Chemically Induced Assay for Centriolar Satellite Mispositioning Reveals Their Functions at The Primary Cilium

by

Şevket Onur Taflan

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Şevket Onur Taflan

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examining committee have been made.

Committee Members:

Asst. Prof. Elif Nur Firat-Karalar (Advisor, Koç University)

Prof. Dr. Halil Kavaklı (Koç University)

Assoc. Prof. Halil Bayraktar (Istanbul Technical University)

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ABSTRACT

Centriolar satellites are membrane-less, electron dense granular structures that localize and move around centrosomes and cilia. The satellite proteome is composed of over 200 protein components, which were implicated in a wide range of functions such as centriole duplication, cell division, cellular signaling, primary cilium biogenesis and microtubule dynamics. Importantly, various proteins mutated in ciliopathies and primary microcephaly were also identified as part of the satellite proteome, suggesting an intimate link between centriolar satellite function and development. Although centriolar satellites have remained as understudied structures since their discovery more than 60 years ago, recent work showed that satellites store, modify and traffic centrosome/cilium proteins and play important roles in the regulation of centrosome/cilium biogenesis and function. To mediate their trafficking function, satellite cluster and move around centrosomes in most cell types. However, satellite distribution varies in response to different stimuli such as cell cycle cues and across different cell types, suggesting context-dependent functions for satellites. Dissecting these spatial and temporal functions have been challenging using traditional approaches such as loss-of-function studies, in particular, in ciliated cells. To overcome these challenges, I developed a chemical based inducible trafficking assay that allows efficient redistribution of centriolar satellites to cell periphery or cell center. Using this assay, I showed that satellite mispositioning disrupts centrosomal targeting of key regulators of cilium biogenesis, identifying a direct role for satellites in centrosomal protein targeting and sequestration. To identify the functional consequences of satellite mispositioning, I used functional assays to probe cilium biogenesis and microtubule dynamics in cells where satellites were mispositioned at the membrane. The results of these assays showed that satellites regulate primary cilium assembly, maintenance and disassembly. Taken together, our results showed a direct link between satellite functions and their pericentrosomal clustering in ciliated cells and also provided a new tool for studying acute functions of satellites in a context-dependent way. Finally, given the crucial roles of the primary cilium as the signaling center for developmentally important signaling pathways such as Hedgehog signaling, our results sheds light into why satellite proteins are mutated in developmental disorders.

ÖZET

Sentriyolar uydular, sentrozomlar ve silyumlar etrafında lokalize olan, hareket eden zarsız, elektron yoğun granüler yapılardır. Sentriyolar uyduların proteomu, sentrozom duplikasyonu, hücre bölünmesi, hücresel sinyalleri, birincil siliyum biyojenez ve mikrotübül dinamikleri gibi geniş bir işlev yelpazesinde yer alan 200'den fazla protein bileşeninden oluşur. Siliyopatilerde ve birincil mikrosefali hastalıklarında sentrozomal uydu yapılarının mutasyona uğramış çeşitli proteinleri tanımlanmış ve gelişme bozuklukları ile sentrozomal uydu proteinleri arasında yakın bir bağlantı olduğunu gösterilmiştir. Sentrozomal uydular, 60 yıldan uzun bir süre önce keşfedilmelerinden bu yana yeterince çalışılmamış yapılar olarak kalsa da, son çalışmalar, uyduların sentrozom / silium proteinlerini depoladığını, değiştirdiğini, taşınmasında görev aldığını ve sentrozom / siliyum biyogenezinin ile işlevinin düzenlenmesinde önemli roller oynadığı gösterilmiştir. Proteinlerin taşınması görevini üstlenmesi için sentrozomal uydu yapıları, sentrozom etrafında kümelenir ve hareket halinde bulunur. Bununla birlikte, uydu yapılarının hücre içinde dağılımı, hücre döngüsü ile ilgili moleküller gibi farklı uyaranlara yanıt olarak ve farklı hücre türleri arasında değişiklik gösterir. Bu da uyduların bulundukları bağlama göre farklı görevler üstlenebildiğini göstermektedir. Bu mekansal ve zamansal işlevleri incelemek, özellikle siliyumlu hücrelerde proteinin silinmesi veya susturulması gibi geleneksel yaklaşımları kullanarak zorlayıcı olmuştur. Bu zorlukların üstesinden gelmek için, sentriyolar uyduların hücre çevresine veya hücre merkezine verimli bir şekilde yeniden dağıtımına izin veren, kimyasal bazlı uyarılabilir bir protein taşınma yöntemi geliştirdim. Bu yöntemle, uydu yapılarının yanlış konumlandırması sağlanarak, sentrozomal protein hedeflemesi ve sekestrasyonunda uydu yapılarının rolünü belirlemekle birlikte, siliyum biyogenezinin anahtar düzenleyicilerinin uydu yapıları olduğunu gösterdim. Yanlış uydu konumlandırmasının işlevsel sonuçlarını belirlemek için, uyduların hücre zarına yanlış konumlandırıldığı hücrelerde, siliyum biyogenezini ve mikrotübül dinamiklerini araştırmak için fonksiyonel analizler kullandım. Bu analizlerin sonuçları, uyduların birincil siliyum oluşumu, korunması ve parçalanmasını düzenlediği gösterilmiştir. Birlikte ele alındığında, sonuçlarımız uydu fonksiyonları ile bunların siliye eden hücrelerde pericentrozomal kümelenmeleri arasında doğrudan bir bağlantı olduğunu göstermiş ve ayrıca uyduların akut fonksiyonlarını içeriğe bağlı bir şekilde incelemek için yeni bir araç sağlamıştır. Son olarak, Hedgehog sinyalizasyonu gibi gelişimsel açıdan önemli sinyal yollarının sinyalleme merkezi olarak birincil siliyerin hayati rolleri göz önüne alındığında,

sonuçlarımız, uydu proteinlerinin gelişimsel bozukluklarda neden mutasyona uğradığına ışık tutuyor.

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NOMENCLATURE

AMP	Adenylyl-Mono-Phosphate
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
AZI1	5-azacytidine-induced protein 1
BBS	Bardet Biedl Syndrome
BBS4	Bardet Biedl Syndrome 4
BBSome	Bardet Biedl Complex
BICD2	Bicaudal D Homolog 2
BioID	Biotin Identification
BirA	Bifunctional Ligase
BSA	Bovine Serum Albumin
CC	Coiled-coil domain
CCDC14	Coiled-coil domain containing protein 14
CCDC66	Coiled-coil domain containing protein 14
CDK5RAP2	CDK5 regulatory subunit-associated protein 2
CEP72	Centrosomal protein of 72 kDa
CEP131	Centrosomal protein of 131 kDa
CEP152	Centrosomal protein of 152 kDa
CEP164	Centrosomal protein of 162 kDa
CEP290	Centrosomal protein of 290 kDa
DAPI	4’6’-diamino-2phenylindole
DMEM	Dulbecco’s Modified Eagle Medium
DMEMF12	DMEM/Nutrient Mixture 12
DMSO	Dimethyl Sulfoxide

DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
FKBP	FK506 Binding Protein 12
FRB	FKBP12-Rapamycin Binding
G0	Gap0
G1	Gap1
G2	Gap2
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
h	Hours
HeLa	Henrietta Lack
IFT	Intraflagellar Transport
Kif5b	Active kinesin-1 motor domain
Kif17	Kinesin-like protein 17
MIB1	Mind bomb homolog-1
MS	Mass Spectroscopy
MTOC	Microtubule Organizing Center
PBS	Phosphate Buffer Saline
PCD	Primary Ciliary Dyskinesia
PCM	Pericentriolar Material
PCM1	Pericentriolar Material 1
PFA	Paraformaldehyde
PLK1	Polo-like kinase-1
RPE1	Retinal Pigment Epithelium 1

SEM	Standard Error of the Mean
siRNA	Small Interference RNA

CHAPTER 1: Introduction

The eukaryotic cytoskeleton is composed of microtubules, actin filaments and intermediate filaments, which play crucial roles in a wide range of cellular processes such as determining the cell shape, organelle positioning, cell migration, protein trafficking and cell signaling. Among the three components of cytoskeleton, microtubules stand out by their functions in protein trafficking and cell division and as the structural components of the centrosomes and the cilia (Goodson H.V and Jonasson E.M., 2018).

Centrosome is the main microtubule-organizing center of animal cells. It is composed of two centrioles and pericentriolar material (PCM) around the centrioles. Centrioles are microtubule-based, evolutionarily conserved structures with a defined size and shape (Piel and Bornens, 2000). Centrioles are cylindrical structures composed of nine-triplet microtubules organized in rotational symmetry (Greenan et al., 2018). The two centrioles of the centrosome are perpendicular to each other where their proximal ends embedded in pericentriolar material. Pericentriolar material (PCM) is the protein matrix around the centrosome that assigns the centrosomes its microtubule-organizing center functions through recruitment of microtubule nucleation and organization such as γ-tubulin, ninein and pericentrin (Muroyama and Lechler, 2017). While PCM have long been considered as an unorganized matrix of proteins, application of super resolution microscopy to the PCM showed that it is composed of ordered layers of protein and complex gel-like scaffolds (Fry et al., 2017). Importantly, the organization and composition of the PCM changes in response to extracellular stimuli such as cell cycle cues, raising questions on mechanisms that mediate their dynamic remodeling.

In non-dividing cells, centrioles migrate to the cell surface and nucleate the formation of cilia, which are microtubule-based cellular extensions surrounded by membranes. Cilia are broadly categorized into two groups: 1) primary cilium, 2) motile cilia and flagella. Primary cilium is the immotile cilium that is present in almost all cells in human body and functions as the signaling center. It receives and transmits developmentally important signaling pathways such as Hedgehog and Wnt signaling (Huang and Schier, 2009). Therefore, structural and functional defects of the primary cilium cause ciliopathies, multisystemic developmental disorders characterized by retinal degeneration, polycystic kidney disease, polydactyly, obesity among others (Waters and Beales 2011). Motile cilia are found in the specialized epithelium lining the trachea, fallopian tube and brain ventricles and functions in movement of extracellular fluid and

particles as a result of their synchronous beating (Croxatto and Ortiz 1975; Ganesan et al. 2013; Brooks and Wallingford 2014). The flagellum of sperm cells generate propeller-like circular motility to propel them into the female reproductive tract. Given their motility-related funcctions, defects in the structure and function of motile cilia and flagella causes various human diseases including motile ciliopathies such as Primary Ciliary Dyskinesia (PCD) and infertility (Brooks and Wallingford 2014). To gain insight into the pathogenesis of sensory and motile ciliopathies and to develop new diagnostics and therapeutics, it is essential first to unravel the mechanisms underlying the assembly and functions of the primary and motile cilia.

In addition to centrosomes and cilia, vertebrate cells have centriolar satellites that localize and move around centrosomes and cilia and is regarded as the third component of the vertebrate centrosome/cilium complex. Although centriolar satellites were described by electron microscopy more than 60 year ago, they have remained as poorly characterized structures until the identification of the link between centriolar satellite abnormalities and human disease. Studies over the last 10 year identified centriolar satellites as important regulators of centrosome and cilium biogenesis and functions (Prosser and Pelletier, 2020). In my thesis, I aimed to address key unknowns about centriolar satellites that pertain to their spatiotemporal functions and molecular mechanism of action.

To investigate the acute functions of centriolar satellites in ciliated cells, I developed a chemically inducible assay to manipulate their cellular positioning and examined the phenotypic consequences of satellite mispositioning. In the first part of my thesis, I will present the development and validation of the chemically inducible satellite trafficking assay, which proved to be a new and powerful tool for studying spatiotemporal functions of satellites. In the second part of my thesis, I will present results of the functional dissection of satellite mispositioning.

CHAPTER 2: Literature Review

2.1 Centrosome

From the beginning of cell biology, many questions raised regarding to the function and importance of centrosome. First theory was proposed by Theodor Boveri (Boveri, 1887; Boveri, 1900) who give the name ‘centrosome’ based on its localization next to the nucleus (Boveri, 1887). According to the Boveri, centrosome is the dynamic center of the cell and other organelles arrange themselves according to the centrosome during cell division (Boveri, 1900). After a couple of years, Henneguy-Lenhossek proposed a different idea about the centrosome related to the the relationship between cilium and centrosome. The Henneguy-Lenhossek (1898) theory said that the centrioles and the basal body are structurally very similar to each other. Following these discoveries, electron microscopy studies first revealed the structure of and then defined the dense fluid-like structure around the centrioles as the pericentriolar material (PCM) (Schatten, 2008).

Unlike other cellular organelles, centrosomes are not surrounded by membranes. They are composed of two perpendicular microtubule-based cylindrical centrioles and associated pericentriolar protein-dense pericentriolar material (Piel et al., 2000). Centrosomes function as the main microtubule organizing center (MTOC) of animal cells by nucleating and organizing microtubules. Through their MTOC function, they play crucial functions during intracellular protein trafficking, cell polarization and cell motility in interphase cell (Muroyama and Lechler, 2017). In cycling cells, centrosomes duplicate and assemble the bipolar mitotic spindle, which is required for equal segregation of genetic material (Winey and O’Toole, 2014). Like DNA replication, centrosome duplication is a highly regulate process coordinated by many protein complexes and signaling pathways (Wong and Stearns, 2003). Defects in centrosome duplication results in centrosome number abnormalities such as supernumerary (extra) centrioles, which is widespread in cancer (Godinho et al., 2014). Extra centrioles were shown to cause cancer by inducing aneuploidy and invasive behavior (Godinho et al., 2014).

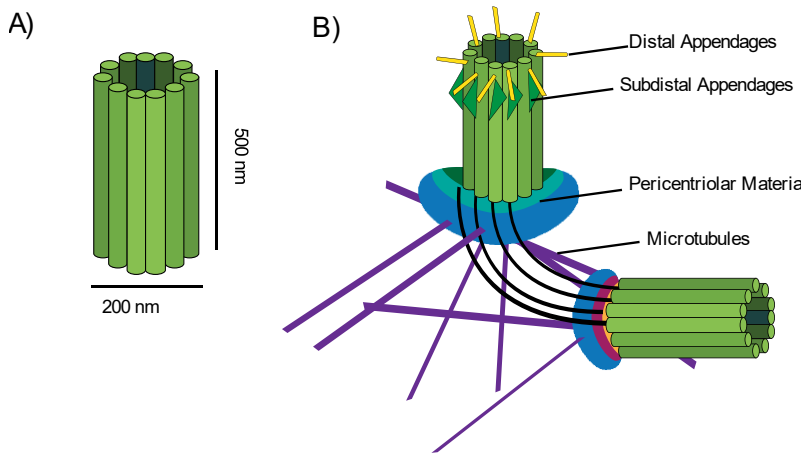

Figure 1: Centrosome Structure A) Centrioles are cylindrical structure with 200 nm in diameter and 500 nm in length. B) Mammalian centrosomes consist of mother and daughter centrioles. Mother centriole has distal and subdistal appendages and larger PCM.

2.1.1 Centrioles

Centrioles are barrel-shaped structures composed of nine triplet microtubules arranged with a rotational symmetry. Unlike cytoplasmic microtubules, centriolar microtubules are highly modified by acetylation, detyrosination, glycylation and polyglutamylation (Janke and Bulinski, 2011), which makes them more resistant and stable. Centrioles are approximately 250 nm in diameter and 500 nm in length (Winney and O'toole, 2014). Their size and shape are evolutionarily highly conserved in eukaryotic cells (Hodges et al, 2010). The two centrioles of the centrosome are perpendicular to each other and abut each other at their proximal ends. In addition to their size and shape, centriole number is also highly regulated. Majority of cells have two centrioles in interphase, which duplicate once and only once during S phase, mature during G2 and are segregated equally to daughter cells by the mitotic spindle during M phase (First-Karalar and Stearns, 2014). Like DNA, centriole number is controlled by various protein complexes and signaling pathways and defects in this regulation causes centriole number abnormalities, which are hallmarks of cancer.

The two centrioles of the centrosome differ from each other in terms of their structure, function and motility behavior (Piel et al, 2000; Andersen, 2000; Loukil et al., 2017). The older centriole is named as the 'mother centriole' and the younger name is named as the 'daughter centriole'. The mother centriole is characterized by subdistal and distal appendages, which are required for microtubule anchoring and cilium assembly, respectively (Uzbekov and Alieva, 2018). The distal appendages mediate the docking of the centrioles to the plasma membrane during ciliogenesis (Garcia-Gonzalo and Reiter, 2012). The composition of the distal and subdistal

appendages were recently defined by proteomics studies and loss-of-function experiments (Tanos et al,2013; Huang et al., 2017). Additionally, super resolution microscopy and cryoelectron microscopy provided important insight into their structural organization (Chong et al., 2020; Nogales et al., 1999). Although the daughter centrioles lacks the appendages, there are proteins specific to daughter centrioles such as Cep120 and Neurl4, which function during centriole duplication and cilium assembly (Winey and O'Toole 2014).. The proximal end of the centrioles are embedded in a protein dense material termed the pericentriolar material (PCM), most of which is associated with the mother centriole.

The two centrioles connected to each other via connecting fiber proteins such as CNAP-1, Rootletin and LRRC45 (Fry et al, 1998; Yang et al, 2012; He et al. 2013). In an interphase cell, these proteins prevent to increase the distance between centrioles by connecting them to organize microtubules from one center. After the cell enters S phase, connecting fiber proteins are degraded and duplicated centriole move towards two poles of the cell to form bipolar spindles. Degradation of connecting fiber starts with phosphorylation of the proteins by centrosomal kinase NEK2 (Rellos et al., 2007). Once the centrioles are duplicated, they moved away to the spindle pole of the cells by kinesin motor proteins in dividing cells (Tanenbaum and Medema, 2010) Mutations in connecting fiber protein cause premature splitting of centriolar before S phase. For instance, in Seckel like syndrome patients, mutation in CNAP-1 is commonly observed which dwarfism and hematological abnormalities seen as phenotype (Floriot et al, 2015).

2.1.2 Pericentriolar Material

Pericentriolar material (PCM) is the protein dense matrix of the centrosome associated with the proximal end of the centrioles. Centrioles function as scaffolds for the recruitment of PCM and consequently centrosome formation. PCM assigns the microtubule-organizing function for the centrosomes by recruitment and organization of proteins required for microtubule nucleation and anchoring (Woodruff et al., 2014). γ-tubulin ring complex (γ-TURC) is essential for microtubule nucleation at the centrosome. This complex anchor the PCM and enables the addition of new α- and β- tubulin monomers for microtubule elongation (Oakley et al., 2015). The size and organization of PCM is dynamically altered in response to extracellular stimuli. For example, its size increases in a process called “centrosome maturation” in order to meet the increasing needs of microtubule nucleation during mitosis. The dynamic changes in the nucleation capacity of the centrosome plays important roles during spindle formation, spindle

orientation, centriole duplication and cytokinesis and is highly regulated by various control mechanisms such as PLK1 and Aurora A kinase activity (Glover et al, 1995; Barr et al., 2004). Technological advances in imaging approaches such as electron microcopy, Forster resonance energy transfer (FRET) and 3D-structured electron microscopy (3D-SIM) dramatically changed the way I think about organization of the PCM (Woodruff et al., 2014). Early electron microscopy studies defined pericentriolar material as 'amorphous mass' of proteins (Gould and Borisy, 1977). What was once considered as a meshwork of protein interactions is now regarded as a highly organized structure as a result of the application of advanced imaging approaches to the PCM. These studies revealed that PCM is organized as 'concentric toroids' with specific diameters for each protein complex around the centrioles (Woodruff et al., 2014). Pericentrin (PCNT) was the first identified component of pericentriolar material, which was followed by identification of many more components such as CDK5RAP2 and AKAP450 (Andersen et al., 2003; Lawo et al., 2012; Doxsey et al., 1994). Majority of the PCM proteins are enriched in coiled-coil domains and are insoluble (Lupas et al., 1991).

2.1.3 Centriolar Satellites

Centriolar satellites (hereafter satellites) are 70-100 nm non-membranous, electron-dense protein complexes that localize and move around the centrosome (Balczon et al., 1994). In most interphase cells, majority of the satellite granules cluster around the centrosome, with a minor pool that are homogenously scattered throughout the cytoplasm. In ciliated cells, they are mostly clustered as the base of the basal body. As cells enter mitosis, they rapidly disperse in a DYRK3-dependent manner and reassemble upon mitotic exit (Rai et al., 2018). Although satellites exhibit pericentrosomal clustering in most cell types, they were also shown to adapt cell type-specific distribution profiles. For example, they are clustered around the nuclear envelope in myoblasts and beneath the basal bodies at the apical side of multiciliated epithelial cells (Vladar and Stearns, 2007; Prosser and Pelletier, 2020). This variation suggests that cellular positioning of satellites are important for their function and that different cell types regulate satellite positioning in order to meet cell type-specific needs.

Proteomic profiling of the satellite interactome by proximity-based labeling methods and affinity purifications identified about 200 proteins (Gheiratmand et al., 2019). Strikingly, about 50% of the centrosome proteome overlaps with the satellite interactome, corroborating the functional relationship between centrosomes and satellites. Satellite interactome also contains proteins mutated in developmental disorders such as sensory and motile ciliopathies and

primary microcephaly, suggesting regulatory roles for satellites during primary cilium assembly and function. The first satellite component that was identified is pericentriolar material 1 (PCM1), which is essential for satellite integrity and thus is considered as the molecular marker and scaffolding protein for satellites (Kubo and Tsukita 2003; Odabasi et al., 2020). While PCM1 exclusively localizes to the satellites, the rest of the satellite residents have additional cellular pools such as the centrosome, cilium and microtubules. Acute loss of PCM1 by siRNA-mediate protein depletion and its knockout by CRISPR-Cas9 genome editing approaches generates satellite-less cells (Odabasi et al., 2019). Phenotypic characterization of these cells by functional assays identified satellite functions during primary cilium assembly, Hedgehog signaling, microtubule organization and mitotic progression (Firat-Karalar, Rauniyar et al. 2014, Tollenaere et al. 2015). While these studies advanced our understanding of cellular satellites functions, they were limited in dissecting the spatiotemporal functions of satellites in ciliated and cycling cells.

Satellites are dynamic granules. Live imaging and particle tracking analysis of satellite dynamics showed that they undergo both molecular motor and microtubule-mediated bimodal motility and Brownian motility (Conkar et al., 2017; Francis et al., 2009). Consistently, satellite interactome contains dynein adaptors (i.e. BICD2, Hook3, ninein) as Ill as various kinesins. Microtubule-mediated satellite motility towards or away for the centrosome suggests trafficking functions for satellites by which they regulate centrosomal and ciliary level of proteins. There are multiple lines of evidence that supports such trafficking functions. First, the centrosomal levels of key regulators of ciliogenesis such as IFT88 and Talpid3 are reduced in satellite-less PCM1-/- cells (Wang et al., 2016; Odabasi et al., 2019). Second, live imaging of the satellite marker PCM1 with its residents revealed their co-localization and movement towards and away from the centrosome (Dammermann and Merdes, 2002). In addition to their trafficking function, satellites are also considered as active scaffolds for mediating posttranslational modifications and proper folding of their residents. Future studies are required to extensively dissect the molecular mechanism of action for centriolar satellites.

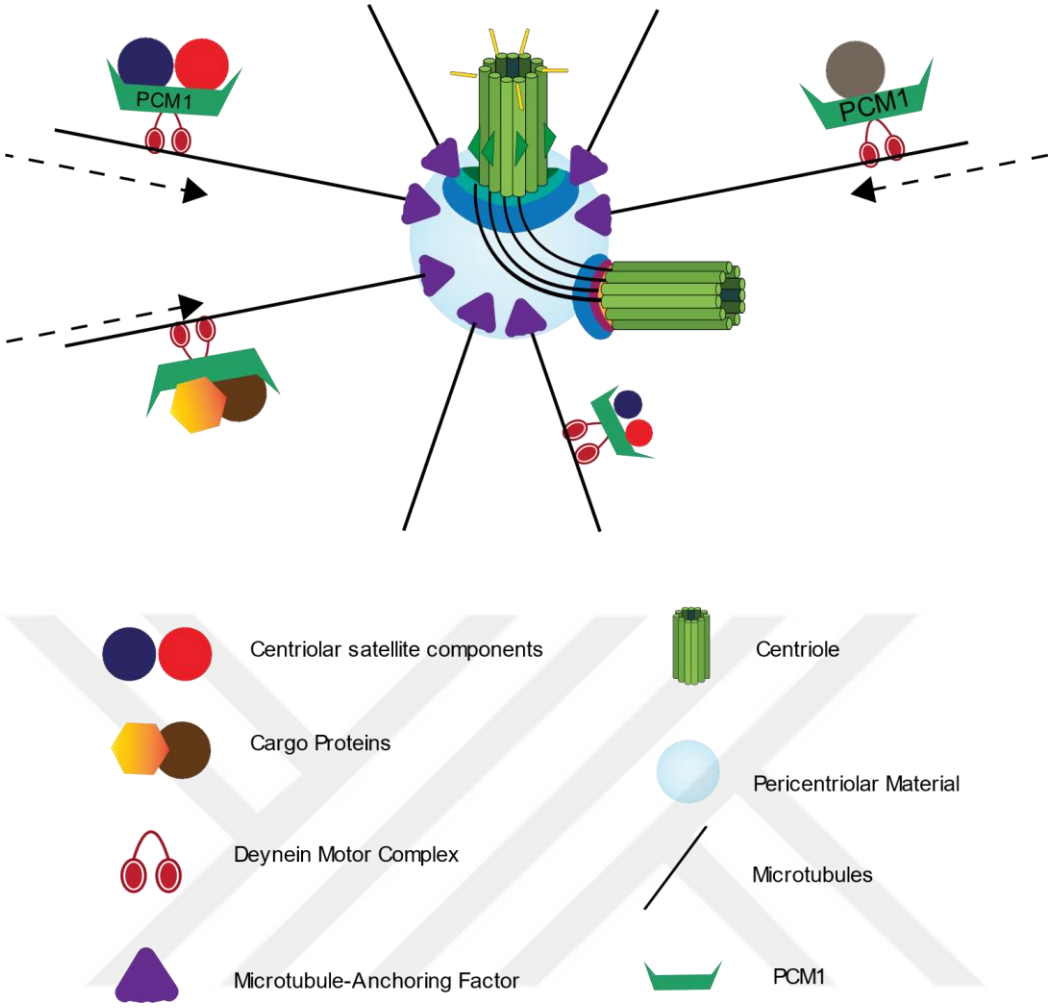

Figure 2: Centriolar satellites are the store, modify and traffic the centrosomal proteins. It also acts as scaffolding protein for the delivery of proteins to the centrosome.

2.1.4 Centrosome and Centriolar Satellite Proteome

Centriolar satellites assembly is scaffolded by PCM1, which is a 2024 amino acid protein highly enriched for coiled coil domains. PCM1 is the only protein that interacts with all other satellite proteins. It is thought to be the scaffold protein for the correct localization and function of other satellite proteins (Balczon et al, 1994). Proteomic profiling of centriolar satellites were performed by proximity-based labeling and affinity purification of PCM1 followed by mass spectrometry (Quarantotti et al., 2019).

Proximity-dependent biotin identification (BioID) is very useful method to study the proteome of centrosome and ciliary proteome (Roux et al., 2012). Mutated form of *E. coli* protein, biotin ligase is used in BioID fused with the protein of interest. In the presence of free biotin at the environment, the mutant form of biotin ligase play role in the formation of biotinoyl-AMP intermediate molecule. The reactive intermediate molecules can react the amine groups of the

polypeptides in the vicinity of the bait protein (Roux et al., 2012). After the reaction occurred, biotinylated proteins collected and solubilized by lysing the cells. Isolation of the biotinylated proteins can be achieved by affinity purification by the help of biotin-streptavidin interaction and then identified by mass spectrometry (Gheiratmand et al., 2019). It is commonly used to characterize protein-protein interactions especially in centrosome and satellite field which are poorly soluble structures (First-Karalar et al., 2014; Gupta et al., 2015; Conkar et al., 2017).

Centriolar satellites are composed of over 200 proteins that are implicated in a wide range of functions. Among these proteins are the ones mutated in ciliopathies such as Cep290, CCDC66 and BBS4 (Kim et al., 2004; Kim et al., 2008; Kodani et al., 2010). Cep290 is also important for recruitment of Rab-8 to primary cilium by interacting PCM1 (Kim et al., 2008). Loss of PCM1 causes pericentrosomal clustering of PCM1 and disrupts microtubule organization, suggesting that Cep290 is required for cellular distribution of satellites (Kim et al., 2008). Given that Cep290 interacts with kinesins, it is possible that is functions in the tug-of-war between kinesins dynein during cellular distribution of satellites (Chang et al., 2006; McEIn et al., 2007). Cep290 also interacts with other satellite proteins (i.e. CCDC66, BBS4, Cep72) and functions during cilium assembly and ciliary signaling (Conkar et al., 2017; Zhang et al., 2013; StoI et al., 2012).

In addition to proteins implicated in primary cilium biogenesis and ciliopathies, PCM1 has extensive interactions with important regulators of centriole duplication and proteins mutated in primary microcephaly. For example, Cep63 was shown to interact with satellite proteins CCDC14 and KIAA0753 and this interaction is required for regulation of centrosomal Cep63 levels and thereby centriole duplication (Firat-Karalar et al., 2014). Cep63 is the centrosomal protein, important in the centriole duplication. PCM1 also interact with structural components of the centrioles such as WDR90 and centrin (Steib et al., 2020; Hori and Toda, 2017). Depletion of PCM1 was shown to cause significant reduction in the centrosomal levels of centrin, suggesting that it is required for centrosomal targeting of centrin (Dammermann and Merdes, 2002). PCM1 also interacts with daughter centriolar proteins such as Cep120, which is important for ciliogenesis and centriole maturation. Depletion of Cep120 is related with increased microtubule nucleation activity, increased dynein activity for centrosomal protein trafficking, abnormal centriolar satellite localization and defects in cilium assembly (Betleja et al., 2018). Finally, satellites interact with proteins required for centrosome cohesion, known as linker proteins. C-Nap1 was the first centrosomal linker protein identified. When cells enter to

the mitosis, C-Nap1 is phosphorylated and the centriolar linker breaks down in order to promote assembly of the bipolar spindle.

Table 1: List of selected centriolar satellites proteins and their associated functions

Protein Name	Function	Localization of Major Pool	Identified Interaction with Satellites
PCM1	Ciliation	Satellites	Yes
Cep290	Ciliation	Satellites	Yes
Cep72	Ciliation	Satellites	Yes
Cep131	Ciliation	Satellites	Yes
Cep164	Centriole Docking and Duplication	Mother Centriole	No
Cep152	Centriole Duplication	Both Centrioles	No
Cep63	Centriole Duplication	Both Centrioles	Yes
Cep120	Centriole Maturation	Daughter Centriole	Yes
C-Nap1	Linker Protein	PCM	No
Centrin	Centriole Duplication	Both Centrioles	Yes
Gamma Tubulin	Microtubule Nucleation	PCM	Yes
MIB1	Post-translational modification of satellites	Satellites	Yes

2.2 Cilia

Cilia are highly conserved microtubule-based structures that protrude from the cell membrane and function in cell signaling and motility (Nachury, 2014). Cilia are composed of the microtubule-associated axoneme that is surrounded by the ciliary membrane that is continuous

with the plasma membrane but has a different composition in order to enable their signaling and motility functions.

The earlier studies on cilia focused on their motility functions (Duldulao et al., 2010). For instance, the unicellular organism *Paramecium* contains high number of cilia motile cilia on their surface, which create a directed flow of the extracellular fluid and are required for their motility. In contrast to Paramecium, sperm cells have a single flagellum for motility that propels them in the female reproductive tract. Finally, the specialized epithelia of the trachea, fallopian tube and brain ventricles contain tens to hundreds of motile cilia that synchronously beat to move extracellular fluid or particles. The discovery of the primary cilium and its functional dissection showed that cilia play important sensory functions in addition to their functions in motility. Specifically, primary cilium functions as the cellular antenna that receives and transmits developmentally important signaling pathways such as Hedgehog (Hh), Wnt and PDGFR-α and cell polarity (Schneider, et al. 2005, Bornens 2018).

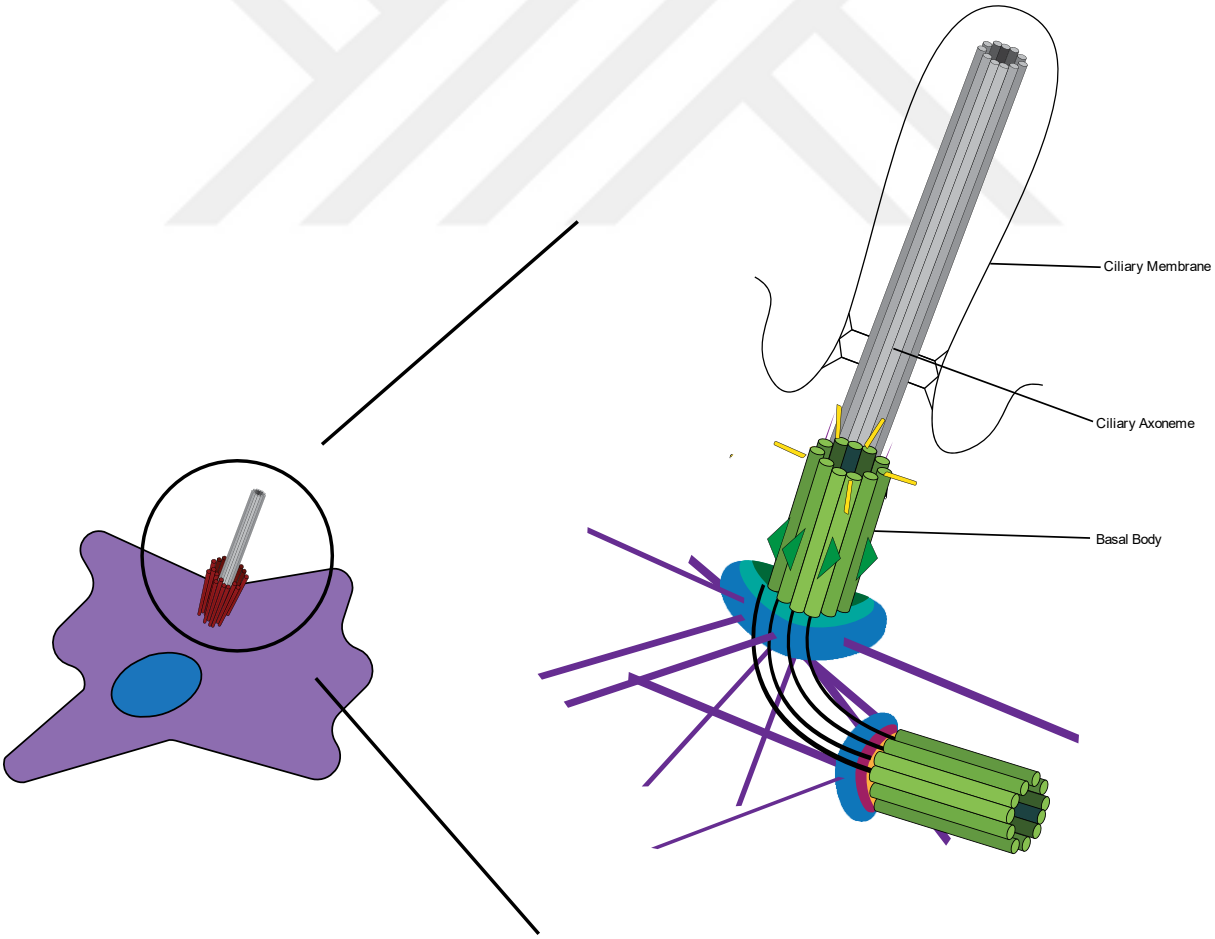

Figure 3: Structure of a cilium conposed of ciliary axoneme, basal body, and ciliary membrane.

Primary cilium assembly is a highly regulated process coordinated by many proteins and signaling pathways. Cells assemble primary cilium during quiescence (i.e. mitogen deprivation). During primary cilium assembly, the mother centriole docks to the plasma membrane to nucleate the formation of the microtubule-based axoneme. The axoneme of the primary cilium extend from the nine-fold microtubule triplets of the basal body and is composed of nine radially organized microtubule doubles (9+0 organization). Unlike cytoplasmic microtubules but like centriolar microtubules, ciliary microtubules are highly modified by posttranslational modifications. Among the most prominent ciliary microtubule modifications are acetylation, glutamylation, tyrosination and glycylation (He et al., 2020). These modifications were shown to be important for ciliary stability and regulation of the interactions between the axoneme and microtubule-binding proteins such as molecular motors.

In ciliated cells, the mother centriole is termed as the basal body, which is connected to the ciliary membrane through specific structures such as the transition fibers (Marshall, 2008; Kobayashi and Dynlacht, 2011). Transition fibers are localized at the proximal end of the primary cilium where they are being at the distal end of the basal body. They are defined by the presence of Y-links and they connect to the microtubule doublets to the ciliary membrane (Reiter et al., 2012). The transition zone (TZ) is the sub compartment between the basal body and the axoneme, which functions as the ciliary gate by regulating the transport of proteins into and out of the cilium (Omran, 2010).

2.2.1 Primary cilium assembly, maintenance, and disassembly

Primary cilium assembles when cells enter quiescence in response to mitogen deprivation and differentiation. Accordingly, it disassembles as cells enter mitosis. Primary cilium assembly and disassembly are highly regulated processes that are coordinated by many organelles, protein complexes and signaling pathways. Defects in primary cilium biogenesis cause developmental disorders known as ciliopathies, which are characterized by defects affecting almost all organs in human body.

Cilium assembles using two different pathways depending on the cell type: 1) intracellular pathway, 2) extracellular pathway (Figure 4) (Pedersen et al., 2012). At the intracellular pathway, a ciliary vesicle interacts with the distal appendage of the mother centriole. The centriolar vesicles fuse and grow and consequentially surround the mother centriole while the mother centriole differentiates into the basal body. As the basal body nucleates the formation

of the axoneme intracellularly, the ciliary vesicle fuses with the plasma membrane and the cilium protrudes from the cell surface. At the extracellular pathway, mother centriole moves towards the plasma membrane and docks to the membrane by the help of its distal appendages. After docking, mother centriole starts differentiates into the basal body and the axoneme grows extracellularly. While fibroblasts and retinal epithelial cells (i.e. RPE1 cells) use the intracellular pathway, kidney epithelial cells (i.e. IMCD3 cells) use the extracellular pathway.

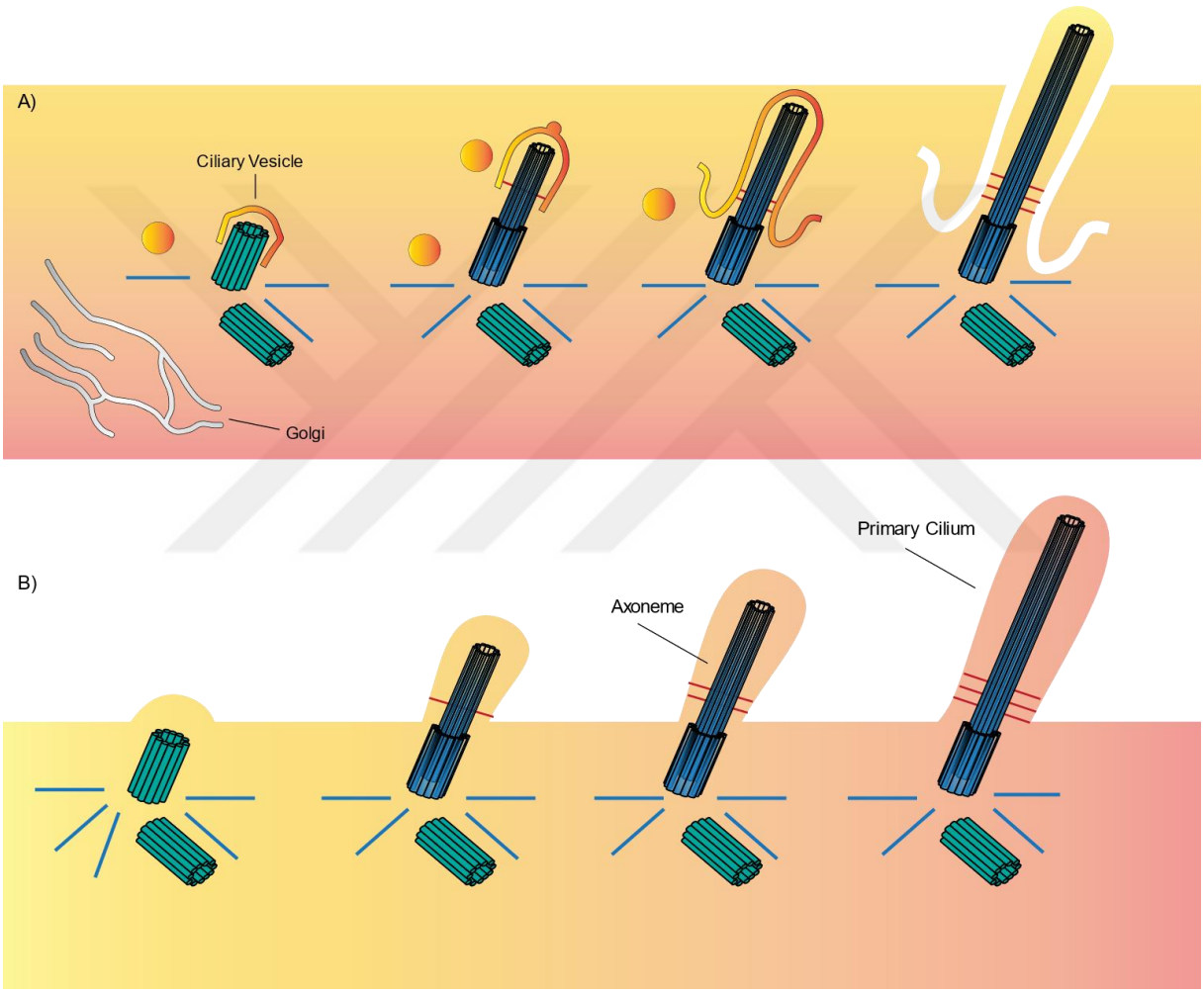

Figure 4: Stages of cilium assembly A) Cilium assembly by intracellular pathway B) Cilium assembly by extracellular pathway

The molecular mechanism that underlies the intracellular pathway has been extensively dissected using a combination of genetic, proteomic and imaging approaches. As cilium assembly is induced by mitogen deprivation or differentiation, `pre-ciliary vesicles` (PCV) originated from the Golgi are recruited to the distal appendages of mother centriole. PCVs fuse to form the ciliary vesicle (CV) in a Rabin8 (Rab8 activator) and Rab8-dependent mechanism

(Sanchez and Dynlacht 2016). CP110 and Cep97 are centriolar capping proteins that are removed from the mother centriole during its differentiation into the basal body (Spektor et al., 2007). is removed which is capping protein, from the basal body. Once the membranous cap formed, microtubules start to polymerize and grow from the distal end to form ciliary axoneme. During axoneme elongation, membranous cap enlarges by vesicle trafficking and axoneme is encapsulated by a second membrane. Eventually, by the fusion of ciliary sheath, the cilium dock to the membrane and protrude from there.

Given that primary cilium is a semi-closed compartment, its content is tightly regulated by multiple mechanisms. This regulation enables the function of the primary cilium as the signaling center by concentration of specific signaling pathway components in response to interacellular and extracellular stimuli (Wheway et al., 2018). The mechanisms that regulate ciliary content include the Intraflagellar transport (IFT) machinery, BBSome complex, vesicular trafficking, transition zone and centriolar satellites (Hsiao et al. 2012; Lechtreck 2015; Garcia-Gonzalo 2017; Liu et al., 2018; Chamling et al. 2014). IFT consist of IFT-A and IFT-B complexes moving anterograde and retrograde, respectively (Eguether et al. 2014). These complexes are train-like structures, moving along axoneme by the help of molecular motors. They carry the ciliary building blocks and signaling molecules toward inside or outside of the cilium according to the complex (Kozminski et al. 1993). Anterograde transport of IFT-A is carried by kinesin motors where the dynein play role in retrograde transport of IFT-B (Pazour et al. 2000; Cole, Diener et al. 1998). In addition to IFT transport, passive diffusion also involves in ciliary transport (De Ighe et al, 2020).

BBSome is a protein complex, composed of BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9 and BBIP10 (Jin and Nachury, 2009). BBSome play role during the during the extension of microtubules and recruit proteins while the cilium grows (Jin et al., 2010). BBS4 is the last component during the assembly of BBSome complex which mediates the recruitment of BBSome to the ciliary base (Zhang et al., 2012). Cep290 and Cep72 are both interacts with BBS4 and this interaction regulates the recruitment of BBSome complex to the ciliary base (Kim et al., 2004; StoI et al., 2012; Kamiya et al., 2008). Cep131 is another centriolar satellite protein, interacts with both BBS4 and PCM1. Knockdown of Cep131 results in accumulation of BB4 at the ciliary transition zone which shows Cep131 negatively regulate the recruitment of BBS4 to the basal body (Chamling et al., 2014).

Primary cilium disassembly can be induced by mitogens, mechanical stress and heat shock (Kim et al., 2015; Miyamoto et al., 2015). Given that posttranslational modifications of the ciliary micorotubules such as acetylation is required for ciliary stability, a major step during cilium disassembly involves deacetylation of ciliary microtubules by the activity of tubulin deaceylases (i.e. HDAC6). Upon induction of primary cilium disassembly, Aurora-A kinase is recruited to the basal body, and activated by phosphorylation (Korobeynikov et al., 2017). Subsequently, Aurora-A phosphorylates histone deacetylase-6 (HDAC6) at the basal body, which initiates deacetylation of axonemal microtubules (Pugacheva et al., 2007). In addition to Aurora-A, another key kinase, PLK1, was shown to play important roles during primary cilium disassembly (Wang et al., 2013). PLK1 phosphorylates Kif2a to stimulate the microtubule depolymerization. After Kif2a and PLK1 activated, Nek2 phosphorylates Kif24 which is another kinesin protein that induces microtubule depolymerization. Kif24 activity disables the cilium assembly (Sanchez and Dynlacht 2016). Despite extensive investigation of primary cilium assembly, its disassembly pathways are still fragmentary and requires further investigation in future studies.

2.3 Tools to Study Organelle Positioning

Compartmentalization of the eukaryotic cells enables them to perform specific functions at the same time with higher efficiency. As part of cellular compartmentalization, different cellular compartments adapt specific subcellular organizations, which must be optimal to receive and transmit the signals from the environment (Bergeijk et al., 2015). Therefore, correct positioning of organelles is also essential for their proper function at the right time and place. Since the cell is a dynamic structure, there are always exchange of materials between different compartments inside the cells and between extracellular matrix. For the proper trafficking of these materials, active or passive transport are utilized. In active transport, motor proteins carry the cargo proteins moving along microtubules or actin filaments by using energy (Vale, 2003). Kinesin and dynein are the two motor proteins that moves along microtubules, which act as cellular tracks. Kinesin and dynein motor proteins move towards the plus and minus end of microtubules, respectively. In addition to their conserved motor domains, these proteins interact with their cargoes through their tail domains (Schlager and Hoogenraad, 2009). To study the functional significance of positioning of organelles that require microtubules and molecular motors, their cellular distribution can be manipulated by disrupting microtubule organization and molecular motor activity. For instance, when a cell treated with the microtubule

depolymerizing reagent nocodazole, cellular positioning of lysosomes and Golgi apparatus changed (Korolchuk et al., 2011; Cole et al., 1996). While drugs that interfere with microtubule organization and motor activity has been useful, they were limited in specificity. Therefore, more specific tools were developed for manipulating organelle positioning to overcome these problems. In one approach, cells are plated on specific micropatterns with cohesive molecules to study the significance of cell morphology and cell polarity to its function (Stoica et al., 2014). In another approach, magnetic nanoparticles are used to manipulate organelles by conjugating them with transport molecules such as Ran-GTP (Hoffmann et al., 2013). Conjugation allows local polymerization and concentration of microtubules to manipulate certain trafficking of specific proteins by providing magnetic field (Hoffmann et al., 2013).

An extensively used organelle mispositioning tool utilizes chemical and protein fusion partners that dimerize (Erhart et al., 2013). To manipulate the position of cellular organelles, motor domains of kinesin or dynein can be used as dimerization partners. To induce dimerization between molecular motors and organelle markers, FKBP (FK506 binding protein) and FRB (FKBP rapamycin binding domain) domains are extensively used as these domains permanently dimerize in the presence of rapalog (Clackson et al., 1998; Kapitein et al., 2010). Fusing one of these domains to the constitutively active motor protein mutants and the other to the marker of select organelles and their subsequent dimerization with rapalog treatment results in forceful movement of organelles towards to minus ends of microtubules that are clustered at the centrosome or plus ends of microtubules that are clustered at the plasma membrane (Kapitein et al., 2010). The chemical organelle trafficking assays has recently been advanced by using light-inducible fusion partners, which results in reversible dimerization and thereby organelle mispositioning in the presence and absence of light (Ballister et al., 2015). By inducing the dimerization of organelles with plus or minus end-directed molecular motors, these new organelle mispositioning assays alloId researchers to address functional questions that cannot be addressed using loss-of-function experiments.

CHAPTER 3: MATERIALS and METHODS

3.1 Cell culture, transfection, and transduction

Human cervical cancer HeLa and human embryonic kidney HEK293T cells were cultured with DMEM medium (Pan Biotech, Cat. # P04-03590) supplemented with 10% FBS and 1% penicillin-streptomycin. Mouse kidney medulla collecting duct cells IMCD3:Flip-In cells were cultured with Dulbecco's modified Eagle's Medium DMEM/F12 50/50 medium (Pan Biotech, Cat. # P04-41250) supplemented with 10% Fetal Bovine Serum (FBS, Life Technologies, Ref. # 10270-106, Lot # 42Q5283K) and 1% penicillin-streptomycin (Gibco, Cat. # 1540-122). All cell lines were authenticated by Multiplex Cell Line Authentication (MCA) and were tested for mycoplasma by MycoAlert Mycoplasma Detection Kit (Lonza).

IMCD3 and HeLa cells were transfected with the plasmids using Lipofectamine 2000 according to the manufacturer's instructions (Thermo Fisher Scientific). For serum starvation experiments, IMCD3 cells were washed twice with PBS and incubated with DMEM/F12 supplemented with 0.5% FBS for the indicated times. For cilium maintenance experiments, IMCD3 cells were serum starved for 48 h, incubated with rapamycin for 1h and fixed at the indicated times. For serum stimulation experiments, ciliated IMCD3 were incubated with rapamycin for 1 h, then with DMEM/F12 50/50 supplemented with 10% FBS and fixed at the indicated times. For rapamycin induction experiments, cells were treated with cells were treated with 100 nm or 500 nm rapamycin (Milipore. Cat#553210) for 1 h followed by 2X PBS washout. To induce microtubule depolymerization, cells were treated with 10 □g/ml nocodazole (Sigma-Aldrich, Cat. #M1404) or vehicle (dimethyl sulfoxide) for one hour at 37°C. For microtubule regrowth experiments, cells were washed 3X with PBS after microtubule depolymerization, incubated in warm media for the indicated times, fixed and stained. For HDAC inhibition, cells were treated with 2 μm tubacin (Cayman Chemicals, Cat#13691) for the indicated times.

3.2 Plasmids

Full-length cDNA of *Homo sapiens* PCM1 was amplified from peGFP-C1-PCM1 and cloned into peGFP-C1 (Clontech) without stop codon using XhoI and KpnI sites. The cDNA of FKBP was amplified from PEX-RFP-FKBP by PCR and cloned into peGFP-PCM1 using KpnI and

BamHI sites. PCM1-FKBP was amplified from the peGFP-C1-PCM1-FKBP by PCR and cloned into pcDNA3.1-myc-BirA* using XhoI and BamHI sites. pC4M-F2E-PEX-RFP-FKBP, PCI-neo-HA-BICD2-N (1-594)-FRB, pCI-neo-HI-Kif5b (1-807)-FRB and pbactin-GFP-FRB-Kif17 (1-546) .

3.3 Immunofluorescence and antibodies

Cells were grown on coverslips, washed twice with PBS and fixed in either ice cold methanol at -20°C for 10 minutes or 4% PFA in cytoskeletal buffer (10 mM PIPES, 3 mM $MgCl_2$, 100 mM NaCl, 300 mM sucrose, pH 6.9) supplemented with 5 mM EGTA and 0.1% Triton X for 15 min at 37 °C. After PBS wash, cells were blocked with 3% BSA (Capricorn Scientific, Cat. # BSA-1T) in PBS + 0.1% Triton X-100 followed by incubation with primary antibodies in blocking solution for 1 hour at room temperature. Cells were washed three times with PBS and incubated with secondary antibodies and DAPI (Thermo Scientific, cat#D1306) at 1:2000 for 45 minutes at room temperature. Following three washes with PBS, cells were mounted using Mowiol mounting medium containing N-propyl gallate (Sigma-Aldrich). Primary antibodies used for immunofluorescence were mouse anti-acetylated tubulin (clone 6-11B, 32270, Thermo Fischer) at 1:10000, mouse anti gamma tubulin (Sigma, clone GTU-88, T5326) at 1:1000, mouse anti GFP (Thermo Scientific, A-11120, clone 3E6) 1:750, mouse anti alpha tubulin (Sigma, DM1A) at 1:1000, rabbit anti CEP152 (Bethyl, A302-480A) at 1:500, rabbit anti-Cep131 (Proteintech, 25757-1-AP) at 1:1000, mouse anti-centrin 3 (Abnova, H0007070-MO1, clone 3E6) at 1:500, rabbit anti-Cep290 (Abcam, ab84870) at 1:1000, anti-rabbit Cep72 (Bethyl, A301-298A) at 1:1000, rabbit anti-Cep63 (Millipore, 61292) at 1:1000, rabbit anti-MIB1 (Sigma, M5948) at 1:1000, mouse anti-C-NAP1 (Santa Cruz, sc 390540) at 1:1000, rabbit anti IFT88 (Proteintech, 13967-1-AP) at 1:250, mouse GT335 (Adipogen, A27791601), rabbit Arl13B (Neuromab, Clone N295B/66) at 1:500,. Rabbit anti-PCM1, anti-Cep120 and anti-GFP antibodies were generated and used for immunofluorescence as described (Firat-Karalar et al., 2014). Secondary antibodies used for immunofluorescence experiments were AlexaFluor 488-, 568- or 633-coupled (Life Technologies) and they were used at 1:2000.

3.4 Microscopy and image analysis

Time lapse live imaging was performed with Leica SP8 confocal microscope equipped with an incubation chamber mounted on an automized stage. For cell cycle experiments, asynchronous cells were plated on LabTek dishes (Thermo Fisher) and imaged at 37°C with 5% CO_2 with a frequency of 4.5 minutes per frame with 1 µm step size and 12 µm stack size in 512 X 512 pixel format at a specific position using HC PL APO CS2 40x 1.3 NA oil objective. For time-lapse imaging experiments, HeLa cells were stained with SIR-Hoechst for visualization of chromosomes (100 nm, Spirochrome). For centrosomal protein level quantifications, images were acquired with Leica DMi8 inverted fluorescent microscope with a stack size of 10 µm and step size of 0.5 µm using HC PL APO CS2 63x 1.4 NA oil objective. Higher resolution images were taken by using HC PL APO CS2 63x 1.4 NA oil objective with Leica SP8 confocal microscope equipped with the SVI Huygens deconvolution suite.

Quantitative immunofluorescence for pericentrosomal levels of select proteins was performed by acquiring a z-stack of control and depleted cells using identical gain and exposure settings. The maximum-intensity projections were assembled from z-stacks. The centrosome region for each cell were defined by staining for a centrosomal marker gamma-tubulin. The region of interest that encompassed the centrosome was defined as a circle 3 μm^2 area centered at the centrosome in each cell. Total pixel intensity of fluorescence within the region of interest was measured using ImageJ (National Institutes of Health, Bethesda, MD) (Schneider et al., 2012). Background subtraction was performed by quantifying fluorescence intensity of a region of equal dimensions in the area proximate to the centrosome. Statistical analysis was done by normalizing these values to their mean. Primary cilium formation was assessed by counting the total number of cells, and the number of cells with primary cilia, as detected by DAPI and acetylated tubulin or Arl13b staining, respectively. Ciliary length was measured using acetylated tubulin or Arl13b as the ciliary length marker. All values were normalized relative to the mean of the control cells (=1).

For functional assays, quantification and analysis were performed only in cells that exhibited complete redistribution of satellites to the cell periphery or center. Complete redistribution to the cell periphery was defined by lack of centrosomal GFP-PCM1-FKBP signal in the pericentrosomal area within 3 μm^2 area centered on the centrosome. Complete redistribution to the cell center was defined by the lack of GFP-PCM1-FKBP signal in the cytoplasm beyond

the 3 μm^2 pericentrosomal area. As controls, cells that were not treated with rapamycin in which satellites display their typical distribution pattern were quantified.

3.5 Statistical analysis

Statistical results, average and standard deviation values were computed and plotted by using Prism (GraphPad, La Jolla, CA). Two-tailed t-tests, one-way and two-way ANOVA tests were applied to compare the statistical significance of the measurements. Error bars reflect SD. Following key is followed for asterisk placeholders for *p*-values in the figures: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $**** P < 0.0001$

CHAPTER 4: RESULTS

4.1 Development and validation of the inducible satellite trafficking assay

Satellites cluster around the centrosome in most cell types, suggesting that their proximity to the centrosomes is important for their functions. To uncover temporal functions of satellites and to elucidate how their pericentrosomal clustering contributes to their functions, I developed an inducible satellite trafficking assay to target satellites away from the centrosomes to the cell periphery and determined the molecular and cellular consequences (Figure 5A). To correlate the associated phenotypes with satellite proximity to the centrosomes, the satellites in parallel targeted to the cell center (Figure5A). Our approach makes use of the inducible dimerization of the FK506 binding protein 12 (FKBP) and FKBP12-rapamycin-binding (FRB) domain of mTOR by rapamycin or its cell-permeable analog rapalog (Clackson et al., 1998; Hoogenraad et al., 2003) Due to the long half-life of rapamycin interaction with FKBP and FRB (about 17.5 hours), the dimerization is essentially irreversible (Hosoi et al., 1999). This FKBP-FRB-based rapamycin-inducible heterodimerization approach was previously used to spatiotemporally manipulate specific cellular structures and catalytic activities such as the removal of IFT20 and IFT74 from cilia and sequestration at the mitochondria, ciliary targeting of CCP5 tubulin deglutamylase, and recruitment of molecular motors to endosomes, peroxisomes and the nucleus (Euguether et al., 2018; Hong et al., 2018; Bentley and Banker, 2015; Kapitein et al., 2010; Wilson et al., 2015).

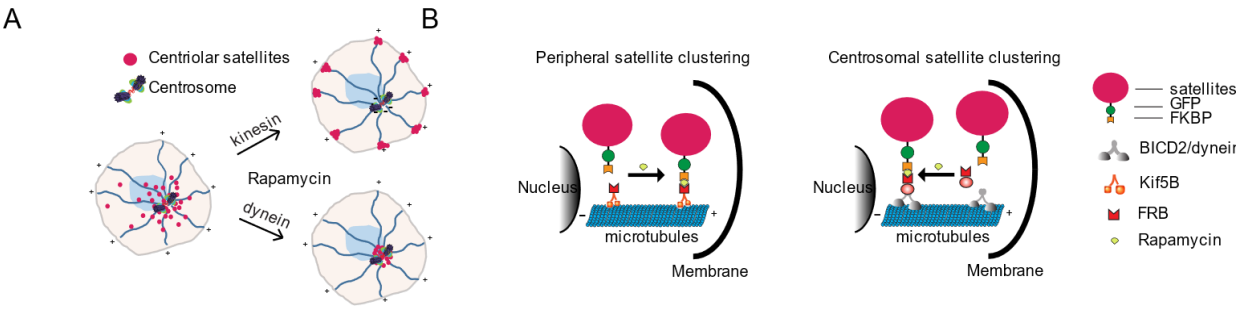

Figure 5: Development of chemically inducible system. A) Representation of satellite redistribution to cell periphery or center upon inducible dimerization of the satellite scaffolding protein PCM1 with the constitutively active plus and minus end–directed molecular motor proteins. (B) Design of the inducible satellite-trafficking assay. PCM1 was tagged with GFP at the N terminus and FKBP at the C terminus. Co-expression of GFP-PCM1-FKBP with HA-Kif5b (1–269 aa)-FRB and subsequent rapamycin induction targets satellites to the cell periphery, where microtubule plus ends are concentrated. Co-expression of GFP-PCM1-FKBP with HA-BICD2 (1–198 aa)-FRB and subsequent rapamycin induction targets satellites to the cell center, where microtubule minus ends are concentrated.

I adapted this assay for satellites by co-expressing FKBP-fusion of satellites with the FRB-fusion of constitutively active plus-end or minus-end directed motor domains (Figure6B). I tagged the satellite-scaffolding protein PCM1 with GFP at the N-terminus for visualization of satellites, and with FKBP at the C-terminus for inducible dimerization with the motor domains (Fig1B). To target satellites to the microtubule plus ends at the cell periphery, I induced their recruitment to the FRB fusion of constitutively active kinesin-1 HA-Kif5b (1-269 a.a.) motor domain (hereafter HA-Kif5b-FRB or Kif5b), which lacks the tail region required for interaction with endogenous cargoes and thus can only bind to satellites via the FKBP-FRB dimerization (Kapitein et al., 2010). To target satellites to the microtubule minus ends at the cell center, I induced their recruitment to the FRB fusion of the constitutively active N terminus of dynein/dynactin cargo adaptor bicaudal D homolog 2 BICD2 (1-198 a.a.) (hereafter HA-BICD2-FRB or BICD2), which interacts with dynein and dynactin but lacks the cargo-binding domain (Kapitein et al., 2010).

To test the feasibility of the inducible satellite trafficking assay, I first determined the localization of satellites in transfected HeLa cells before and after rapamycin addition by quantitative immunofluorescence. In cells co-expressing GFP-PCM1-FKBP and HA-Kif5b-FRB, satellites had their typical pericentrosomal clustering pattern in the absence of rapamycin (Figure6A).

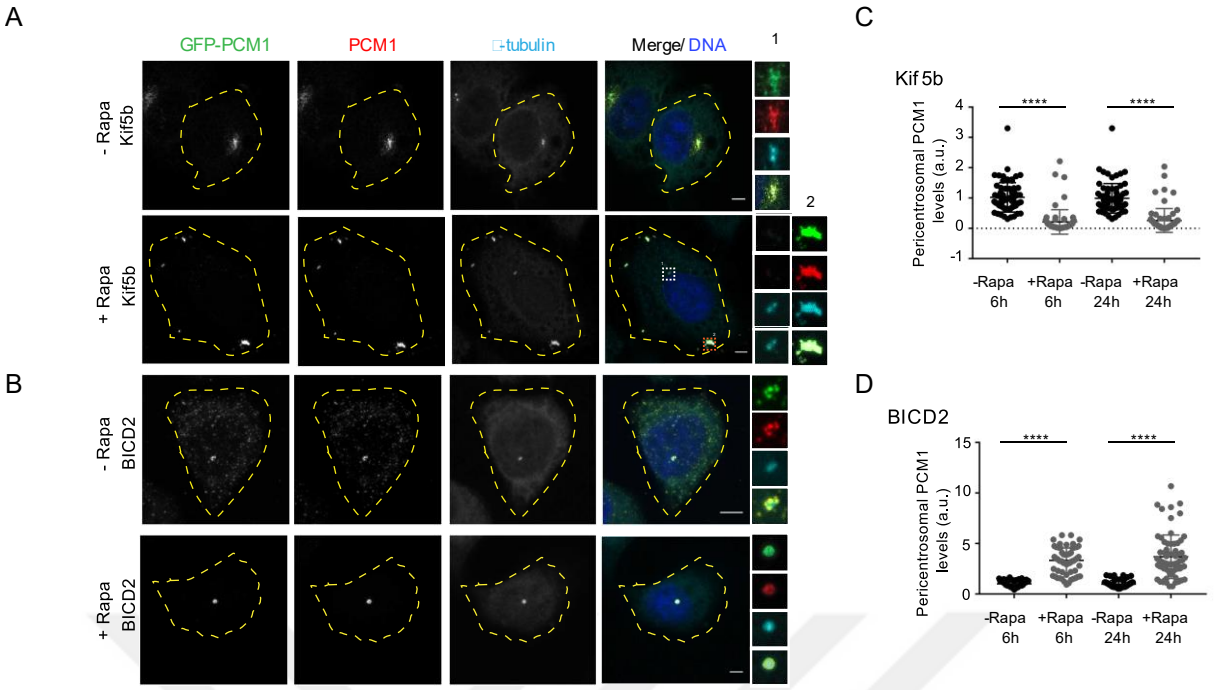

Figure 6: Redistribution of centriolar satellites with addition of rapamycin (A, B) Representative images for satellite distribution in control and rapamycin-treated HeLa cells. Cells co-expressing GFP-PCM1-FKBP with HA-Kif5b-FRB or HA-BICD2-FRB were treated with rapamycin for 1 hour. Twenty-four hours after rapamycin induction, cells were fixed and stained with antibodies against GFP, PCM1, and gamma-tubulin. DNA was stained with DAPI. Cell edges are outlined. Scale bar, 5 μm. (C, D) Endogenous PCM1 exhibits the same localization pattern as GFP-PCM1-FKBP. Pericentrosomal PCM1 levels was quantified at 6 hours and 24 hours after rapamycin treatment of cells expressing GFP-PCM1-FKBP with (E) HA-Kif5b-FRB or (F) HA-BICD2-FRB. Average mean intensity of pericentrosomal levels in control cells were normalized to 1. n ≥ 25 cells per experiment. Data represent mean value from two experiments per condition, ±SD (****$p < 0.0001$).γ

Upon rapamycin addition to cells expressing HA-Kif5b-FRB and GFP-PCM1-FKBP, satellites were targeted to the cell periphery where the majority of microtubule plus ends localize (Figure6A). In contrast, in cells expressing HA-BICD2-FRB and GFP-PCM1-FKBP, rapamycin addition resulted in clustering of satellites at the centrosome (Figure6B). Satellite localization ranged from clustering around the centrosomes to scattering throughout the cytosol in cells co-expressing GFP-PCM1-FKBP and HA-BICD2 depending on ectopic BICD2 expression levels (FigureFig 9B).

Importantly, localization of endogenous PCM1 tightly correlated with the localization of GFP-PCM1-FKBP before and after rapamycin addition (Figs 6A and 6B), which is in agreement with PCM1 self-dimerization through its N-terminal coiled-coil domains (Kubo and Tsukita, 2003). To determine the targeting efficiency of endogenous PCM1, I quantified its pericentrosomal levels by measuring fluorescence signal intensities within a 3 μm2 circle

encompassing the centrosome. I found that pericentrosomal levels decreased significantly in Kif5b-expressing (peripherally targeted) cells (6 h: Control: 1 ± 0.53, Rapa: 0.25 ± 0.46, p value <0.0001, 24 h: Control: 1.1 ± 0.55, Rapa: 0.3 ± 0.43, p<0.0001) and increased significantly in BICD2-expressing (centrosomally targeted) cells (24 h: Control: 1 ± 0.07, Rapa: 4.8 ± 1.9, p<0.0001) at 24 h after rapamycin induction (Figure6C and 6D) that shows the efficient re-localization of satellites to cell periphery or cell center with rapamycin induction. Together, these results validated rapid and efficient redistribution of satellites to the cell periphery or center upon rapamycin-induced dimerization.

HA-Kif5b-FRB localized to the cytosol and did not affect the distribution of satellites (Figure9B). Analogous to GFP-PCM1 and endogenous PCM1, GFP-PCM1-FKBP localized to satellites in transfected human cervical carcinoma (HeLa) cells without altering their distribution (Figure9A). HA-BICD2-FRB localized diffusely throughout the cytosol in low-expressing cells and formed granules in high-expressing cells (Figure7B). In contrast to HA-Kif5b, I noticed that HA-BICD2 expression by itself resulted in satellite dispersal throughout the cytosol (Figure 7B), likely through outcompeting endogenous activating adaptors that couple satellites to dynein-dynactin complex. Of note, this is consistent with its previous characterization as a dominant-negative mutant that impairs dynein-dynactin function (Guardia et al., 2019; Hoogenraad et al., 2001; Teuling et al., 2008). Although the activating adaptors required for minus-end-directed movement of satellites are not known, our results validate that chemical dimerization of BICD2 with satellites is sufficient to induce their centrosomal clustering (Figure 6B).

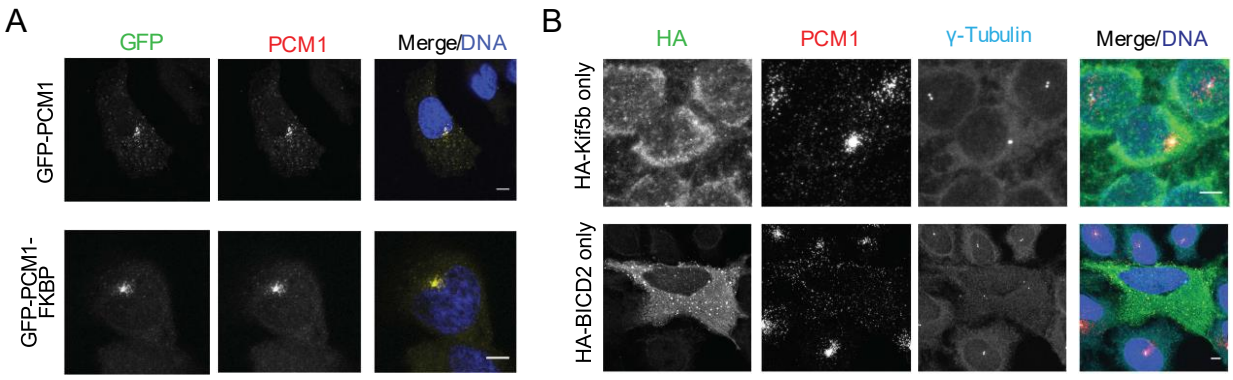

Figure 7: Distribution of satellites in cells only transfected with GFP-PCM1-FKBP, HA-BICD2-FRB and HA-Kif5b-FRB **(A)** Localization of GFP-PCM1 and GFP-PCM1-FKBP in cells. HeLa cells were transfected with GFP-PCM1 or GFP-PCM1-FKBP, fixed after 24 hours, and stained for GFP, PCM1, and DAPI. (B) Effects of HA-BICD2-FRB or HA-Kif5b-

FRB expression on satellite distribution. HeLa cells were transfected with HA-BICD2-FRB or HA-Kif5b-fRB, fixed after 24 hours, and stained for HA, PCM1, gamma-tubulin, and DAPI. Scale bars, 10 μm

As controls, rapamycin treatment of control cells or cells expressing only GFP-PCM1-FKBP did not perturb satellite distribution in cells, as assessed by staining cells with anti-PCM1 antibody (Figure 8).

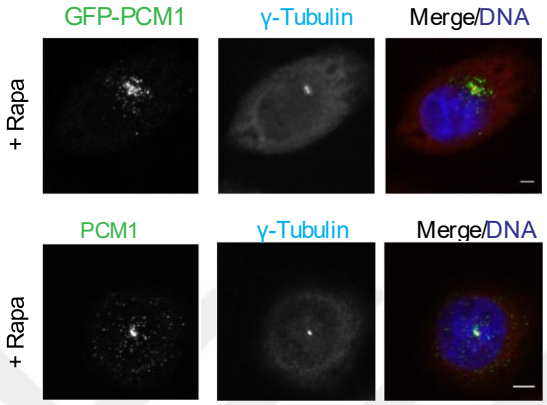

Figure 8: Effect of Rapamycin itself on satellite distribution**.** Rapamycin treatment did not perturb satellite distribution in wild-type cells and cells expressing only GFP-PCM1-FKBP. Cells were treated with rapamycin for 1 hour, fixed after 24 hours, and stained for GFP or PCM1, gamma-tubulin, and DAPI. Scale bar, 10 μm

When the cells were co-transfected with GFP-PCM1-FKBP and HA-BICD2-FRB or HA-Kif5b-FRB, the kinesin protein dispersed at the cytosol while BICD2 range from scattering through the cytosol or concentrate at the centrosome due to the ectopic expression of BICD2 (Figure 9A, 9B).

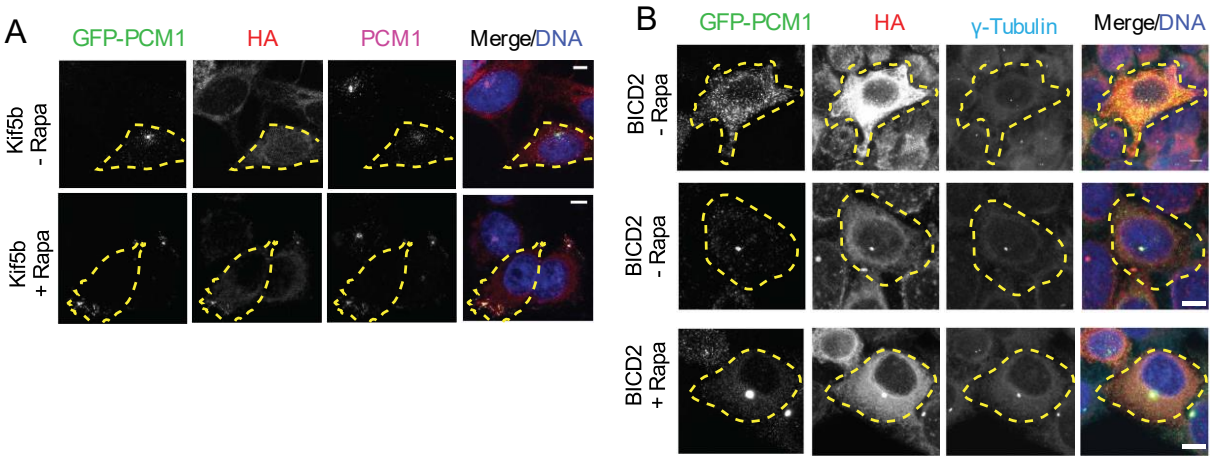

Figure 9: Validation of GFP-PCM1-FKBP and endogenous PCM1 mispositioning upon rapamycin-induced dimerization in cells. Hela cells co-expressing GFP-PCM1-FKBP with (A)

HA-Kif5b-FRB or (B) HA-BICD2-FRB were treated with rapamycin for 1 hour, fixed 24 hours after transfection, and stained for GFP, HA, PCM1, and DAPI. Cells that were not treated with rapamycin were processed in parallel as controls. Scale bar: 10 μm

Notably, I observed partial distribution of both GFP-PCM1-FKBP and PCM1 to the cell periphery or center upon rapamycin addition in a subset of cells, likely due to insufficient expression of the active motors relative to the satellites (Figure 10). This difference in expression levels causes the range of satellite localization from clustering around the centrosome to scattering through the cytosol. Partial distribution likely due to insufficient expression of the active motors relative to the satellites (Figure 10).

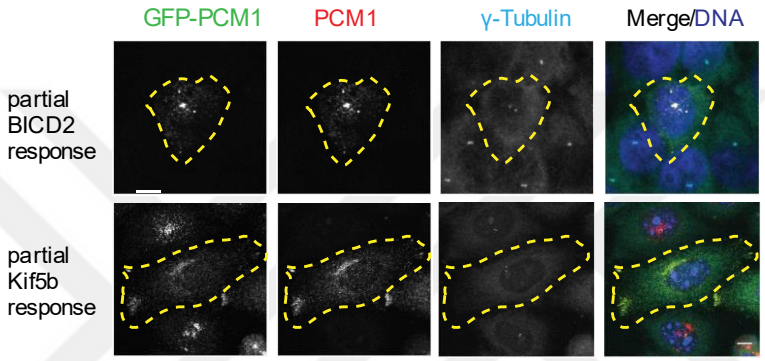

Figure 10 Representation of partial distribution of satellites upon rapamycin induction. HeLa cells co-expressing GFP-PCM1-FKBP with HA-Kif5b-FRB or HA-BICD2-FRB were treated with rapamycin for 1 hour, fixed 24 hours after transfection, and stained for GFP, HA, PCM1, and DAPI. Partial distribution was defined by GFP-PCM1-FKBP signal in the pericentrosomal area in Kif5b-expressing cells and signal in the region excluding the centrosomal area in BICD2-expressing cells. Scale bar, 10 μm

In Kif5b-expressing cells, satellites formed large clusters that were heterogeneously distributed at the peripheral cell protrusions, whereas in BICD2-expressing cells, they formed a single cluster at the centrosome (Figure 6A and 6B) A similar heterogeneous distribution pattern at the periphery was also observed when GFP-PCM1-FKBP was co-expressed with the motor domain of another kinesin Kif17 (Figure 11)

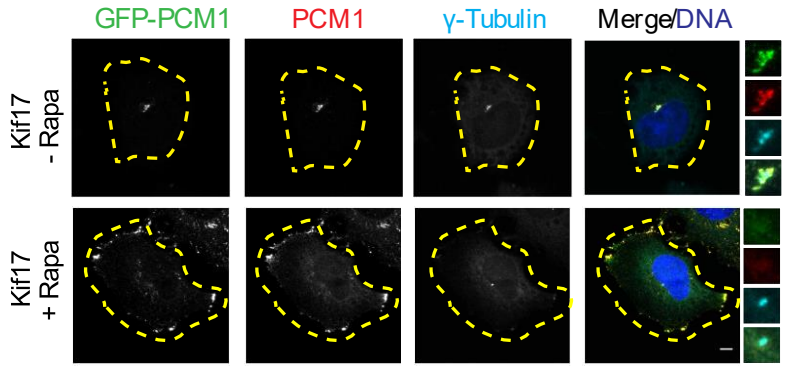

Figure 11: Redistribution of satellites with Kif17-FRB and GFP-PCM1-FKBP co-epression. Co-expression of GFP-PCM1-FKBP with the constitutively active HA-Kif17 (1–181 aa)-FRB targets satellites to the cell periphery, where satellite clusters are heterogeneously distributed. Transfected HeLa cells were treated with rapamycin for 1 hour, fixed after 24 hours, and stained for GFP, PCM1, gamma-tubulin, and DAPI. Scale bar, 10 μm; all insets show 4× enlarged centrosomes.

The integrity and organization of the microtubule network remained unaltered in both cases upon rapamycin-induced redistribution of satellites (Figure 12).

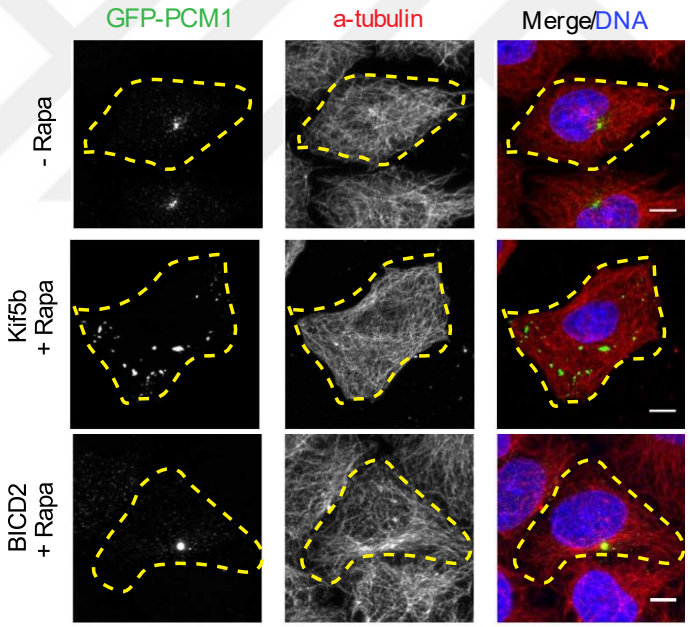

Figure 12: Effect of GFP-PCM1-FKBP with HA-Kif5b-FRB or HA-BICD2-FRB to microtubule network**.** Expression of GFP-PCM1-FKBP with HA-Kif5b-FRB or HA-BICD2-FRB and their redistribution upon rapamycin induction do not perturb the microtubule network. Cells were stained for GFP, alpha-tubulin, and DAPI, Scale bar, 10 μm.

Staining of BICD2-expressing cells for satellites and the mother centriole protein Cep164 revealed tight clustering of satellites around the mother centriole (Figure 13).

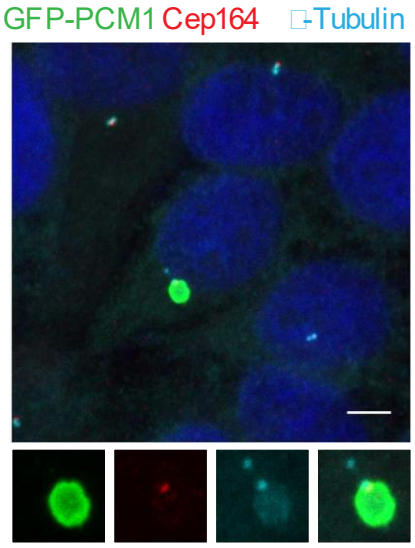

Figure 13: Satellites concentrate around mother centriole in cells co-expressing GFP-PCM1-FKBP with HA-BICD2-FRB after rapamycin treatment. Cells were stained for GFP, mother centriole marker Cep164, and gamma-tubulin. DNA was stained with DAPI. Cell edges are outlined. Scale bar, 5 μm

Given that a subset of centrosomal microtubules are anchored at the subdistal appendages of the mother centrioles, this localization pattern suggests preferential trafficking of satellites along these microtubules (Piel et al., 2000). Because satellite targeting to the cell periphery or center was dependent on microtubules, I next examined whether these satellite cluster(s), once they formed, were maintained independently of microtubules or not. The clusters remained mostly intact and did not split into smaller granules upon depolymerization of microtubules by nocodazole treatment, suggesting that satellite granules fuse into stable solid-like clusters when forced into close proximity (Figure 14). Together, these results show that rapamycin-induced dimerization of satellites with molecular motors irreversibly perturbs their distribution and size, and thus this assay provides a tool to investigate satellite functions in a temporally-controlled manner.

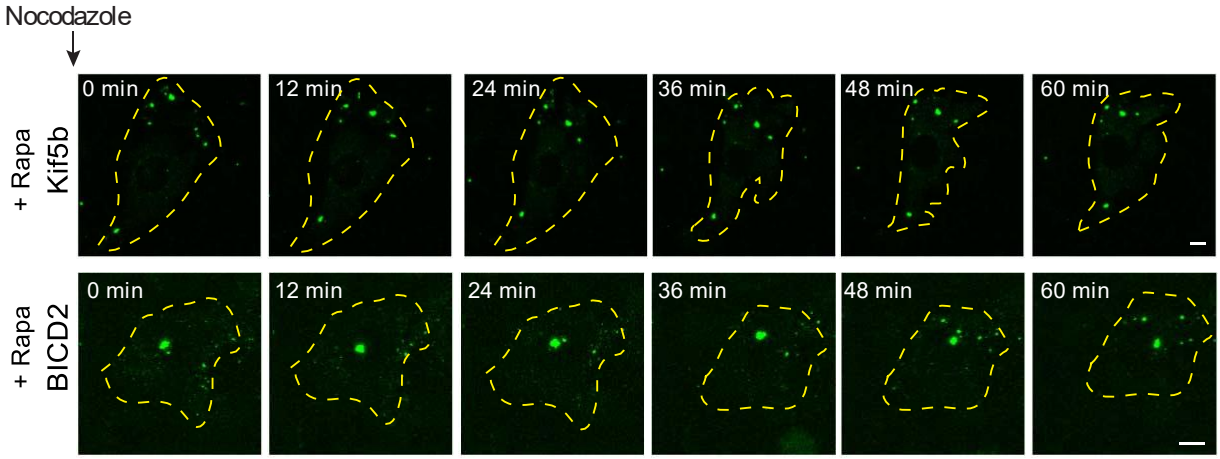

Figure 14: Peripheral and centrosomal satellite clusters were maintained upon microtubule depolymerization. Cells with peripheral or centrosomal satellite clusters were treated with nocodazole for 1 hour and imaged every 3 minutes by time-lapse microscopy. Representative still frames from time-lapse experiments were shown. Cell edges are outlined. Scale bar, 5 µm

Once the satellite granules cluster with each other, they become into solid-state like structure which with previous results, it is shown that satellites are irreversibly loses the distribution and size with rapamycin-induced dimerization but can be controlled temporarily.

4.2 Functional dissection of satellite mispositioning

4.2.1 Effect of PCM1 Re-distribution to Its Residents

Acute or chronic depletion of PCM1, alters the centrosomal levels and dynamics of a subset of proteins (Hames et al., 2005; Dammermann and Merdes 2002; Odabasi et al., 2019). Given that satellites interact with and store a wide range of centrosome proteins, their pericentrosomal localization is likely required for efficient protein targeting to the centrosomes. To test this and to determine which proteins are regulated by satellites, I co-transfected HeLa cells with the indicated constructs and used quantitative immunofluorescence to measure the pericentrosomal levels of various known satellite proteins by measuring fluorescence signal intensities within a 3 µm2 circle encompassing the centrosome. I chose satellite proteins that also localize to different parts of the centrosome (e.g., pericentriolar material, distal appendages, centriole linker) and are implicated in different centrosome/cilium-associated cellular functions (Figure 15 and Table 2).

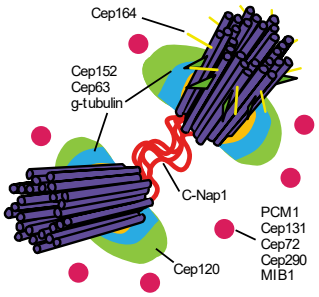

Figure 15: Spatial localization of the selected proteins at the centrosome.

As controls, I quantified the transfected cells in which GFP-PCM1-FKBP localized like endogenous PCM1 and excluded the ones that were impaired for pericentrosomal satellite clustering due to overexpression of molecular motors. For quantifying centrosomal levels in cells with peripheral or centrosomal satellite clustering after rapamycin treatment, I only accounted for the cells that exhibited complete redistribution to the cell center or periphery.

Table 2: Summary of the results for the changes in the pericentrosomal abundance of the indicated proteins and their associated functions and spatial localizations at the centrosome and cilia. "Localization at peripheral clusters" represents the group of proteins that concentrate with PCM1 at the periphery upon rapamycin addition to cells co-expressing GFP-PCM1-FKBP and HA-Kif5b-FRB.

Function-Spatial Localization of the Protein	Protein Name	Localization at Peripheral Clusters	Function-Spatial Localization of the Protein	Protein Name	Localization at Peripheral Clusters
Ciliation	PCM1	+	Centriole Duplication	Cep63	-
Ciliation	Cep290	+	Daughter Centriole	Cep120	-
Ciliation	Cep72	+	Linker	CNAP-1	-
Ciliation	Cep131	+	E3 ubiquitin-protein ligase	MIB1	+
Distal Appendage Protein	Cep164	-	MT Nucleation	Gamma Tubulin	+
Centriole Duplication	Cep152	-		Centrin	+

The pericentrosomal levels of the ciliogenesis factors, including the transition zone component Cep290, the E3 ubiquitin ligase Mib1, Cep131 and Cep72, increased significantly in centrosomally-targeted BICD2-expressing cells and decreased significantly in peripherally targeted Kif5b-expressing cells at 24 h after rapamycin treatment (Figure 16A). I note that these proteins were also recruited to the PCM1-positive clusters at the cell periphery in Kif5b-expressing cells (Figure 16A and 16B). In contrast, there was no significant change in the pericentrosomal levels of the centriole duplication factors Cep63 and Cep152, the distal appendage protein Cep164, the centriole component centrin and the centrosomal linker protein C-Nap1 in cells with peripheral satellite clustering (Figure 16A). Additionally, except for

centrin, these proteins were not recruited to the peripheral aggregates in Kif5b-expressing cells (Figure 16A).

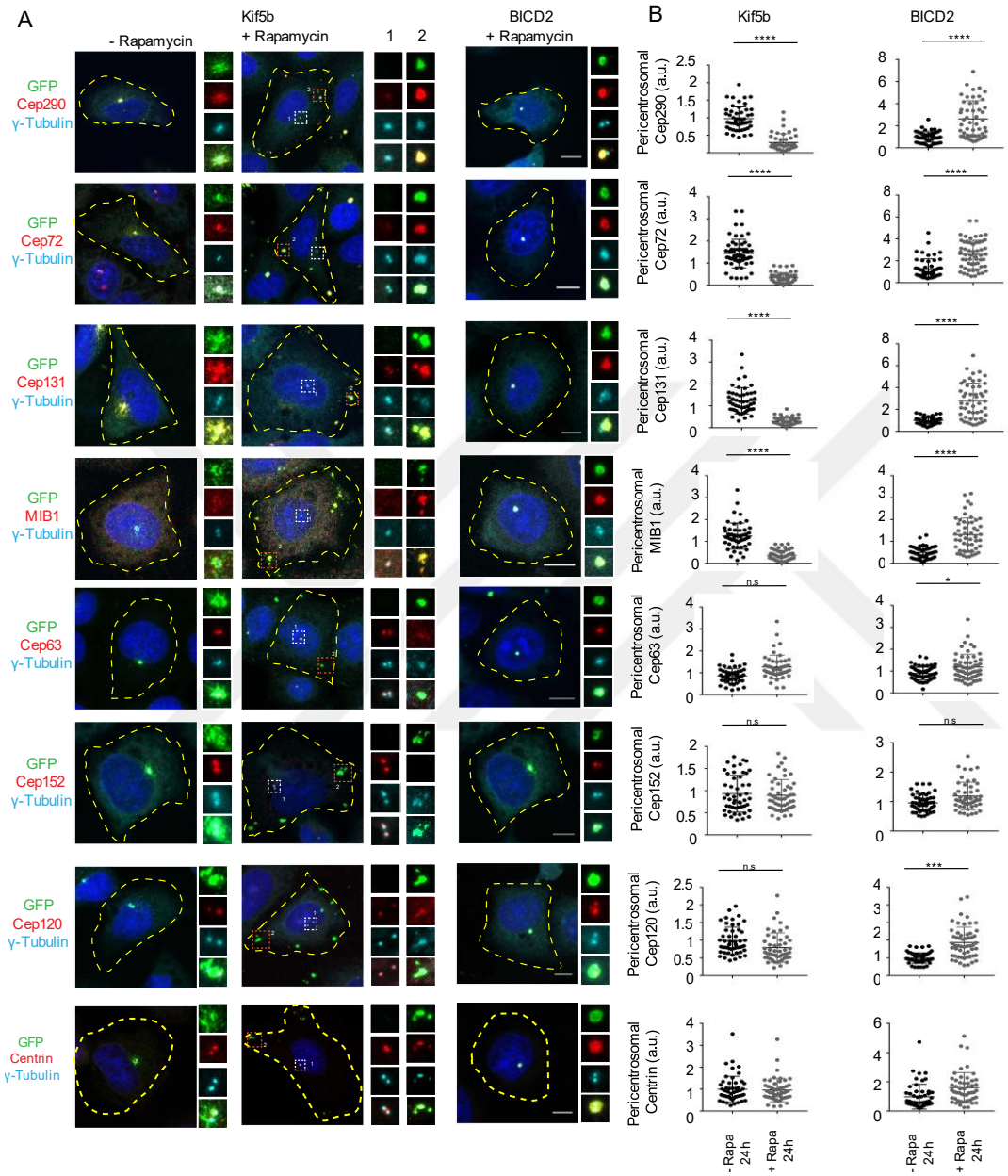

Figure 16: Effects of satellite redistribution to the cell periphery or center on pericentrosomal abundance of multiple satellite residents with varying functions and subcentrosomal localizations A) HeLa cells co-expressing GFP-PCM1-FKBP with HA-Kif5b-FRB or HA-BICD2-FRB were treated with rapamycin for 1 hour followed by fixation at 24 hours. Cells that were not treated with rapamycin were processed in parallel as controls. Cells were stained with anti-GFP to identify cells with complete redistribution to the cell periphery or center, and anti-gamma-tubulin to mark the centrosome and antibodies against the indicated proteins. Images represent centrosomes in cells from the same coverslip taken with the same camera settings. DNA was stained by DAPI. B) Fluorescence intensity at the centrosome was

quantified, and average means of the levels in control cells at 6 hours were normalized to 1. n ≥ 25 cells per experiment. Data represent mean value from two experiments per condition, ±SD (***p < 0.001, ****p < 0.0001). Cell edges are outlined. Scale bars, 10 μm, all insets show 4× enlarged centrosomes. Error bars = SD

I next examined the potential role of satellite positioning in regulating the daughter centriole composition by using the rapamycin-treated BICD2-expressing cells, which leads to satellite accumulation around the mother centriole. I chose the daughter centriole protein Cep120 for further study as it was identified in the satellite proteome and 60% of its centrosomal pool was shown to be mobile (Quarantotti et al., 2019; Betleja et al., 2018).

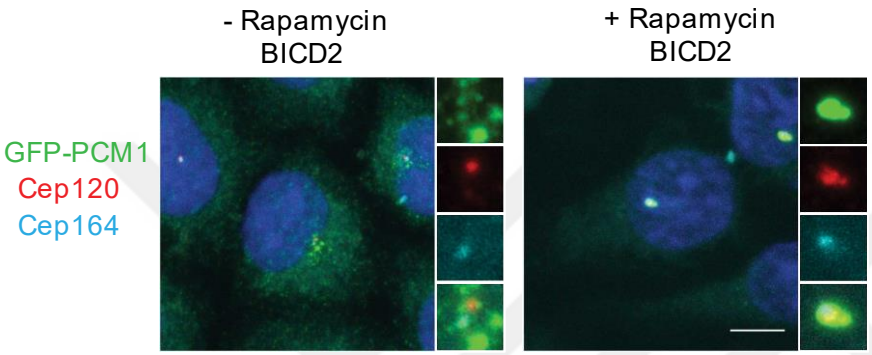

Figure 17: The daughter centriole protein Cep120 was redistributed to the mother centriole in BICD2-expresing cells with centrosomal satellite accumulation. HeLa cells co-expressing GFP-PCM1-FKBP with HA-BICD2-FRB were treated with rapamycin for 1 hour, fixed at 24 hours, and stained for GFP, Cep120, Cep164, and DAPI. Cells that were not treated with rapamycin were used as a control. Scale bars, 10 μm; all insets show 3× enlarged centrosomes.

I stained HeLa cells co-expressing GFP-PCM1-FKBP and HA-BICD2 before and after rapamycin addition with Cep120, the centrosome marker gamma-tubulin, and the mother centriole marker Cep164 (Figure 17). Despite its enrichment in the daughter centriole in control cells, Cep120 was strongly enriched in the mother centriole in BICD2-expressing cells (Figure 17). This indicates that satellite positioning regulates the enrichment of Cep120 at the daughter centriole.

Satellite interactome is highly enriched in microtubule-associated proteins including the key regulators of microtubule nucleation and dynamics such as gamma-tubulin and the components of the HAUS/Augmin complex (Gheiratmand et al., 2019; Quarantotti et al., 2019). While the centrosomal gamma-tubulin levels did not change in cells with peripheral satellite clustering, gamma-tubulin concentrated at the peripheral satellite clusters (Figure 18)

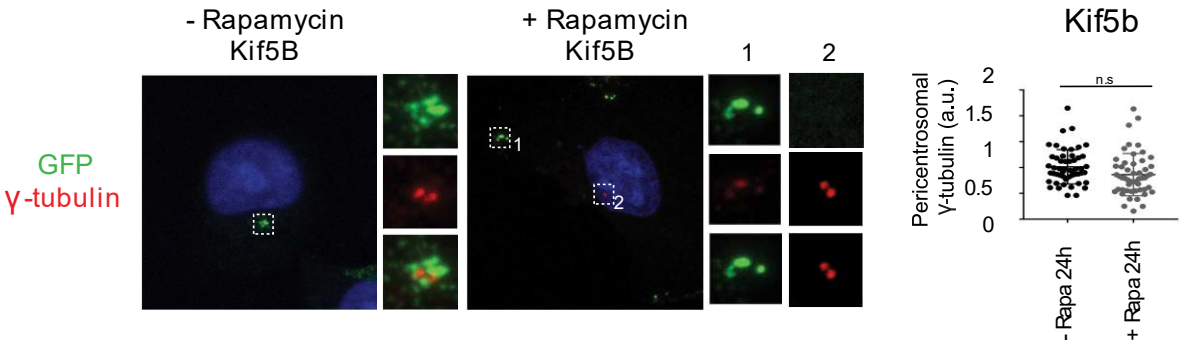

Figure 18: Gamma-tubulin localization in control cells and in Kif5b-expressing cells with peripheral satellite clustering. HeLa cells co-expressing GFP-PCM1-FKBP with HA-Kif5b-FRB were treated with rapamycin for 1 hour, fixed at 24 hours, and stained for GFP, gamma-tubulin, and DAPI. Images represent centrosomes in cells from the same coverslip taken with the same camera settings. Cells that were not treated with rapamycin were processed in parallel as a control. Fluorescence intensity at the centrosome was quantified, and average mean of the levels in control cells were normalized to 1. n ≥ 25 cells per experiment. Data represent mean value from two experiments per condition ± SD (n.s. nonsignificant, ****p < 0.0001). Error bars = SD

To test whether recruitment of gamma-tubulin to these clusters results in microtubule nucleation, I performed microtubule regrowth experiments in IMCD3 cells stably expressing GFP-PCM1-FKBP and HA-Kif5b. After inducing peripheral accumulation of satellites by rapamycin, microtubules were depolymerized by nocodazole treatment. Following nocodazole washout, cells were fixed and stained for alpha tubulin at the indicated time points (Figure 19).

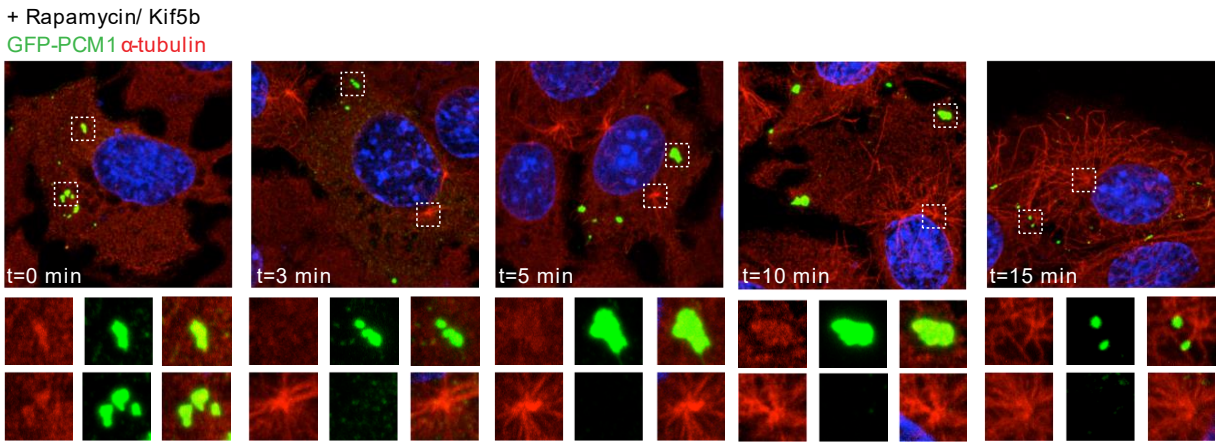

Figure 19: Effect of gamma-tubulin accumulation at the peripheral satellites on microtubule nucleation. Rapamycin-treated IMCD3peripheral cells were treated with DMSO or 10 µg/mL nocodazole for 1 hour. After microtubule depolymerization, cells were washed, incubated with complete media for the indicated times, fixed, and stained for GFP, alpha-tubulin, and DAPI. Scale bars, 10 µm; all insets show 3× enlarged centrosomes

Despite prominent microtubule nucleation at the centrosomes, microtubule nucleation was not initiated at the peripheral gamma-tubulin-positive clusters. Thus, the concentration of gamma-tubulin at the peripheral satellites does not assign them MTOC activity, which might be due to lack of modifications or regulatory proteins required for microtubule nucleation.

4.2.2 Effect of Peripheral Distribution of Centriolar Satellites to Cilium Assembly, Maintenance and Disassembly

Given our results thus far showed that satellites and associated centrosome proteins can be targeted to the periphery rapidly and efficiently in an inducible way, I employed this assay to investigate temporal satellite functions in ciliated and proliferating cells. Satellite-less PCM1-/- kidney and retina epithelial cells had significant defects in their ability to assemble primary cilia (Odabasi et al., 2019; Wang et al., 2016). I therefore first examined whether pericentrosomal clustering of satellites is also required for cilium assembly. To this end, I generated inner medullary collecting duct (IMCD3) cells that stably express GFP-PCM1-FKBP and HA-Kif5b-FRB (hereafter IMCD3^{peripheral} cells). Rapamycin induction resulted in localization of satellites at the cell periphery in both cell lines (Figure 20).

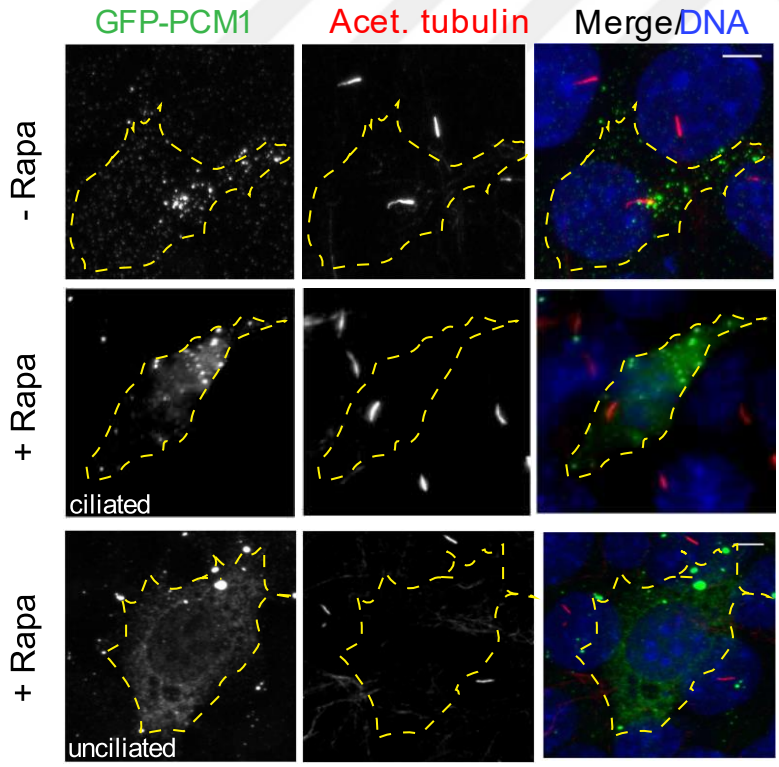

Figure 20: Representative images of ciliated and unciliated cells with peripheral satellite clustering relative to ciliated control IMCD3 cells. Cell edges are outlined. Scale bar, 10 µm.

I were unable to generate cells stably co-expressing GFP-PCM1-FKBP and HA-BICD2-FRB, likely due to the lethality of constitutive BICD2 expression and subsequent inhibition of dynein activity. Therefore, I investigated the consequences of perturbing satellite proximity to the centrosome on cilium assembly only in cells with peripheral satellite clustering. To this end, IMCD3^{peripheral} cells were treated with rapamycin for 1 h, serum-starved for 48 h, and the percentage of ciliated cells was quantified by staining cells for Arl13b, a marker for the ciliary membrane, and acetylated tubulin, a marker for the ciliary axoneme (Figure 21).

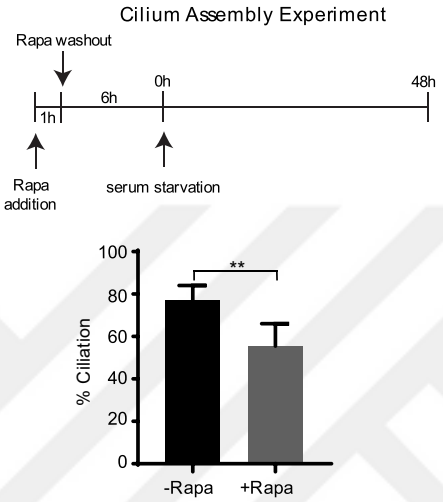

Figure 21: Quantification of the percentage of ciliogenesis in control and rapamycin-treated IMCD3^{peripheral} cells 48 hours after serum starvation. Results shown are the mean of two independent experiments ± SD (= 50 cells/experiment, **p < 0.01)

IMCD3^{peripheral} cells had a significant reduction in their ciliation efficiency relative to control cells (control: 77.35% ± 1.65%, IMCD3^{peripheral}: 54.4% ± 1.4) ($p<0.01$) (Figure 21 As a control for the possible effects of rapamycin on cilium assembly, I performed these experiments in IMCD3 cells treated with rapamycin and found that the concentrations and incubation times I used did not affect ciliogenesis efficiency ($p=0.87$) (Figure 22).

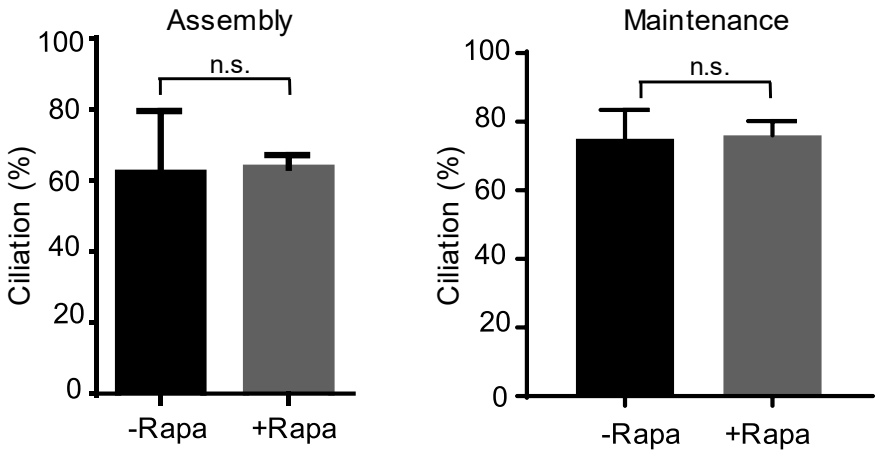

Figure 22: Rapamycin treatment by itself in IMCD3 cells does not affect the efficiency of ciliogenesis and cilium maintenance. The experiments were performed in IMCD3 cells following the experimental outline for ciliogenesis and maintenance experiments in Figure 21 and 23, the fraction of ciliated cells were quantified after 48 hours of serum starvation for the cilium assembly experiment and 24 hours after rapamycin treatment for maintenance experiments. Results shown are the mean of two independent experiments ± SD (= 200 cells/experiment).

The inducible nature of the satellite trafficking assay enabled us to study the temporal function of satellites in ciliated cells during primary cilium maintenance and disassembly, which could not have been tested in satellite-less PCM1-/- cells (Wang et al., 2016; Odabasi et al., 2019) To study the function of satellites in regulation of steady-state cilia stability, cells were serum starved for 48 h, treated with rapamycin for 1 h, and the percentage of cilia was quantified in a time-course manner after rapamycin washout (Figure 23). As expected, the percentage of ciliation did not change over 24 h in control cells (Figure 22 and 23). However, induction of peripheral satellite clustering resulted in a 22% decrease in the percentage of ciliated cells at 6 h after rapamycin treatment (control: 69% ± 0.5%, Rapa:47.6% ± 3.3, $p<0.0001$). The fraction of ciliated cells continued to decrease over time until only 38% ± 1% of cells retained primary cilia after 24 h of rapamycin treatment, compared to the stable 69.5% ± 0.5% of control cells ($p< 0.0001$) (Figure 23). Rapamycin treatment had no effect on maintenance of control cells (Figure 22).

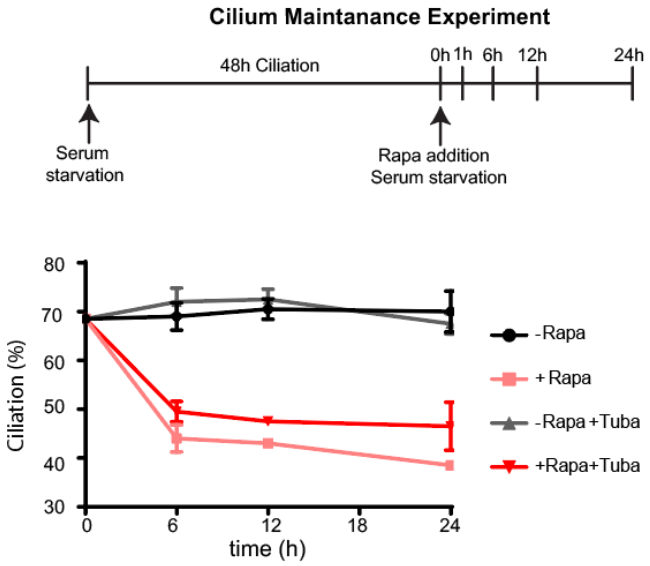

Figure 23: Effect of peripheral satellite clustering on cilium maintenance. Cells were serum-starved for 48 hours, treated with rapamycin for 1 hour, and percentage of ciliated cells was determined over 24 hours by staining for acetylated tubulin. Cells that were not treated with rapamycin were used as a control. The same experiments were also performed in the presence of 2 μM tubacin. Data represent the mean value from two experiments per condition.

Next, I examined whether satellite mispositioning can affect cilium disassembly after serum re-stimulation. To this end, cells were serum starved for 48 h, treated with rapamycin for 1 h, and the percentage of cilia was quantified over a 6h serum-stimulation time course (Figure 24). In control cells, the percentage of ciliation decreased from 68.5% ± 0.5 to 56% ± 1 at 2 h, 46% ± 0 at 4 h and to 38% ± 4.5 at 6 h (Figure 24). In rapamycin-treated cells, there was a rapid and significantly greater decrease in the fraction of ciliated cells at 2 h, from 68.5% ± 0.5 to 45% ± 0 (Figure 24), which corresponds to the first wave of cilium disassembly (Mirvis et al., 2019; Sanchez and Dynlacht, 2016). After 6 h post serum addition, only 30.5% ± 2.5 of cells with peripheral satellite localization had cilia, compared to 38% ± 4 of control cells (p=0.15) (Figure 24). The enhanced deciliation and disassembly phenotypes upon peripheral satellite clustering identified satellites as regulators of cilium maintenance and disassembly in addition to their reported functions in assembly.

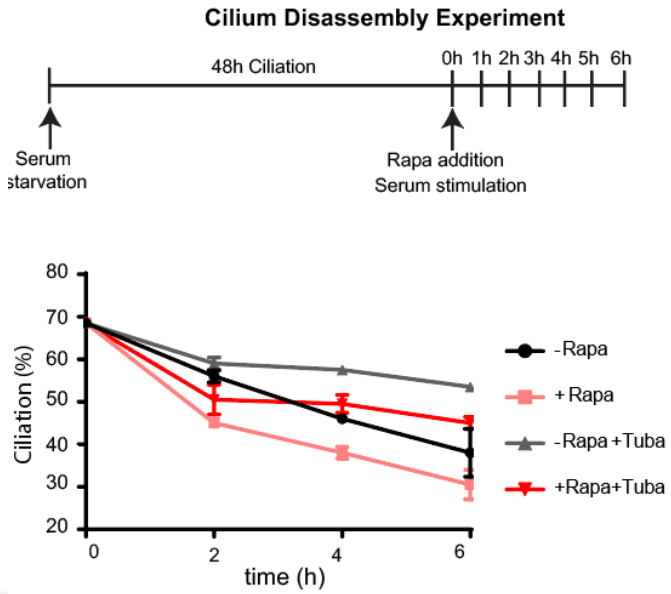

Figure 24: Effect of peripheral satellite clustering on cilium disassembly. Cells were serum-starved for 48 hours, treated with rapamycin for 1 hour, induced by serum-stimulation, and the percentage of ciliated cells was determined over 6 hours by staining for acetylated tubulin. Cells that were not treated with rapamycin were used as a control. The same experiments were also performed in the presence of 2 μM tubacin. Data represent mean value from two experiments per condition.

The maintenance and disassembly of cilium requires modifications of the axonemal tubulins. A major event that promotes cilium disassembly is the phosphorylation of histone deacetylase 6 (HDAC6) by Aurora A kinase and subsequent deacetylation of the modified tubulins of the ciliary axoneme and cortactin (Pugacheva et al., 2007; Ran et al., 2015). To test whether satellites regulate cilium maintenance and disassembly in a HDAC6-dependent manner, I performed cilium maintenance and disassembly experiments in control and rapamycin-treated cells using tubacin as a potent and selective inhibitor of HDAC6 deacetylase activity (Ran et al., 2015). Analogous to control cells, tubacin treatment rescued the enhanced cilium disassembly phenotype of rapamycin-treated cells at 4 and 6 h (4 h: Rapa: 38% ± 4.5%, tubacin+Rapa: 49.5 ± 1.5% ($p<0.01$); 6h: Rapa: 30.5% ± 2.5%, tubacin+rapa: 45% ± 1% , $p<0.01$), but did not rescue the cilium maintenance defects at 6,12 or 24 h.($p>0.05$) (Figs 23 and 24). These results show differential requirements for regulation of cilium maintenance and disassembly and suggest that satellites function in cilium disassembly by regulating the structural integrity of the ciliary axoneme upstream of HDAC6.

5. DISCUSSION

5.1 Development and validation of the inducible satellite trafficking assay

Chemically inducible satellite trafficking assay was developed that allows to target satellites rapidly and specifically to the cell periphery. This method is used to study satellite functions in a temporally controlled way in ciliated and proliferating cells. Application of this assay in epithelial cells for the first time identified functions for satellites not just for cilium assembly, but also for cilium maintenance and cilium disassembly. Targeted and systematic quantification of the satellite proteome at the cell periphery suggested defective centrosomal targeting of proteins implicated in these processes as possible mechanisms underlying these phenotypes. In addition to probing temporal satellite functions, our results for the first time also revealed a direct link between satellite functions and their cellular positioning. Importantly, the satellite-trafficking assay provides a powerful tool for future studies aimed at investigating cell-type specific functions and mechanisms of satellites. Its use in specialized cell types, in particular the ones with non-centrosomal MTOCs such as differentiated muscle cells and polarized epithelial cells, has the potential to provide new insight into why and how satellite distribution varies in different contexts.

5.2 Functional Dissection of Satellite Mispositioning

Inducible peripheral satellite targeting in ciliated cells revealed novel functions for satellites as positive regulators of cilium maintenance and disassembly. Cilium disassembly upon serum stimulation in IMCD3 cells occurs predominantly by whole-cilium shedding, which requires HDAC6 activity (Mirvis et al., 2019). Enhanced cilium disassembly defect of rapamycin-induced IMCD3^{peripheral} cells was partially restored by HDAC6 inhibition, suggesting that satellites function upstream of HDAC6. Because HDAC6 was not identified in the satellite interactome, it is likely that satellites are not direct regulators of HDAC6 activity and that they might take part in cilium disassembly through regulating other disassembly regulators such as PLK1 (Gheiratmand et al., 2019; Quarantotti et al., 2019). Of note, PLK1 is part of the peripheral satellite proteome I generated in this study. Importantly, tubacin treatment did not rescue the cilium maintenance defect, highlighting differential regulation of cilium maintenance and disassembly through independent and/or overlapping mechanisms. Given that I identified the IFT-B components including IFT20, IFT81, IFT57, IFT46 and IFT74 in the proteome of peripheral satellites in Kif5b-expressing cells, I propose that perturbation of IFT trafficking through sequestration of IFT components at the peripheral satellite clusters might underlie the

cilium maintenance defects. Supporting this mechanism, sequestration of IFT20 and IFT74 at the mitochondria using rapamycin-dimerization approach in ciliated fibroblasts resulted in a similar defect in cilium maintenance (Eguether et al., 2018). Defective IFT-B complex targeting could also explain the cilium assembly and shorter cilia phenotypes associated with peripheral satellite clustering. With identified regulatory roles for satellites in IFT88 targeting to the basal body and cilia (Odabasi et al., 2019), it is indicated the presence of a critical, but poorly understood regulatory relationship between satellites and the IFT-B complex. Whether IFT-B complex is regulated at the assembly, activity and/or targeting level by satellites remain as critical outstanding questions.

Depletion or deletion of various centrosome proteins perturbs cellular positioning of satellites either by inducing their dispersal throughout the cytoplasm or tighter concentration at the centrosome (Prosser and Pelletier, 2020; Hori and Toda, 2017). The satellite distribution defects have been proposed to underlie the phenotypes associated with loss of these proteins such as defective ciliogenesis. However, these loss-of-function studies remained insufficient in directly testing this proposed link. The results of our study provide direct evidence for the functional significance of proper satellite positioning during primary cilium-associated processes, and thus strengthens the conclusions of the previous work on how perturbed satellite localization might contribute to the function of their residents.

Satellites are proposed to mediate their functions in the context of centrosomes and cilia by trafficking proteins to or away from the centrosome and by sequestering centrosome proteins to limit their recruitment at the centrosome (Prosser and Pelletier, 2020; Hori and Toda, 2017). Using quantitative analysis of pericentrosomal levels of multiple centrosome proteins upon satellite redistribution, I found that only a subset of proteins required proper satellite distribution for their centrosomal localization. While these results provide further support to the functions of satellites in protein targeting, they also raise questions that pertains to why and how satellites do not regulate all their residents at this level. To gain insight into the rules that govern satellite selectivity, I categorized proteins regulated by satellites based on their evolutionary conservation pattern, functions, subcentrosomal localization and interactions. These analyses did not reveal a tight relationship between these properties and satellite-mediated regulation. The only relationship that stood out was the one between proteins that interact and regulate the same cellular process such as Cep72-Cep290 ciliogenesis module (Firat-Karalar et al, 2014; Kodani et al., 2015; StoI et al., 2012). Co-regulation of these proteins suggest functions for

satellites in mediating assembly of these complexes and/or their targeting to the centrosome as a complex.

Microtubules and molecular motors were shown to cooperate with satellites to regulate active protein targeting of satellite residents to the centrosome (Prosser and Pelletier, 2020). Because our results revealed a new functional relationship between proper pericentrosomal satellite positioning and centrosome/cilia-mediated processes, I propose a complementary way by which microtubules and motors regulate satellite-mediated functions. Our results suggest that they cooperate to maintain satellite proximity around the centrosome and that this is required for timely and efficient exchange of proteins between centrosomes and satellites. Notably, satellite granules fuse and form stable aggregates when forced into close proximity at the cell center or periphery, suggesting that cells have mechanisms to inhibit aggregation of satellites. Future studies are required to address several key questions that pertains to satellite mechanisms and cellular distribution: What is the precise mechanism that establishes and maintains pericentrosomal satellite clustering and inhibit their aggregation? Do satellites exchange material with the centrosome through active transport or simple diffusion? What are the upstream signaling pathways that induce protein release from satellites?

The direct link between cellular satellite positioning and their functions revealed by this study corroborates the notion that functions associated with different cellular compartments are regulated by their distinct spatial distribution within cells (van Bergejk et al., 2015). This relationship has been reported for multiple organelles and functions. One example is the nutrient-induced peripheral and starvation-induced perinuclear positioning of lysosomes, which was proposed to coordinate mTOR activity and autophagosome biogenesis during cellular anabolic and catabolic responses (Korolchuk et al., 2011). Another example is the requirement for proper mitochondrial positioning during T cell activation to allow sustained local Ca+2 influx and during neuronal differentiation to allow terminal axon branching (Courchet et al., 2013; Schwindling et al., 2010).

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