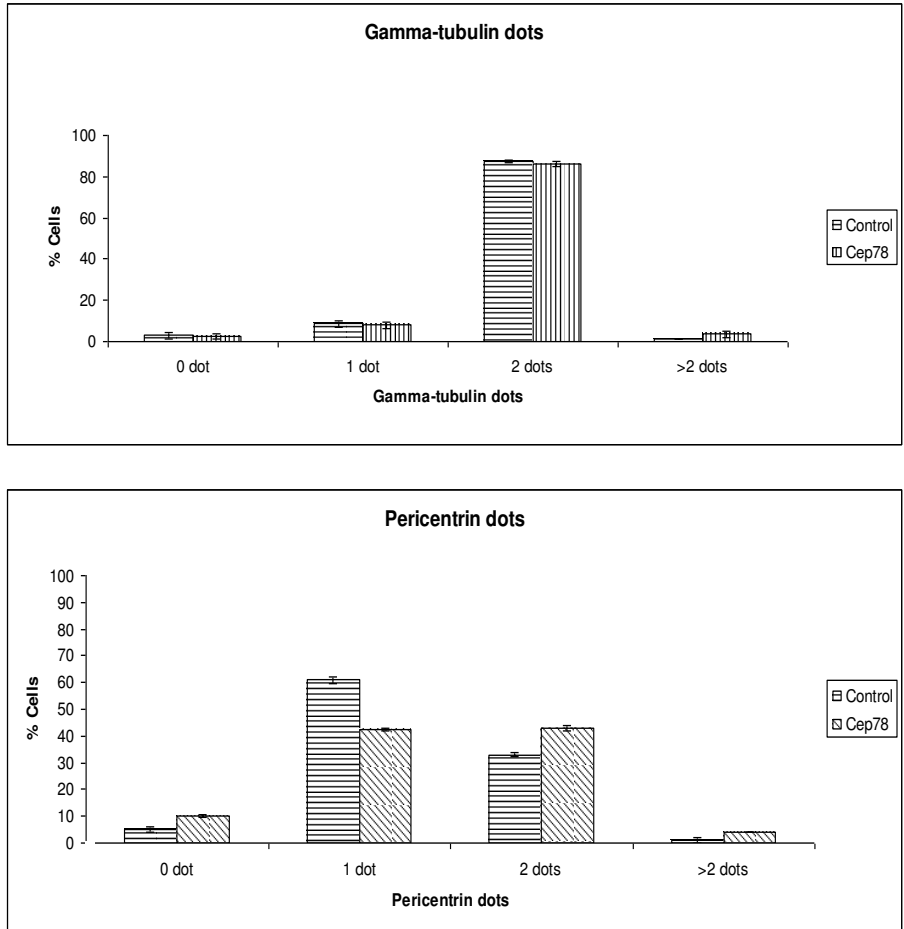
Figure 11: CEP78 overexpression does not affect Gamma-tubulin and Pericentrin dots. RPE cells are transfected with flag tagged full length CEP78 and CAIP (control), fixed and stained with flag and Gamma-tubulin amd Pericentrin.

3.8 CEP78 overexpression reduces the number and intensity of CEP170, a sub-distal appendage protein.

The only protein that showed a difference after CEP78 overexpression was CEP170 which is a sub-distal appendage marker. Following the overexpression of the full length CEP78 in RPE cells, an immunofluorescence assay was carried out and the cells were stained for CEP170. Comparing control and transfected cells showed that the number of the transfected cells not having CEP170 dots increased. In fact 22% of the transfected cells did not have CEP170 dot compared to 4% in control [Figure 12a]. Also, the intensity of CEP170 dots decreased significantly in the transfected cells. Measuring the intensity of CEP170 dots by fluorescence microscopy and the software Velocity, indicated a decrease of about 3.7 times in the transfected cells. A similar procedure had already been used to compare the intensity of CEP78 dots on mother and daughter centrioles. Next, the expression level of CEP170 was checked in transfected cells. For this purpose, the full length CEP78 was expressed in 293 cells and a western blot was carried out. The comparison between the control sample expressing Flag and CEP78 overexpressing samples did not show a decrease in the expression level of CEP170 [Figure 12b]. Finally, the interaction between CEP78 and CEP170 was studied by expressing the full length CEP78 in 293 cells, doing flag immunoprecipitation and western blotting for CEP170. No

interaction between the 2 proteins was observed [Figure 10b]. Overexpressing GFP-CEP170 in 293 cells, immunoprecipitation and western blotting for CEP78 did not show an interaction either [Figure 12c]. Since CEP170 is a sub-distal appendage protein, it was necessary to check the effect of CEP78 overexpression on other sub-distal appendage proteins. One of these proteins is Ninein. Once again, the full length CEP78 was expressed in RPE cells and an immunofluorescence assay was carried out to stain them for Ninein. The results indicated that unlike CEP170, the number of Ninein dots did not decrease and their intensity did not change either [Figure 13A]. Also the possible interaction between CEP78 and Ninein was studied by the same method used for CEP170. No interaction was observed between the two proteins [Figure 13b]. So the results indicate that CEP78 overexpression decreases both the number and intensity of CEP170 dots but does not decrease its expression level. This implies that CEP78 does not regulate the expression level of CEP170 but it might affect the recruitment of CEP170 to centrosome. This result was not confirmed for Ninein, another sub-distal appendage protein. Also, there was no interaction between CEP78 and CEP170 or Ninein.

Figure 12: CEP78 overexpression a) decreases the number of CEP170 dots. RPE cells are transfected with flag tagged full length CEP78 and CAIP (control), fixed and stained with flag and CEP170. b) does not decrease CEP170 expression level. RPE cells are transfected with flag tagged full length CEP78 and flag (control) and western blots were carried out for CEP170. c) CEP170 does not interact with CEP78. 293 cells are transfected with GFP-tagged CEP170 and GFP (control), immunoprecipitated for GFP and western blots were carried out for CEP78. IN represents input and IP represents immunoprecipitation.
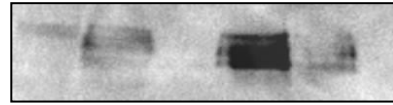
a
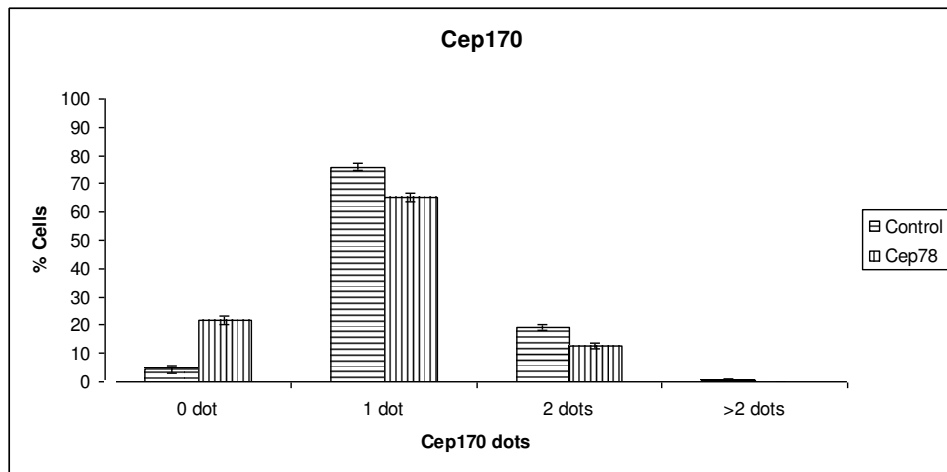
b
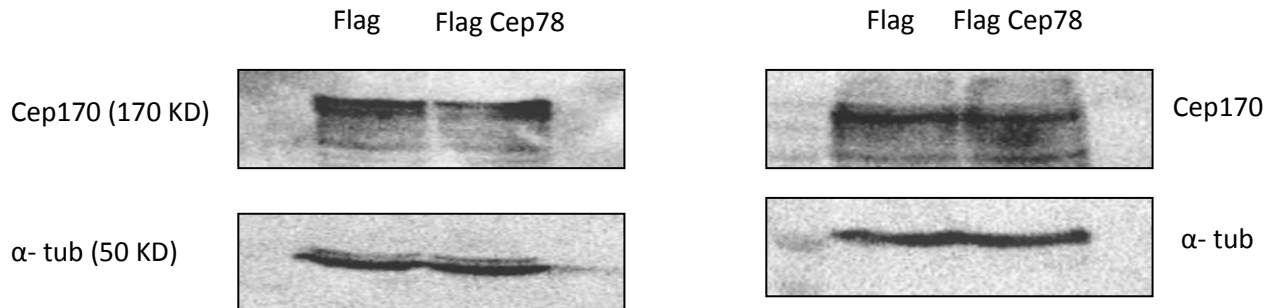
C

GFP GFPCep170   GFP GFPCep170

Cep78 (78 KD)

Cep170 (170 KD)
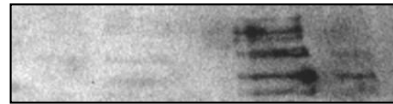
GFP

In   IP

Figure 13: a) CEP78 overexpression does not decrease the number of ninein dots. RPE cells are transfected with flag tagged full length CEP78 and CAIP (control), fixed and stained with flag and Ninein. b) CEP78 does not interact with CEP170, Ninein and α-tubulin.293 cells are transfected with flag- tagged CEP78 and flag (control), immunoprecipitated for flag and western blots were carried out for CEP170, Ninein and α-tubulin. IN represents input and IP represents immunoprecipitation.

a
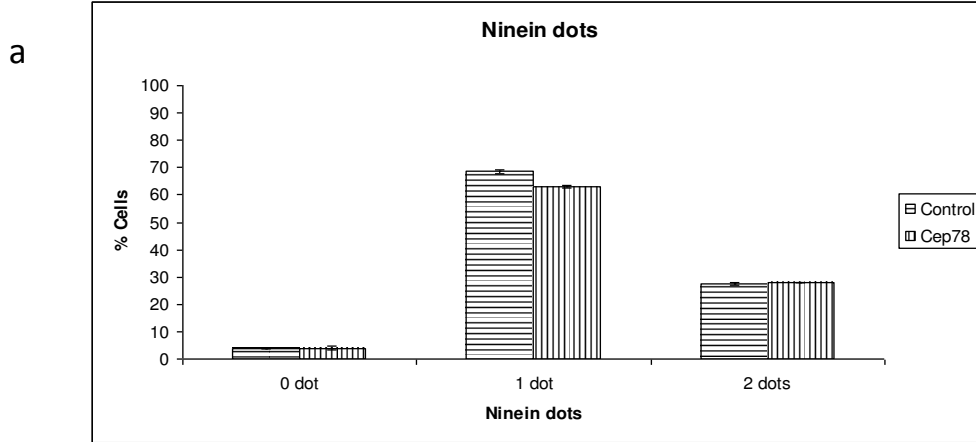
b
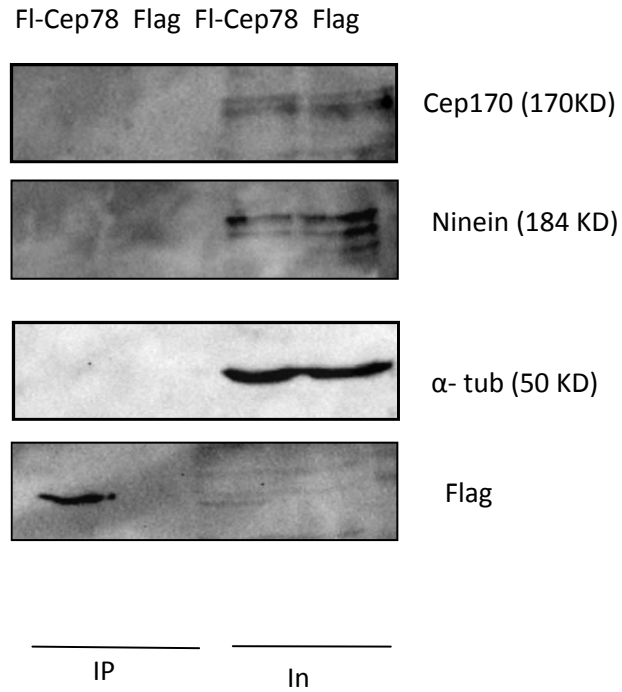
3.9 CEP78 overexpression stabilizes microtubules after nocodazole treatment.

Since RPE cells overexpressing full length CEP78 and some CEP78 fragments showed microtubule binding pattern, a microtubule assay was carried out to see the effect of nocodazole on transfected cells. For this assay, cells were treated with nocodazole and after the removal of the nocodazole from the wells and replacing the media, they were incubated at 37°C for 2, 5 and 20 minutes. The purpose of this experiment was to study the effect of CEP78 overexpression on microtubule stability and repolymerization following nocodazole treatment. The cells that were not transfected and also the cells transfected for full length NPHP5 were used as a control for this experiment. The reason NPHP5 was used as control is that its overexpression does not have any effect on microtubule stability. Finally, the cells were stained for α-tubulin by immunofluorescence assay and studied by fluorescent microscopy. Before the treatment, both the controls and CEP78 overexpressing cells had a net α-tubulin pattern due to intact microtubules. The results showed that following nocodazole removal, the controls had depolymerized microtubules and their net pattern was not observed anymore whereas 86% of the cells overexpressing full length CEP78, still had their microtubules. This could be because CEP78 binds to microtubules and prevents their depolymerization. After 2 and 5

minute incubation at 37°C, some filaments started to appear in all samples but these filaments were significantly more in CEP78 overexpressing cells. Finally, after 20 minutes, asters started to form in controls whereas in cells overexpressing full length CEP78, these asters were not as clear. This could be because the majority of microtubules did not depolymerize after the treatment [Figure 14]. In order to study the possible interaction between CEP78 and alpha-tublin, full length CEP78 was expressed in 293 cells, a flag immunoprecipitation was carried out and the membrane was stained for alpha-tubulin. No interaction was observed (Figure 13b). Next, to make sure the observed pattern is not exclusive to RPE cells, nocodazole treatment was also carried out on ARPE cells expressing full length CEP78. ARPE cells are derived from RPE cells and contain a spontaneous mutation. The results indicated that the microtubule binding pattern was observed in 74% of the transfected ARPE cells as well. Next, CEP78 fragments were studied after nocodazole treatment. The fragments that already showed microtubule binding pattern (1-445, 221-445, 221-722, Δ450-497), were able to stabilize microtubules after nocodazole treatment as well [Figure 15]. This could help identify the region of CEP78 protein involved in microtubule binding and stabilization.

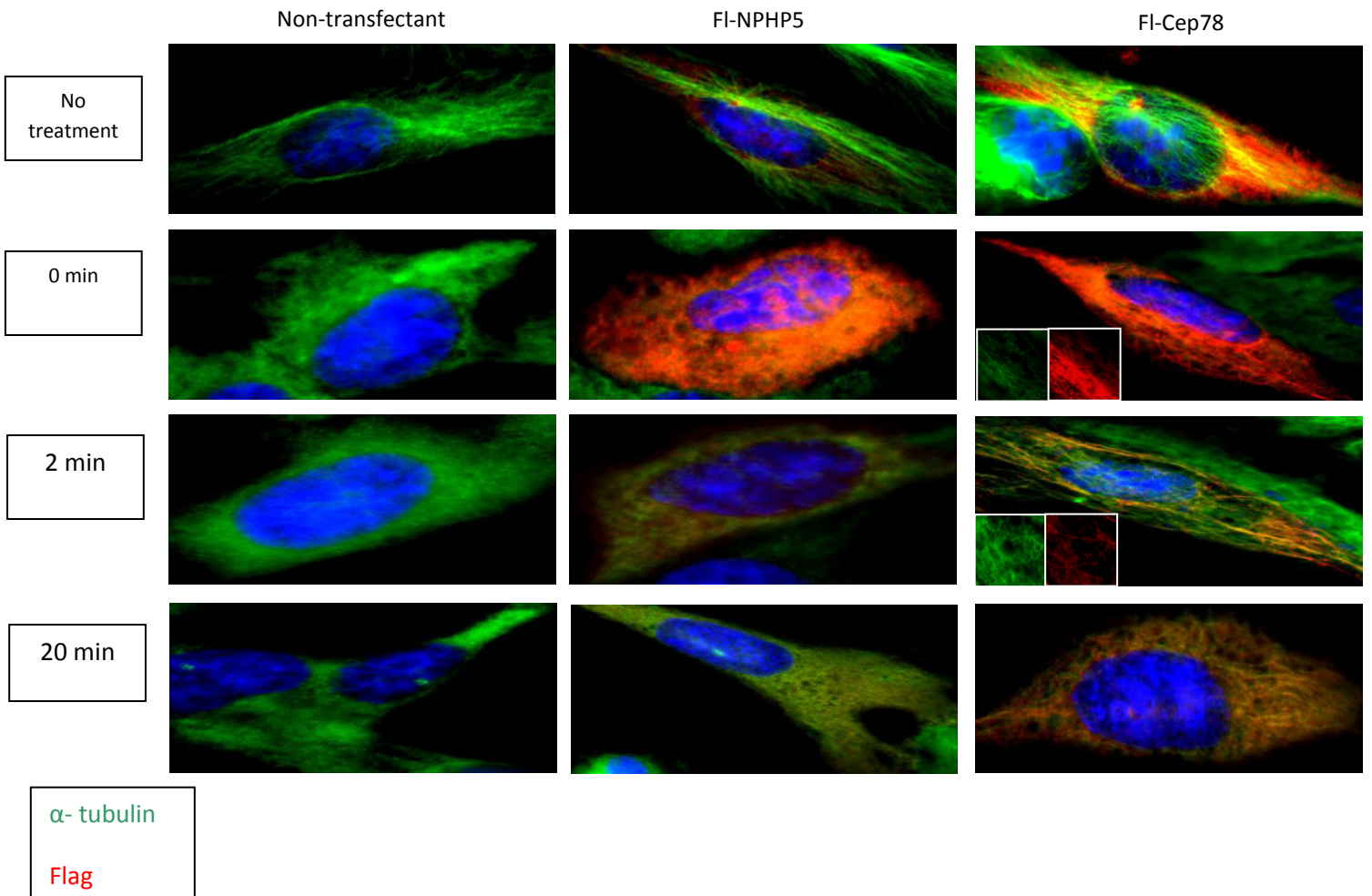|	Non-transfectant	Fl-NPHP5	Fl-Cep78
No treatment			
0 min			
2 min			
20 min			

α- tubulin

Flag

Figure 14: CEP78 overexpression stabilizes microtubules after nocodazole treatment in RPE cells. RPE cells are transfected with full length CEP78 and full length NPHP5 (control), treated with nocodazole for 1 hour, incubated back at 37°C for 0, 2 and 20 minutes, fixed and stained with α-tubulin and flag.
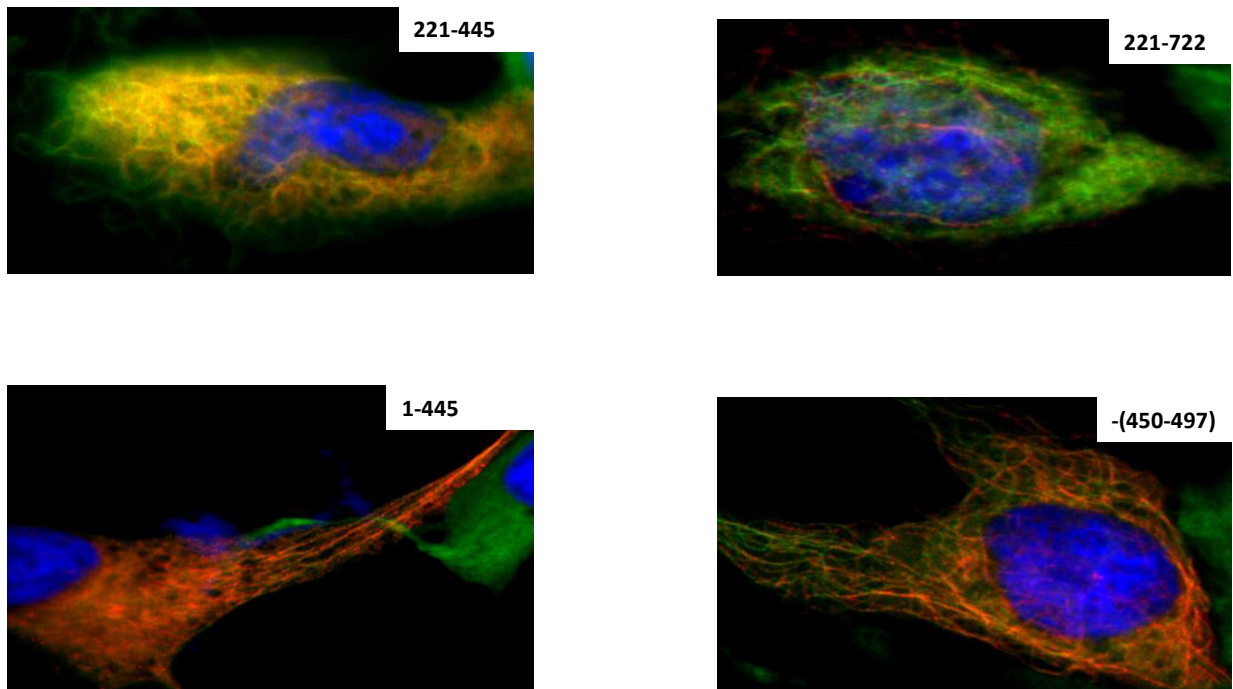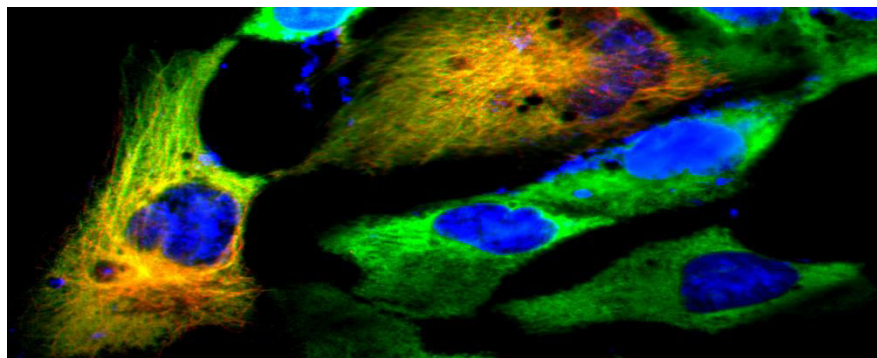
a

α- tubulin

Flag

b

α- tubulin

Flag

Figure 15: a) Some CEP78 fragments stabilize microtubules after nocodazole treatment in RPE cells. RPE cells are transfected with CEP78 fragments (221-445, 221-722, 1-445 and Δ (450-497)), treated with nocodazole for 1 hour, fixed and stained with α-tubulin and flag b) ARPE cells overexpressing CEP78 also keep their microtubules after nocodazole treatment. ARPE cells are transfected with full length CEP78, treated with nocodazole for 1 hour, fixed and stained with α-tubulin and flag

Chapter 4 Discussion

The cell cycle is an important cellular process modulated and regulated by different molecules, pathways and organelles and its misregulation could result in several diseases. One cellular organelle crucial in the cell cycle regulation is the centrosome which consists of two centrioles embedded in an amorphous proteinacious material called pericentriolar material (PCM). Around 500 centrosomal proteins have been identified so far and any abberations in these proteins can cause defect in the number or structure of centrosome and lead to several diseases including cancer, ciliopathies and brain disorder syndromes. Therefore, it is vital to study the function of novel centrosomal proteins for potential therapeutic applications.

The objective of this research was to study the localization and function of CEP78 as a novel centrosomal protein. CEP78 was first identified by Anderson et al. in 2003 (Andersen, Wilkinson et al. 2003). They isolated centrosome from the cells in the interphase and did a mass-spectrometry analysis to identify any new centrosomal proteins. Following this analysis, they studied the discovered proteins by correlation with already known centrosomal proteins and also their *in vivo* localization. They identified 23 new centrosomal components including CEP78. This

78kDa protein has several isoforms with the longest one consisting of 722 amino acids. It also has orthologs in mouse, chicken, lizard, tropical clawed frog, zebrafish and fruit fly. The human *cep78* gene is located on chromosome 9.

As it was mentioned in the previous paragraph, CEP78 had already been discovered as a centrosomal protein but further studies were necessary to confirm whether it is a permanent component of centrosomes and also where in centrosome it localizes. Depolymerization of RPE microtubules by nocodazole indicated that CEP78 is a stable centrosomal protein that does not require microtubules for its centrosomal localization. Also, colocalization studies between CEP78 and other centrosomal proteins showed that this protein does not colocalize with the proximal centriolar proteins such as C-Nap1 or Polyglutamylated tubulin. On the other hand, the proteins localizing to the distal end of centrioles such as CEP170, Centrin and POC5 showed a close colocalization with CEP78. These results suggest that CEP78 is a permanent component of the distal end of centrioles. For further studies, electron microscopy can be carried out to determine whether CEP78 is a distal or sub-distal protein.

Centrosomal proteins show different patterns during the cell cycle based on their functions and studying this pattern could provide

us with more information regarding the function of a new protein. Thus CEP78 was studied during different stages of the cell cycle by colocalization with the centriolar marker, Centrin, and using DAPI to identify the nuclei cycle. The results indicated that there are two CEP78 dots on the mother and daughter centrioles in G1, S and early G2 phases of interphase, with the intensity of the one on the mother 1.9 times stronger than the one on the daughter. Late G2, the intensity of the two existing dots becomes equal and two new weak CEP78 start to appear. The new dots get stronger as the cell goes through mitosis and by the end of the telophase each daughter cell has two CEP78 dots. The colocalization studies with Centrin showed that CEP78 only localizes to the mature centrioles, mother and daughter, and not the procentrioles. Since late G2 coincides with centriole maturation phase of centrosome cycle during which the daughter centriole acquires appendages to become mother and also procentrioles elongate, the increase in the intensity of the daughter centriole and appearance of the two new CEP78 dots suggest the possible role of CEP78 in centriole maturation process. To further study this hypothesis, more experiments were planned and carried out. First, the effect of the overexpression of CEP78 on another protein involved in centriole maturation, POC5, was studied. Next, the possible interaction between CEP78 and the two proteins POC5 and PLK1 was studied. It has been shown that POC5 gets recruited to centrosome during G2/M and is involved in centriole elongation (Azimzadeh, Hergert et al. 2009). This protein has a similar cell cycle pattern to CEP78.

So we first studied the effect of CEP78 overexpression on the number of POC5 dots. Our results showed that the overexpression had no significant effect on the number of POC5 dots and most transfected cells showed similar results to the control. One possible explanation could be that CEP78 is downstream to POC5 hence does not affect POC5. Therefore, it would be a good idea to study the effect of POC5 overexpression/depletion on the number of CEP78 dots as well. Moreover, in the research on POC5, the distance between the distal ends of the mother and daughter centrioles was measured to study the effect of POC5 depletion on centriole elongation by high resolution microscopy. It would be interesting to measure this distance following CEP78 overexpression/knockdown as well. POC5 is a distal centriolar protein which colocalizes quite well with CEP78. So for the next step, the interaction between CEP78 and POC5 was studied in 293 cells using immunoprecipitation. Despite what we expected, no interaction was observed between the two proteins. There are other proteins involved in centriole maturation as well including PLK1. This protein plays a role in centriole maturation by recruitment of Aurora A to the centrosome (Korzeniewski, Hohenfellner et al. 2013). This persuaded us to study the possible interaction between this protein and CEP78 in 293 cells as well. However, our studies did not show an interaction between CEP78 and PLK1. So far, our results do not show a role for CEP78 in centriole maturation but more experiments need to be done to

confirm this result. For instance, it would be interesting to study the effect of PLK1 inhibition on the number of CEP78 dots.

Structurally, CEP78 consists of 4-6 Leucin rich repeats (LRRs), amino acids 147-308, and one coiled coil domain, amino acids 450-497. In order to study the importance and function of each of these domains, fragments with deletions in one or some of these domains were expressed in RPE cells and their expression patterns were more closely studied. Our results indicated that the LRRs are essential for CEP78 localization to centrosome and any deletions in them would prevent the protein from its centrosomal localization. This could be due to an unidentified protein that binds to the LRRs of Cep78 and brings Cep78 to the centrosome. In order to identify this protein a mass-spectrometry analysis can be done to study the possible proteins interacting with CEP78. As for the coiled-coil domain, since these domains are usually involved in the regulation of gene expression, CEP78 coiled-coil domain might play a similar role in its expression level as well.

Overexpression of full length CEP78 and the CEP78 fragments containing the three middle LRRs in RPE cells showed a microtubule binding pattern. To further study this phenotype and the effect of CEP78 overexpression on microtubule stability and nucleation, both RPE and ARPE cells were transfected with full

length CEP78 and then treated with Nocodazole. Contrary to what we expected, the transfected cells maintained their microtubule network even after the depolymerizing treatment. One explanation for this phenotype could be that CEP78 when overexpressed, covers microtubules and does not allow nocodazole to depolymerize them. Also, carrying out this experiment for CEP78 fragments showed that the fragments containing the three middle LRRs, amino acids 221-445, had the ability to stabilize microtubules as well. It seems that the region responsible for this phenotype is somewhere in the three middle LRRs but still more fragments are necessary to identify the exact region involved in this phenotype. The next question to answer was whether CEP78 stabilized microtubules by binding α-tubulin directly. For this purpose, full length CEP78 was expressed in 293 cells and immunoprecipitation assay was carried out. Western for α-tubulin did not show an interaction between CEP78 and α-tubulin. However, it is possible that CEP78 interacts with microtubules that are polymers of tubulins instead and therefore further studies are necessary to confirm the microtubule binding activity of CEP78 by an in vitro microtubule binding assay.

In a meeting held on "Building a Centrosome" in 2013, it was suggested that CEP78 interacts with PLK4, CP110 and CEP97 (Baffet, Martin et al. 2013). These proteins are involved in centriole duplication and cooperate to regulate this process. In

order to study the possible role of CEP78 in centriole duplication or accumulation, several centriolar proteins including CEP164 (mother centriole marker), SA6 (procentriole marker) and Centrin (mother, daughter and procentriole marker) were studied after overexpressing CEP78 in RPE cells. Our results indicated no increase/ decrease in the number of centrioles and the number of mother, daughter or procentrioles did not change. This suggests that CEP78 overexpression does not result in centriole overduplication or accumulation. Also, there is no significant change in the number or intensity of CEP78 dots at S phase during which the centriole duplication occurs.

Studying the effect of CEP78 overexpression on various centrosomal components in RPE cells showed that the only affected protein was CEP170 which is a sub-distal appendage protein. This protein localizes only to the mother centriole and its overexpression shows a microtubule binding pattern similar to CEP78 (Guarguaglini, Duncan et al. 2005). The results of our overexpression studies indicated that the RPE cells expressing full length CEP78 did not have CEP170 or the intensity of this protein was too weak in them. Despite the observed phenotype, the expression level of CEP170 did not decrease in 293 cells overexpressing full length CEP78. This could mean that CEP78 does not regulate the expression of CEP170 but instead might play a role in its recruitment to the centrosome. This could be due

to cell line-specific differences as well so it would be a good idea to study the effect of CEP78 overexpression on the number and expression level of CEP170 in other cell lines. We were also curious to see if this phenotype is caused by the direct interaction of CEP78 and CEP170. For this purpose, the interaction between CEP78 and CEP170 was studied by co-immunoprecipitation and contrary to what we expected no interaction was observed between the two proteins. Next, we looked at the effect of CEP78 overexpression on another sub-distal appendage marker, Ninein. Unlike CEP170, there was no decrease in the number of Ninein dots in RPE cells overexpressing full length CEP78. Furthermore, there was no interaction between CEP78 and Ninein in 293 cells overexpressing full length CEP78. For future direction, it would be interesting to study more sub-distal appendage proteins as well as the effect of their overexpression on the number of CEP78 dots and determine whether Cep78 plays a role in the formation/maintenance of sub-distal appendages, a hallmark of centrosome maturation.

As it was mentioned before, it is important to study the function of novel centrosomal proteins in order to use this information for possible therapeutic applications in the future. As for the clinical studies on CEP78, there have been very few papers published so far. In 2007, a study was carried out by Nesslinger et al. on the effect of standard treatments, hormone and radiation therapy, on

immune responses in prostate cancer patients (Nesslinger, Sahota et al. 2007). They used SEREX immunoscreening of a prostate cancer cDNA expression library and discovered several treatment associated autoanitigens including CEP78. In their SEREX antigen array analysis, CEP78 was negative in the pretreatment sample but was seroreactive following hormone therapy. This could imply the possible importance of CEP78 in prostate cancer. All our studies were carried in RPE, ARPE and 293 cells. For future direction, it would be a good idea to use a prostate cancer cell line as well to study CEP78 overexpression pattern and its possible phenotype. Also, comparing the mRNA/protein levels of Cep78 in a prostate cell line and other cell lines would be interesting. Finally, it is a good idea to study the effect of CEP78 knockdown in a prostate cell line and see if these cells stop growing.

Finally, there are two studies suggesting a possible role for CEP78 in ciliogenesis. In a study on the components of the human centrosome for which homologs are still present in planarians, Azimzadeh et al. observed a locomotion defect in planarians knocked down for CEP78 (Azimzadeh, Wong et al. 2012). Since planarians lack centrosomes but still own cilia, it is suggested that CEP78 is rather involved in ciliogenesis. In another study, it was observed that CEP78 expression is upregulated 5 fold by noise stress in rat cochleae (Han, Hong et al. 2012). A possible explanation could be the effect it has on the cochleae cilia. Although our preliminary experiments did not show any change in the number of cilia following CEP78 overexpression, further

studies including knocking down CEP78 are still necessary to confirm these results.

In order to confirm the results of the overexpression studies including the observed decrease in CEP170, the knockdown of CEP78 was required. For this, several siRNA oligos, transfection reagents and incubation times were used but none was efficient. Previously, Azimzadeh et al. used a double knockdown procedure to knock down CEP78 (Azimzadeh, Wong et al. 2012). Although we used the same oligos and procedure, the knockdown was not as good as we expected. For future direction, it would be a good option to use shRNA for CEP78 knockdown. The advantage of using shRNA is that its effect can be more specific and last longer than siRNA.

In summary, I found that CEP78 decreases the number and intensity of CEP170 and it helps stabilize the microtubule network in RPE cells treated with nocodazole. My findings contribute to our understanding of the role of centrosome in the cell cycle and cytoskeleton.

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