

**Molecular and Functional Dissection of the Retinal Degeneration Protein
CCDC66**

by

Deniz Çonkar

A Dissertation Submitted to the
Graduate School of Sciences and Engineering
in Partial Fulfillment of the Requirements for
the Degrees of
Doctor of Philosophy

In
Molecular Biology and Genetics



July 2020

Molecular and Functional Dissection of the Retinal Degeneration Protein CCDC66

Koç University

Graduate School of Sciences and Engineering

This is to certify that I have examined this copy of a doctoral dissertation by

Deniz Çonkar

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

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

Assoc. Prof. Nurhan Özlü (Koç University)

Prof. Dr. Arzu Karabay Korkmaz (Istanbul Technical University)

Prof. Dr. Uygur Tazebay (Gebze Technical University)

Asst. Prof. Oktay İsmail Kaplan (Abdullah Gül University)

Date: _____



*To my dearest family and my true friends,
they go beyond space and time...*

ABSTRACT

Mammalian centrosome/cilium complex is a signaling hub which is essential for many cellular processes during embryonic development and tissue homeostasis. Defects in its structure and/or function may lead to cancer and ciliopathies. Centriolar satellites are protein-based granules that localize and move around the centrosome/cilium complex in a microtubule-dependent manner. They are implicated to play roles in centrosome duplication, centrosomal and ciliary protein trafficking and cilium formation. However, the molecular mechanism of this regulation remains poorly understood.

In the first chapter of my thesis, I studied the role of satellites during ciliogenesis, by determining the proximity partners of a known satellite protein CEP72 by Biotin Identification (BioID). Focusing on one of its proximity partners, I identified the retinal degeneration gene product CCDC66 as a microtubule-associated protein that localizes to the centrosome, centriolar satellites and the primary cilium. Like BBS4, CCDC66 relocated from satellites to the primary cilium during ciliogenesis. Proximity mapping of CCDC66 revealed extensive proximity interactions with centriolar satellite proteins and co-immunoprecipitation experiments identified interactions between CCDC66, CEP290 and PCM1. Loss of CCDC66 caused defects in ciliogenesis, ciliary recruitment of BBS4 and centriolar satellite organization. My findings identified CCDC66 as a targeting factor for centrosome/cilium proteins.

In the second chapter of my thesis, I focused on understanding protein targeting mechanisms to centrosome and cilia through studying CCDC66 dynamics by Fluorescent Recovery After Photobleaching (FRAP). My analysis showed that CCDC66's centrosomal pool was dynamic and its ciliary pool associated with the ciliary axoneme and was stable. By perturbing microtubule dynamics through different drug treatments, I showed that centrosomal CCDC66 abundance and dynamics required microtubule-dependent active transport and tethering, and was inhibited by sequestration at satellites. Systematic quantitation of satellite dynamics identified only a small fraction to display microtubule-based bimodal motility, consistent with trafficking function. Majority displayed diffusive motility with unimodal persistence, supporting sequestration function. My findings revealed new mechanisms of communication within the centrosome/cilium complex compartments.

ÖZET

Sentrozom/silyum kompleksi, embriyonik gelişim ve doku homeostazı sırasında meydana gelen hücrel olaylar için önemli bir sinyal mekanizmasıdır. Yapısındaki ve/veya fonksiyonlarındaki problemler kansere ve silyopatilere sebep olabilir. Sentriyolar satelitler, sentrozom/silyum kompleksine yerleşen ve onun etrafında mikrotübüllere bağlı şekilde hareket eden protein bazlı granüllerdir. Sentriyolar satelitlerin sentrozom bölünmesi, sentrozom ve silyum proteinlerinin taşınması ve silyum oluşmasında görev aldığı düşünülmektedir. Fakat, bu olayların altında yatan moleküler mekanizma bilinmemektedir.

Tezimin ilk bölümünde, sentriyolar satelitlerin silyum oluşumu sırasındaki görevini bilinen bir satelit proteini olan CEP72'nin proksimite partnerlerini BioID proksimite işaretleme yöntemini kullanarak belirledim. CEP72'nin proksimite partnerlerinden biri olan CCDC66'ya odaklanarak CCDC66'nın mikrotübül-ilişkili bir protein olduğunu, sentrozoma, silyuma ve sentriyolar satelitlere yerleştiğini gösterdim. BBS4 gibi CCDC66 da silyum oluşması sırasında satelitlerden silyuma yerleşti. CCDC66'nın BioID analizi, çoğunluğu satelit proteinlerinden oluşan bir proksimite partner haritası ortaya çıkardı. Ko-immünopresipitasyon deneyleri CCDC66, CEP290 ve PCM1 arasındaki fiziksel etkileşimi belirledi. CCDC66 ifadesinin eksikliği silyum oluşumunda, BBS4'ün silyuma iletilmesinde ve sentriyolar satelit organizasyonunda problemlere yol açtı. Bulgularım, CCDC66'nın sentrozom/silyum proteinleri için hedef belirleyici bir faktör olduğunu gösterdi.

Tezimin ikinci bölümünde, sentrozom ve silyuma proteinlerin nasıl taşındığını CCDC66 dinamiklerini FRAP metodu ile çalışarak irdeledim. Analizlerim, CCDC66'nın sentrozom havuzunun dinamik olduğunu, silyum havuzunun ise stabil olup akzonem ile etkileştiğini gösterdi. Değişik ilaçlar ile mikrotübül dinamiklerini değiştirerek sentrozomdaki CCDC66 miktarı ve dinamiklerinin mikrotübüle bağlı aktif transporta ihtiyaç duyduğunu ve satelitler tarafından depolanmasıyla engellendiğini gösterdim. Satelit dinamiklerinin sistemik kantitatif analizi ile küçük bir satelit grubunun, satelitlerin protein taşıma fonksiyonları ile uyumlu olarak, mikrotübüle bağlı çift modlu harekete sahip olduğunu belirledim. Geriye kalan çoğunluğun ise satelitlerin protein depolama fonksiyonu ile uyumlu olarak tek modlu bir şekilde pasif difüzyon hareketi yaptığını gösterdim. Bulgularım, sentrozom/silyum kompleksi içerisinde yeni iletişim mekanizmaları ortaya çıkardı.

Work presented in this thesis has been published in the following books/journals:

Introduction:

Chapter 6 - Structure and Function of the Primary Cilium. Flagella and Cilia: Types, Structure and Functions. *Nova Science Publishers*. pp 129-167 (2018)

Chapter 1:

The centriolar satellite protein CCDC66 interacts with CEP290 and functions in cilium formation and trafficking. *Journal of Cell Science*. (2017)

Chapter 2:

Centrosomal and ciliary targeting of CCDC66 requires cooperative action of centriolar satellites, microtubules and molecular motors. *Scientific Reports* (2019)

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my supervisor Dr. Elif Nur Fırat-Karalar. Working with her has been an intense journey of growth as a scientist. Her enthusiasm, resilience and ambition has carved a unique road in my life and will lead me for the rest of my scientific career.

I would like to next thank to my thesis monitoring committee members, Dr. Nurhan Özlü and Prof. Dr. Arzu Karabay Korkmaz for their valuable comments and contributions during the formation of my thesis work and to the other members of my thesis committee, Prof. Dr. Uygur Tazebay and Dr. Oktay İsmail Kaplan for their critical reading of my thesis and their comments.

I would also like to thank to Dr. Sedat Nizamoğlu, we have been collaborating during my doctoral work, I have learnt a lot from his decent and honest personality as a scientist.

I would like to acknowledge the financial support from Scientific and Technological Research Council of Turkey (TÜBİTAK) and Koç University during my doctoral studies.

Next, I would like to express my gratitude to my dearest labmates Ezgi Odabaşı, Efraim Culfa, Melis Dilara Arslanhan, Özge Zelal Aydın, Onur Taflan, Dila Gülensoy, Umut Batman, Ebru Topçu, Fatmanur Tiryaki, Deniz Örnek and Jovana Deretic, I am very lucky to have worked with such brilliant people. We shared many precious moments of laughter and sorrow and faced hard realities of becoming who we are. Especially, with Ezgi and Efraim, we went over many obstacles together, day and night, since the beginning; with Ezgi, Melis and Özge, we had the most fun and craziest moments while holding onto one another during stressful times. I also thank all of my labmates for their valuable discussions and contributions to my thesis work during our lab meetings.

Lastly, I would like to thank to my family, my mother Hülya, my father Rindol and my brother Oğuz, for always being there for me, encouraging me and supporting me no matter what happens in life.

TABLE OF CONTENTS

ABSTRACT.....	iv
ÖZET	v
ACKNOWLEDGEMENTS	vii
LIST OF FIGURES.....	xi
NOMECLATURE	xiii
Chapter 1: INTRODUCTION.....	1
1.1 The Primary Cilium Structure	1
1.1.1 Ciliary Compartments	4
1.2 Primary Cilium Assembly and Disassembly	5
1.2.1 Parts List for the Primary Cilium	8
1.3 Mechanisms for Ciliary Compartmentalization	11
1.3.1 Transition Zone	12
1.3.2 Intraflagellar Transport	15
1.3.3 BBSome Complex.....	17
1.3.4 Centriolar Satellites	19
1.3.5 Vesicular Trafficking.....	20
1.3.6 Ectosomes.....	20
1.4 Primary Cilium as the Sensory Organelle.....	22
1.5 Conclusion	25
Chapter 2: CCDC66 INTERACTS WITH CEP290 AND FUNCTIONS IN CILIAM FORMATION AND TRAFFICKING.....	26
2.1 Introduction.....	26
2.2 Literature Review	27
2.3 Materials and Methods.....	30
2.3.1 Cell Culture and Transfection	30
2.3.2 RNAi and Rescue Experiments.....	30
2.3.3 Immunoprecipitation and BioID pulldowns.....	30
2.3.4 Microtubule Pelleting Assays.....	31
2.3.5 Mass Spectrometry and Data Analysis	31
2.3.6 Immunofluorescence, microscopy and quantitation.....	32
2.3.7 Statistical Analysis	32

2.4 Results	33
2.4.1 Proximity mapping identified new centriolar satellites proteins	33
2.4.2 CCDC66 Localizes to The Centrosome/Cilium Complex and The Centriolar Satellites	37
2.4.3 Analysis of CCDC66 Deletion Mutants for Localization To The Centriolar Satellites and The Centrosome	47
2.4.4 CCDC66 is a Microtubule-Associated Protein	49
2.4.5 Overexpression of CCDC66 Disrupts Organization of Centriolar Satellites and Inhibits Primary Cilium Formation	56
2.4.8 CCDC66 is Required for Relocalization of BBS4 From Centriolar Satellites to Primary Cilia During Ciliogenesis	71
2.5 Discussion	73
Chapter 3: CENTROSOMAL AND CILIARY TARGETING OF CCDC66 REQUIRES COOPERATIVE ACTION OF CENTRIOLAR SATELLITES, MICROTUBULES, AND MOLECULAR MOTORS	76
3.1 Introduction	76
3.2 Literature Review	77
3.3 Materials and Methods	
3.3.1 Immunofluorescence, antibodies and microscopy	81
3.3.2 Fluorescence Recovery After Photobleaching	82
3.3.3 Cell lysis and Immunoblotting	82
3.3.4 Immunoprecipitation	83
3.3.5 In vitro Microtubule Pelleting Assay	83
3.3.6 Statistical Tests	84
3.4 Results	85
3.4.1 Centriolar Satellites Negatively Regulate CCDC66 Centrosomal Recruitment and Kinetics	85
3.4.2 Centrosomal CCDC66 Recruitment Requires an Intact and Dynamic Microtubule Network	91
3.4.3 The Dynein Complex Interacts with CCDC66 and Mediates Centrosomal Recruitment and Dynamics of CCDC66	96
3.4.4 Ciliary CCDC66 Is Not Dynamic and Its Ciliary Recruitment Is Regulated By Satellites	103
3.4.5 Centriolar Satellites Exhibit Both Diffusive and Directional Motility	111
3.4.6 The Directed Motility and Random Movement of Satellites are Mediated by Microtubules	118

3.5 Discussion 122

Bibliography..... 127

Vita..... 147

Appendix A..... 148

Appendix B..... 149



LIST OF FIGURES

Figure 1. Primary cilium structure

Figure 2. Primary cilium assembly pathways

Figure 3. Identification of the ciliary proteome by APEX2-based proximity labeling

Figure 4. Overview of trafficking pathways involved in ciliary compartmentalization

Figure 5. Ectosome release from the ciliary tip

Figure 6. Hedgehog signaling pathway

Figure 7. Localization, activity and proximity interactors of BirA*-tagged CEP72

Figure 8. Localization and interaction analysis of CCDC66 deletion mutants

Figure 9. Endogenous CCDC66 expression and localization in cultured RPE1 cell line

Figure 10. Expression and cell cycle dependent localization of GFPCCDC66 in RPE1 cells

Figure 11. CCDC66 localization in serum starved RPE1 cells

Figure 12. Analysis of CCDC66 and its deletion mutants for colocalization with centrosomes and centriolar satellites

Figure 13. CCDC66 overexpression associates with and results in microtubule bundles

Figure 14. CCDC66 associates with microtubules in vitro

Figure 15. C terminal of CCDC66 binds to microtubules

Figure 16. CCDC66 overexpression disrupts organization of centriolar satellites

Figure 17. Localization and proximity interactors of BirA*-tagged CCDC66 and CCDC66-RD

Figure 18. CCDC66 interactions with CEP290 and CEP72

Figure 19. CCDC66 regulates cilium formation

Figure 20. CCDC66 regulates centriolar satellite distribution

Figure 21. CCDC66 regulates organization of interphase microtubules

Figure 22. CCDC66 regulates LAP-BBS4 recruitment to the cilium

Figure 23. Centriolar satellites inhibit CCDC66 dynamic localization at the centrosome.

Figure 24. FRAP in PCM1 depleted cells

Figure 25. CCDC66 accumulates at centriolar satellites in CEP290 and CEP72 depleted cells

Figure 26. Steady state CCDC66 levels are regulated by microtubule network

Figure 27. An intact and dynamic microtubule network is required for CCDC66 dynamic localization at the centrosome

Figure 28. The dynein complex interacts and co-localizes with CCDC66

Figure 29. Activity of molecular motors are required for CCDC66 dynamic localization at the centrosome.

Figure 30. Ciliary CCDC66 is not dynamic.

Figure 31. Whole and partial FRAPs of ciliary GFP-CCDC66 and GFP-BBS4.

Figure 32. CCDC66 recruitment to primary cilium is regulated by satellites.

Figure 33. CCDC66-positive centriolar satellites exhibit both diffusive and microtubule-mediated directional motility

Figure 34. Simulation of motility patterns observed for CCDC66 and PCM1 satellites.

Figure 35. GFP-CCDC66 exhibits fusion and splitting with centrosome and other satellites.

Figure 36. PCM1-positive centriolar satellites exhibit both diffusive and microtubule-mediated directional motility

Figure 37. Different modes of CCDC66 targeting to the centrosome

Table 1. Proximity interactors of CCDC66 and CCDC66-RD

NOMECLATURE

AMP-PNP	Adenylyl-imidodiphosphate
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
AZI1	5-azacytidine-induced protein 1
BAC	Bacterial Artificial Chromosome
BBS	Bardet Biedl Syndrome
BBS4	Bardet Biedl Syndrome 4
BBSome	Bardet Biedl Syndrome Complex
BICD1	Bicaudal D Homolog 1
BICD2	Bicaudal D Homolog 1
BioID	Biotin Identification
BirA	Bifunctional ligase
BRB80	PEM (PIPES, EGTA, Magnesium) buffer
BSA	Bovine Serum Albumin
C	Centroid
CC	Coiled-coil domain
CCDC11	Coiled-coil domain containing protein 11
CCDC13	Coiled-coil domain containing protein 13
CCDC14	Coiled-coil domain containing protein 14
CCDC66	Coiled-coil domain containing protein 66
CDK5RAP2	CDK5 regulatory subunit-associated protein 2
CEP131	Centrosomal protein of 131 kDa
CEP152	Centrosomal protein of 152 kDa
CEP162	Centrosomal protein of 162 kDa
CEP290	Centrosomal protein of 290 kDa
CEP55	Centrosomal protein of 55 kDa

CEP72	KIAA0753
D	Centrosomal protein of 72 kDa
DAPI	Direct distance
DMEM	4',6-diaminodino-2-phenylindole
DMEM12	Dulbecco's Modified Eagle Medium
DMSO	DMEM/Nutrient Mixture-12
DsRed	Dimethyl sulfoxide
DYNC1IC1	Red fluorescent protein derived from <i>Discosoma sp.</i>
DYNLL1	Cytoplasmic dynein 1 intermediate chain 1
EGTA	Cytoplasmic dynein light chain 1
FBS	Ethylene glycol-bis(2-aminoethylether)-N,N,N'
FGFR1	Fetal Bovine Serum
FOP	Fibroblast Growth Factor Receptor 1
FRAP	FGFR1 Oncogene Partner
G0	Fluorescence Recovery After Photobleaching
G1	Gap0
G2	Gap1
GAPDH	Gap2
GFP	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	Green Fluorescence Protein
GTP	G-protein coupled receptor
h	Guanosine Triphosphate
HEK293T	Hours
HeLa	Human Embryonic Kidney
hTERT	Henrietta Lacks
HTR6	Human Telomerase reverse transcriptase
IFT88	Serotonin receptor 6
IP	Intraflagellar Transport Protein 88
IS	Immunoprecipitation
	Initial Sample

Moonraker	ROI
KIF14	Kinesin-like protein KIF14
KIF20A	Kinesin-like protein KIF20A
KIF7	Kinesin-like protein KIF7
LAP	The localization and affinity purification
LPC	Leupeptin, Pepstatin, Chymostatin
MBP	Maltose Binding Protein
Mib1	Mind bomb homolog 1
mRNA	messenger RNA
MS	Mass Spectrometry
NINL	Ninein like protein
NSAF	Normalized Spectral Abundance Factor
NUMA	Nuclear mitotic apparatus protein 1
OFD1	Prolyl 3,4-dihydroxylase ofd1
P	Pellet
p150 ^{glued}	150 kDa dynein-associated polypeptide
p38	Mitogen-activated kinase 11
Par6alpha	Partitioning defective 6 homolog alpha
PBS	Phosphate-buffered saline
PCM	Pericentriolar Material
PCM1	Pericentriolar Material 1
PEI	Polyethylenimine
PFA	Paraformaldehyde
Pfam	Protein family database
PIPES	1,4-Piperazinediethanesulfonic acid
PLK1	Polo-like kinase 1
PMSF	Phenylmethylsulfonyl Fluoride
RD	Retinal Degeneration
RNAi	RNA interference
RNase A	Ribonuclease A

Region of Interest

RPE1

Retinal Pigment Epithelium 1

RPM

Revolutions Per Minute

S

Supernatant or Synthesis

SDS-PAGE

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SEM

Standard Error of the Mean

siRNA

Small interference RNA

SSTR3

Somatostatin receptor 3

STED

Stimulated emission depletion

T

Total distance

TBST

Tris-Buffered Saline, 0.1% Tween®

Chapter 1: INTRODUCTION

1.1 The Primary Cilium Structure

The primary cilium is a non-motile microtubule-based structure that protrudes from the apical surface of cells during growth arrest. Sorokin and colleagues first described primary cilia on fibroblasts and smooth muscle cells. Since then, these structures have been observed on almost all mammalian cells (Sorokin, 1962). Ultrastructural studies of the primary cilium have revealed unprecedented complexity for its structure and morphology (Fisch & Dupuis-Williams, 2011). The primary cilium size usually varies in length from 1 to 5 μm with a diameter of approximately 0.2 μm that gets narrower towards its tip (Scherft & Daems, 1967). Very short potentially mechanosensitive primary cilia were described in osteocytes (Uzbekov et al., 2012). Their small length (below 300 nm) was probably associated with the hard bone environment of these cells.

At the core of the primary cilium is a microtubule-based axoneme composed of nine radially arranged doublet microtubules (Figure 1). Surrounding the ciliary axoneme is the ciliary membrane that is continuous with the plasma membrane, but molecularly distinct from it in terms of protein and lipid content. The organization of the ciliary microtubules is traditionally abbreviated as $(9 \times 2 + 0)$ axoneme, referring to nine peripheral doublet and no central singlet microtubules. This organization is distinct from the $(9 \times 2 + 2)$ axoneme of motile cilia. A microtubule doublet consists of a complete A-tubule of 13 protofilaments and an adjacent incomplete B-tubule of 10 protofilaments (Pedersen et al., 2012). The protofilaments are polymerized from alpha-beta-tubulin heterodimers longitudinally in the presence of GTP. Ciliary tubulins are modified by a combination of various posttranslational modifications including acetylation, glutamylation and detyrosination, which together regulate ciliary stability (Janke & Bulinski, 2011).

The primary cilium protrudes from the apical side of quiescent cells and is composed of a basal body, an axoneme and a ciliary membrane. The centrosome is composed of two centrioles and the associated pericentriolar material (PCM). Mother centrioles are the older of the two centrioles that bear distal and subdistal appendages. Basal bodies are mother centrioles that have been modified by the addition of the accessory structure and are connected to the younger daughter centrioles by striated rootlets at their proximal ends. During ciliogenesis, the 9-fold symmetrical microtubule triplets of the basal body nucleate the microtubule doublets, which transition to singlet microtubules towards the distal part of the cilium. Distal to the singlet microtubules is the ciliary tip, which is a specialized compartment for regulating ciliary length, trafficking and signaling. The ciliary gate is the most proximal region of the primary cilium and is composed of the transition fibers and the transition zone. Transition fibers originate from the distal appendages of the basal body and connect the outer surface of the triplet microtubule barrel to the plasma membrane. Distal

to the transition fibers is the transition zone, which is composed of the Y-shaped linkers and ciliary necklace and functions as a diffusion barrier and selective gate for the transport of soluble and membrane-bound components into and out of cilia. Y-shaped linkers connect the outer doublet microtubules of the axoneme to the ciliary membrane and the ciliary necklace consisting of circumferential parallel strands of intramembrane particles encircle the base of the axoneme. The proximal region of the cilium is surrounded by a cytoplasmic invagination of the periciliary membrane, termed the ciliary pocket, which exhibits endocytic activity and interacts with the actin cytoskeleton.

The ciliary axoneme is nucleated by the older of the two centrioles of the centrosome, the mother centriole, which transforms into a basal body during primary cilium formation. The interphase centrosome is composed of two orthogonally arranged centrioles that are connected by 2-4 striated rootlets at their proximal ends (Figure 1) (Mónica Bettencourt-Dias et al., 2011; S. Kim & Dynlacht, 2013). The centrioles are evolutionarily conserved symmetrical cylindrical structures of nine triplet microtubules. At the onset of ciliogenesis, the centrosome migrates to the cell surface and the 9-fold symmetrical radial microtubule triplets of the mother centriole template the ring of the nine axonemal microtubules. Interestingly, the microtubule triplets of the basal body transition to the microtubule doublets at the distal end of the basal body, which forms the core of the ciliary axoneme (Figure 1). Why and how this transition from microtubule triplets to doublets happens during ciliogenesis is not known. Moreover, in most cilia, the B-tubules of the microtubule doublets terminate towards the distal part of the cilium, which leaves singlet A-tubules. The length of this singlet region varies between cell types (Bertelli & Regoli, 1994; Satir, 1965, 1968). Distal to the singlet region of microtubules is the ciliary tip, which has been implicated in regulation of ciliary length, IFT remodeling and Hedgehog signaling.

Despite their highly conserved size and structures, the two centrioles of the centrosome differ in structural details, age and function (Erich A. Nigg & Stearns, 2011). The older (mother) centriole, but not the younger (daughter) centriole, has unique structural components that contribute to the primary cilium structure (Figure 1). First, the mother centriole bears nine distal appendages that interact with the apical plasma membrane, and nucleates the formation of the ciliary axoneme that pushes the plasma membrane outward to form the primary cilium as it grows. Second, proximal to the distal appendages of the mother centriole are from 1 to 13 (usually 2-5) the subdistal

appendages (Uzbekov and Alieva, 2013), that project laterally from the sides of the triplet microtubule barrel. The basal feet of the basal bodies in the ciliary epithelia are homologues of the subdistal appendages. Transmission Electron Microscopy (TEM) studies identified microtubule ends embedded in the ends of the basal feet, which suggests a function for these structures in anchoring a subset of microtubules to the base of the cilium (Michel Bornens, 2002).

1.1.1 Ciliary Compartments

Several distinct compartments in the primary cilium have been defined based on morphology and localization of specific proteins and here we will discuss the structure and function of three major compartments, which are ciliary gate, ciliary pocket, ciliary tip.

The “ciliary gate” is the most proximal region of the primary cilium and is composed of two distinct structural domains: the transition fibers and the transition zone (Figure 1). The transition fibers originate from the distal appendages of the basal body and connect the outer surface of the triplet microtubule barrel to the plasma membrane (Anderson, 1972). In TEM cross-sections, transition fibers are visualized as a pinwheel-like structure. Although the precise function and composition of transition fibers is not known, they are proposed to serve as a pore complex for regulating transport of vesicles and macromolecules in and out of cilia or as a docking platform for the transport factors of the ciliary building blocks (Deane et al., 2001; Rosenbaum & Witman, 2002). Distal to the transition fibers is the transition zone, where the microtubule triplets of the basal body transitions to the microtubule doublets of the axoneme. The transition zone is composed of the “Y-shaped linkers” that connect the outer doublet microtubules of the axoneme to the ciliary membrane, and the ciliary necklace consisting of circumferential parallel strands of intramembrane particles that encircle the base of the axoneme (G. D. Gupta et al., 2015; Szymanska & Johnson, 2012). Like transition fibers, the transition zone is proposed to regulate ciliary composition by regulating intracellular trafficking to and from the cilium.

The proximal region of the cilium is surrounded by a cytoplasmic invagination of the periciliary membrane, termed “ciliary pocket” in many cell types and organisms (Figure 1) (Ghossoub et al., 2011; Molla-Herman et al., 2010). Ciliary pocket is reminiscent of the flagellar pocket, a plasma membrane invagination at the base of the flagellum specialized in vesicular trafficking to and from the flagella in Trypanosomatids (Field & Carrington, 2009). In mammalian cells, the ciliary pocket is an endocytic membrane compartment that interacts with the actin

cytoskeleton. Importantly, clathrin-coated vesicles form around the ciliary pocket, suggesting that this compartment is important for the docking of the vesicles originated from the secretory pathway or from endosomes (Molla-Herman et al., 2010).

The distal region of the cilium is a specialized compartment called the “ciliary tip” (Figure 1). This compartment has recently emerged as an important regulator of cilium assembly by adding and removing tubulin subunits at the plus ends of axonemal microtubules, ciliary trafficking by remodeling IFT and ciliary signaling by recruiting the Hedgehog signaling components Gli2/3 and SuFu upon Hedgehog pathway activation (Satish Tammanna et al., 2013). Despite its implication in these important processes, the ultrastructure, composition and dynamics of the ciliary tip remain poorly understood.

1.2 Primary Cilium Assembly and Disassembly

The key steps of ciliogenesis have been described by numerous ultrastructural studies that date back to 1959 to the description of this pathway in vertebrate ciliary photoreceptors (Tokuyasu & Yamada, 1959). Based on the model suggested by Sorokin and colleagues in 1962, the first step in ciliogenesis is the localization of a large Golgi-like vesicle, ciliary vesicle, to the distal end of the mother centriole (Sorokin, 1962). This vesicle flattens to form a double membrane sheath around the axoneme and later fuses with the plasma membrane. Subsequent studies added more steps to this pathway and identified the molecular components and dynamics of these different steps.

Two distinct ciliogenesis pathways have been described in mammalian cells, which are distinguished by whether the basal body migrates to the cell surface or not at the onset of ciliogenesis (Figure 2). In the “extracellular” pathway, which is described for epithelial cells, the basal body migrates and docks to the apical surface of the plasma membrane. This precedes the assembly of the axoneme that pushes the plasma membrane out to form the cilium. In the “intracellular pathway, which is described for mesenchymal cells, fibroblasts and neuronal precursors, ciliary vesicles associate with the distal appendages of the mother centrioles and axoneme elongates concomitantly with the recruitment and fusion of ciliary vesicles with the plasma membrane, which protrudes the cilium from the cell surface.

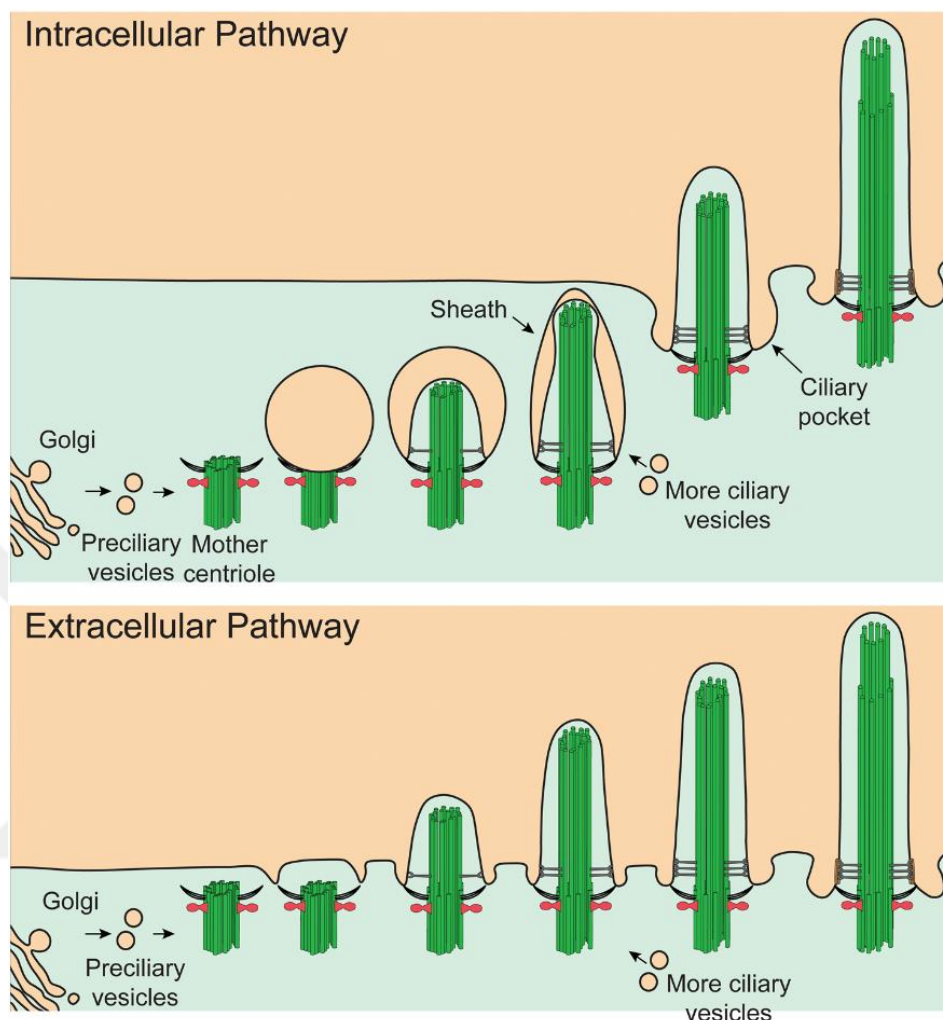


Figure 2. Primary Cilium Assembly Pathways.

There are two distinct ciliogenesis pathways described in mammalian cells. The cilium assembly pathway is a highly ordered process composed of distinct phases and is divided into two different pathways based on whether or not the basal body migrates to the cell surface or not at the onset of ciliogenesis. In the intracellular cilium assembly pathway, at the onset of ciliogenesis, Golgi-derived small periciliary vesicles accumulate around the distal appendages of the mother centriole. Periciliary vesicles fuse to form the ciliary vesicle and concomitantly the axoneme elongates from the mother centriole through microtubule polymerization. The growing nascent axoneme is encased by a double membrane structure termed the ciliary sheath. When the nascent axoneme migrates to the plasma membrane, the ciliary sheath fuses with the plasma membrane for extending the cilium from the cell surface towards the extracellular environment. In the extracellular pathway, the basal

body migrates and docks to the apical surface of the plasma membrane followed by the assembly of the axoneme that pushes the plasma membrane out to form the cilium.

The cilium assembly pathways are highly ordered processes composed of distinct phases (Figure 2) (Pedersen et al., 2012; L. Wang et al., 2016). Cilium assembly initiates when cells exit the mitotic cycle in response to mitogen deprivation and differentiation cues. In the intracellular cilium assembly pathway, several Golgi-derived small periciliary vesicles accumulate around the distal appendages of the mother centriole at the onset of ciliogenesis. A recent study showed that dynein mediates Myosin Va-associated periciliary vesicle transportation to the pericentrosomal region along microtubules, followed by myosin Va-mediated periciliary vesicle transportation to the distal ends of the mother centrioles along the centrosome-associated branched network (Wu et al., 2018). Periciliary vesicles fuse to form the “ciliary vesicle” on the distal end of the mother centriole followed by elongation of the axoneme underneath this cap. As vesicles fuse with the ciliary vesicle, the growing nascent axoneme is encased in a double membrane containing the ciliary sheath. Finally, this nascent cilium docks to the plasma membrane through fusion with the ciliary sheath to form a cilium protruding from the cell surface. In contrast to the intracellular pathway, the extracellular pathway starts with docking of the mother centriole to the plasma membrane, where the cilium grows directly towards the extracellular space through axoneme elongation (Figure 2).

Cilium disassembly is initiated by mitogen stimulation that results in entry into cell cycle. Studies in mammalian cells identified two distinct phases of the cilium assembly pathway, the first one that occurs in G1 phase shortly after cells exit cell cycle and the second one that occurs prior to mitosis (L. Wang et al., 2016). The scaffolding protein HEF1 and calcium-calmodulin activate Aurora A kinase, which then phosphorylates and stimulates the histone deacetylase HDAC6. This results in cilium disassembly through two major activities (Plotnikova et al., 2012; Pugacheva et al., 2007). First, it deacetylates modified, stabilized tubulin within the ciliary axoneme. Second, it deacetylates cortactin that then enhances actin polymerization (Ran et al., 2015). Complementing the HDAC6-mediated destabilization, two members of the Kinesin 13 family of the depolymerizing kinesins Kif2a and Kif24 were also implicated in depolymerization of axonemal microtubules (Kobayashi et al., 2011; Miyamoto et al., 2015). In contrast to cilium assembly, much less is understood about the mechanism of cilium disassembly. Future studies on the high-resolution

spatiotemporal imaging of cilium disassembly will be critical in visualizing the dynamics of this process. Moreover, development of genetic tools or chemical inhibitors will be necessary in order to conclude whether cell cycle reentry is the cause or consequence of cilium disassembly.

1.2.1 Parts List for the Primary Cilium

Precise understanding of the structure, function and regulation of the primary cilium requires the identification and characterization of the ciliary proteome. Once the complete ciliary proteome is identified, time-resolved interaction maps between the ciliary proteins are required to elucidate the underlying mechanisms of the dynamic ciliary processes like ciliary trafficking and signaling. A combination of bioinformatics, genomic and proteomics approaches and genetic screens over the years identified over 800 proteins associated with primary cilium.

Majority of the ciliary proteins were identified using multiciliated and flagellated cells and tissues as starting material using a combination of approaches. First, the proteomic analysis of cilia purified from human bronchial epithelial tissue culture (Ostrowski et al., 2002) and *Tetrahymena thermophile* cells (Smith et al., 2005), and flagella purified from *Chlamydomonas reinhardtii* (Pazour et al., 2005) and *Trypanosoma brucei* (Broadhead et al., 2006) together helped define the ciliary parts list and identified the genetic causes of ciliopathies. Second, transcriptomic profiling of cells (Oliver E. Blacque et al., 2005) and bioinformatics analysis of the *Caenorhabditis elegans* genes regulated by Regulatory Factor X (RFX)-type transcription factor or harboring X-box consensus sequence in their promoter identified a catalogue of ciliary genes (Efimenko et al., 2005; Swoboda et al., 2000). Finally, comparative genome analysis of ciliated and non-ciliated organisms (Tomer Avidor-Reiss et al., 2004; Mykytyn et al., 2004) identified the list of proteins that are required for cilia assembly and function independent of their localization to cilia. Although the comparative genomics of ciliated and non-ciliated organisms were successful in identifying the highly conserved structural proteins of the cilia, they were not able to identify many signaling components including the Hedgehog pathway components, as these pathways are not conserved beyond animals. A recent study by Sigg and colleagues overcame this problem by identifying and comparing the ciliary proteomes from sea urchins, sea anemones and choanoflagellates, which are organisms at the key phylogenetic nodes of animal evolution (Monika Abedin Sigg et al., 2017). This evolutionary proteomics study identified a core, shared ciliary proteome that illuminated the ancestry of ciliary signaling proteins including G protein Coupled Receptor (GPCR), Hedgehog

and Transient Receptor Potential (TRP) channel signaling pathways. While these studies together contributed significantly to the components of the primary cilium that are shared with the motile cilia such as the structural components of the axoneme, they were not sufficient to identify the primary cilium-specific components that are required for its specialized sensory functions.

Identification of the mammalian primary cilia proteome studies faces two significant challenges: First, due to the low abundance of ciliary material, biochemical purifications of the intact primary cilium in sufficient quantities and purity for mass spectrometry analysis have been difficult. Second, cilium is a very dynamic structure and most of the ciliary interactions are transient and insoluble. These interactions cannot be detected with standard biochemical approaches. The first proteomic analysis of the primary cilia from mammalian cells was reported only after primary cilia from mouse kidney cells were purified by developing a purification approach based on a previously published calcium-shock method (Hiroaki Ishikawa et al., 2012; Mayer et al., 2008; Rhein & Cagan, 1980). Multidimensional protein identification technology (MudPIT), protein correlation profiling (PCP) and subtractive proteomic analysis of the purified cilia identified 195 proteins as part of the ciliary proteome (Hiroaki Ishikawa et al., 2012). The mammalian ciliary proteome was complemented with the proteomic analysis of specialized sensory cilia from the mouse photoreceptor cells (Q. Liu et al., 2007) and rat olfactory cells (Mayer et al., 2008, 2009), and the unique, multiple (9x2 + 0) cilia in choroid plexus epithelial cells (CPECs) of the juvenile swine (Narita et al., 2012) to comprehensively identify over 2000 candidate proteins as part of non-motile cilia. Interestingly, the proteome of CPECs and photoreceptor cilia had a significant overlap with each other, but not with the proteome of motile cilia, indicating that this group might represent sensory cilia-specific proteins. Among the proteins identified by proteomics studies of the sensory cilia and the mammalian primary cilia, the signaling factors and membrane proteins of the cilia including the BBSome complex components, polycystin-1 and polycystin-2 were underrepresented, suggesting that this approach had sensitivity problems in detecting transient and insoluble ciliary components and that ciliary components had been lost during biochemical purifications. The challenges in identification of such interactions were in part overcome by the application of the innovative proximity-labeling approaches for the identification of the ciliary proteome.

Proximity labeling is an emergent technology that makes use of enzymes that generate free biotin radicals for temporally and spatially labeling the proximity partners of proteins of interest fused with these enzymes (C.-L. Chen & Perrimon, 2017). The mutant biotin ligase BirA* and the engineered ascorbate peroxidase APEX2 are two of these enzymes that have been successfully applied for proximity mapping of different cellular compartments. APEX2-catalyzed proximity labeling has been successfully used for identifying the ciliary proteome by two different studies (Kohli et al., 2017; David U. Mick et al., 2015). Both studies made use of cilia-targeted labeling enzymes, the fusions of ciliary targeting sequences of the G-protein-coupled receptor Htr6 or NPHP3 with the APEX2 enzyme. Biotinylation of the ciliary proteins by activation of cilia-targeted-APEX2 with H₂O₂ and biotin-phenol incubation followed by streptavidin pulldowns and mass spectrometry identified about 200 candidate ciliary proteins (Figure 3). While the APEX2-fused ciliary targeting sequence of NPHP3 probed both the ciliary membrane and the shaft during biotinylation, APEX2-fused Htr6 specifically probed the interactions at the ciliary membrane, and thus these two proteomes is a valuable resource for compiling the dynamic ciliary proteome.

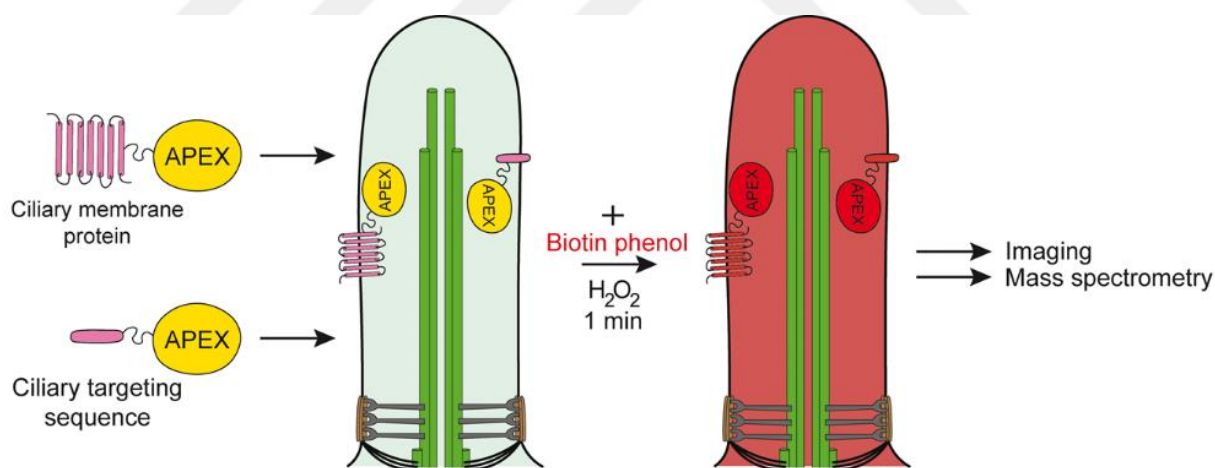


Figure 3. Identification of the ciliary proteome by APEX2-based proximity labeling.

The engineered ascorbate peroxidase APEX2 is fused to a ciliary targeting sequence or a ciliary membrane protein and targeted to the cilium. Biotinylation of the ciliary proteins are activated in cells expressing this fusion protein by incubation with H₂O₂ and biotin-phenol for one minute, which is followed by streptavidin pulldowns and mass spectrometry.

The ciliary proteome identified by proximity labeling include the stable or transient ciliary proteins, including components of the Intraflagellar transport (IFT) and Bardet Biedl Syndrome (BBSome) complexes that mediate intraciliary trafficking, structural components and ciliary membrane proteins, as well as previously unrecognized interactions. One of the novel ciliary signaling mechanisms revealed by cilia-APEX studies is the intraciliary AC6/cAMP/PKA signaling axis, where PKA regulates Hedgehog activity by phosphorylating Gli3 in the primary cilium (David U. Mick et al., 2015). Another novel mechanism revealed by cilia-APEX studies is the identification of a number of actin-binding proteins in the cilium, which might be required to stabilize cilia and the ciliary membrane during dynamic changes in ciliary length (Kohli et al., 2017). Moreover, these approaches proved to be successful in the quantitative analysis of the proteomic composition of the cilium in *Ift27*^{-/-} mouse kidney cells and in cells treated with actin depolymerization drugs. These analyses revealed new mechanisms for the pathways regulated by *Ift27*-mediated trafficking and actin-regulated ciliary processes (Kohli et al., 2017; David U. Mick et al., 2015). Taken together, proximity-based proteomics of the cilia has provided to be highly efficient and sensitive and holds great promise for future studies in screening for changes in the ciliary interior upon stimulation with different signals and in affected ciliopathy tissues.

1.3 Mechanisms for Ciliary Compartmentalization

Primary cilium plays important roles in dynamic cellular processes and its proper function in these processes require tight spatiotemporal regulation of the ciliary content. The unique ciliary content likely enables efficient molecular interactions, which is critical for initiation of signal transduction in response to extracellular stimuli. The ciliary membrane is continuous with the plasma membrane, yet the cilium has a unique protein and lipid composition that dynamically changes in response to mechano- and chemo-signals. Moreover, protein synthesis does not occur inside the cilium and thus active protein trafficking into and out of the cilium is required for assembling and maintaining a functional cilium. Since the identification of the cilium as a sensory organelle, there has been a lot of effort in elucidating the mechanisms that makes the primary cilium a specialized compartment and the current picture is highly complex that integrates coordination of multiple mechanisms for protein entry into the cilium, protein exit out of the cilium and protein retention at the cilium, which are discussed below:

1.3.1 Transition Zone

Transition zone functions as a diffusion barrier and selective gate for the transport of soluble and membrane-bound components into and out of the cilia (Figure 4). The first evidence for the gatekeeper function of the transition zone for the cilium was reported by Spencer and colleagues, whose work aimed to study the dynamic behavior of rhodopsin between the inner and outer segment of the vertebrate photoreceptor cells (Spencer et al., 1988). Although rhodopsin normally functions at the outer segment and is restricted there, it freely diffused to the inner segment when photoreceptor cells were mechanically disrupted to lose their separation by the connecting cilium, demonstrating that an intact transition zone limits this diffusion. This result was corroborated by the studies on the *Chlamydomonas* gametes, which adhered to each other through the active agglutinins in their flagella, but not through the ones found on the body (Hunnicuttt et al., 1990). The presence of two different agglutinin pools in *Chlamydomonas* is the classical evidence for the presence of a functional barrier at the base of the cilium. Supporting these results are the subsequent studies in different model systems including the fluorescence recovery after photobleaching (FRAP) analysis of ciliary protein dynamics that identified no fluorescence recovery for these proteins when the whole cilium is bleached in contrast to their fast recovery when part of the cilium is bleached (Chih et al., 2011; Qicong Hu et al., 2010), as well as altered ciliary protein content when the transition zone proteins were disrupted in mammalian cells by and *C. elegans* by RNAi and knockouts (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Williams et al., 2011). The crucial function of the transition zone as a selective diffusion barrier, which was convincingly demonstrated by various experiments, has led to questions on the underlying molecular mechanism of this function and also how disruption of this function causes ciliopathies.

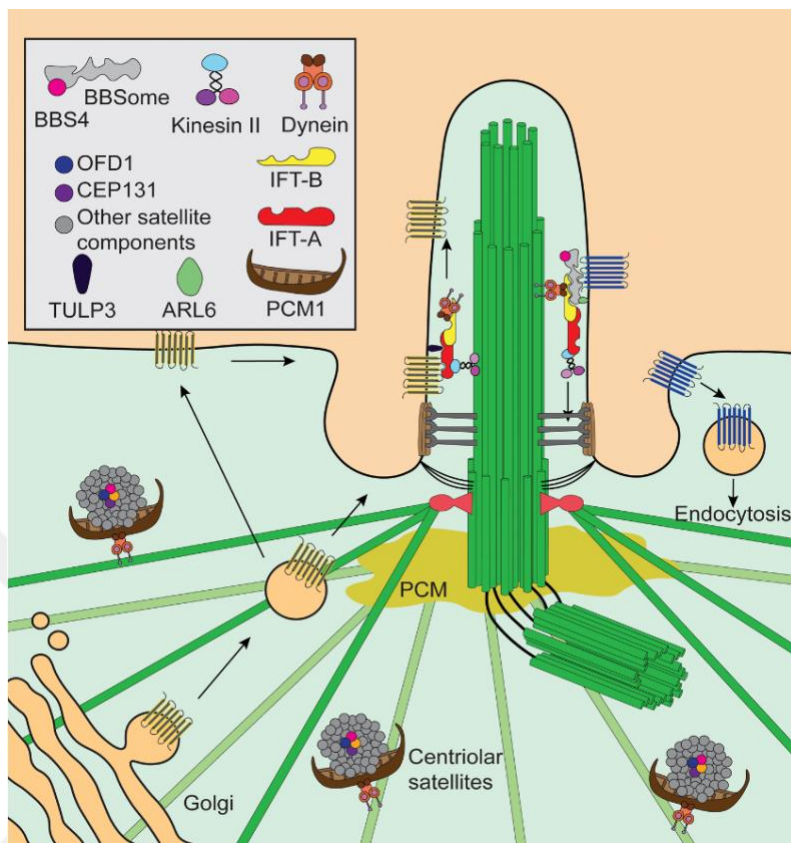


Figure 4. Overview of Trafficking Pathways involved in Ciliary Compartmentalization.

The assembly, maintenance and disassembly of the primary cilium require integration of multiple pathways that mediate entry of proteins to the cilium exit of proteins from the cilium and retention of proteins at the cilium. Most ciliary proteins are recruited to the periciliary membrane by vesicular trafficking, recycling endosomes and lateral diffusion. Vesicular trafficking delivers vesicles from the trans Golgi network (TGN) to the ciliary base, where vesicles fuse with the periciliary membrane in a SNARE-dependent manner. Recycling endosomes serve as an intermediate sorting compartment and segregates proteins to the ciliary pocket or to the apical plasma membrane. Finally, some proteins are recruited to the ciliary membrane through lateral diffusion from the plasma membrane to the periciliary membrane. When proteins are at the ciliary base, they enter the cilium through intraflagellar trafficking (IFT). IFT pathway starts with binding of ciliary cargoes to anterograde IFT-A/TULP3 trains near the basal body, their movement to the ciliary tip by IFT-A/TULP3 and release from the IFT-A/TULP3 at the tip and ends with the trains reorganizing at the tip and returning to the cell body by retrograde IFT-B with new cargo. The eight subunit BBSome complex functions in regulated exit from cilia. Activated GPCRs leads to accumulation

of BBSome at the ciliary tip and processive retrograde BBSome trains form in an Arl6-dependent manner and activated GPCRs are removed from the cilium by a BBSome-mediated retrieval pathway. The least understood ciliary recruitment pathway is mediated by the centriolar satellites, an array of granules that localize and move around the centrosome/cilium complex in a microtubule and molecular motor-dependent manner. Satellites macromolecular complex assemblies that are scaffolded by PCM1 and they function as intermediate sites in delivering proteins such as BBS4 to the cilium and ODF1 to the centrosome.

MKS, NPHP and CEP290 are the three main modules of the transition zone that were identified by genetic studies in *C. elegans* and the biochemical characterization of protein-protein interactions (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Sang et al., 2011). These modules are composed of multiple soluble and membrane proteins that interact and depend on each other for localization to the transition zone for assembling and maintaining a functional transition zone. Adding to the list of transition zone components are the proteomics studies of the transition zones isolated from cell walls of *Chlamydomonas* after axoneme disassembly by biochemical purifications (Diener et al., 2015) and from Trypanosome by affinity purifications (Dean et al., 2016). *Chlamydomonas* and Trypanosome transition zone proteomes identified proteins specific to these organisms as well as orthologs of candidate ciliopathy genes. Finally, the Biotin Identification (BioID) proximity screens performed in non-ciliated and ciliated human embryonic kidney epithelial cells with transition zone proteins as baits identified extensive proximity interactions for these proteins with components of other centrosomal and ciliary structures. This screen showed that some of these interactions were detected independent of cilia formation and interactions between the three transition zone modules increased in ciliated cells (Gagan D. Gupta et al., 2015). The identification of a nearly complete parts list for the transition zone through these studies has provided the leads for the research on elucidating how these components come together through spatial and temporal interactions to assemble a functional transition zone.

Emergent technologies in high-resolution imaging have proven to be very important in visualizing the structures like transition zone that are below diffraction limit of conventional microscopes; thus, their application to the known transition zone components revealed their spatial organization in different cell types. Spatial locations of CEP290, RPGRIP1L, MKS1, TCTN2, CBY and TMEM67 relative to each other were determined to generate an architectural map of the

transition zone using stimulated emission depletion (STED) superresolution microscopy combined with electron microscopy in RPE1 cells (Yang et al., 2015) and 3D superresolution structured illumination microscopy (3D-SIM) in *Drosophila* spermatocytes (Pratt et al., 2016). Analysis of the Trypanosome transition zone by fluorescence microscopy and electron microscopy determined the presence of distinct domains within the transition zone (Dean et al., 2016), in agreement with the different transition zone complexes revealed by the BioID proximity mapping studies (Gagan D. Gupta et al., 2015). Future studies are required to determine how these interactions change temporally in response to signals, in ciliated cells of different tissues and in ciliopathy patient samples.

1.3.2 Intraflagellar Transport

Intraflagellar transport (IFT) is the movement of material into and out of the cilium and is a critical process for assembling, maintaining and disassembling cilia. IFT is highly conserved among a wide range of eukaryotes. Defects in IFT structure and function disrupt biogenesis of cilia and flagella. IFT was first observed as the bidirectional movement of granule-like particles along the *Chlamydomonas* flagellum using differential interference contrast (DIC) microscopy (Kozminski et al., 1993) and subsequent work using various imaging approaches identified IFT in other model systems including *C. elegans* (Hao et al., 2009), *Tetrahymena* (Y.-Y. Jiang et al., 2015), *Trypanosoma* (Santi-Rocca et al., 2015) and mammalian cells (Williams et al., 2014). Electron microscopy studies showed that “trains” of IFT particles that were observed as linear arrays of granules move between the ciliary membrane and the underlying axonemal outer doublet microtubules. A combination of fluorescence and electron microscopy analysis of *Chlamydomonas* flagella showed that IFT particles move anterogradely along B-microtubules of the outer doublets, whereas retrogradely along A-microtubules, explaining in part why these complexes simultaneously traffic inside cilia without crashing into each other (Stepanek & Pigino, 2016).

The components of the IFT machinery were identified mostly using mutants in various model organisms, such as the flagellar assembly (*fla*) mutants in *Chlamydomonas* (G. M. Adams et al., 1982; Huang et al., 2013), chemosensory (*che*), osmotic avoidance (*osm*), dye-filling (*dyf*) and dauer-formation (*daf*) mutants in *C. elegans* (Inglis et al., 2007). Recent work on biochemical mapping and the structural studies of the complex interactions has contributed to an emerging model for how the complex subunits interact to assemble functional trains (Nakayama & Katoh,

2018). IFT is composed of two distinct multisubunit subcomplexes termed IFT-A and IFT-B (Figure 4). IFT-B is a complex of 16 subunits and mediates anterograde trafficking from the ciliary base to the tip by heterotrimeric kinesin-2 motor proteins. IFT-A is a complex of 6 subunits plus TULP3 and mediates retrograde trafficking by cytoplasmic dynein-2 complex. Although the emerging model for IFT pathway is becoming more complex with each new advance in the field, the basic IFT pathway starts with binding of ciliary cargoes to IFT near the basal body, their movement to the ciliary tip by anterograde IFT and release from the IFT at the tip and ends with the trains reorganizing at the tip and returning to the cell body by retrograde IFT with new cargo.

Protein transport by IFT can be divided into four steps, which are cargo loading, admission into the cilium, translocation along the shaft, and unloading. IFT cargoes are defined as proteins that move to cilia by IFT but are not required for IFT assembly and function themselves (Figure 4). They include proteins that traffic from the cell body to their ciliary assembly sites including tubulin, radial spoke proteins, inner- and outer-dynein arms, IFT motor proteins including dynein and kinesin, and proteins associated with ciliary signaling. Given that anterograde transport is critical in supplying the proteins required for cilia biogenesis, disruptions of IFT-B complex proteins result in cilia formation defects. Moreover, defects in IFT proteins that function in the transport of signaling molecules are associated with developmental defects. For example, IFT88 and IFT21 mice mutants have Hedgehog-related phenotypes due to defects in ciliary localization of Smoothened, Gli2 and Gli3 proteins. Also, IFT122 mutants are defective in Opsin transport in mouse photoreceptors that results in retinal defects (Huangfu et al., 2003; Huangfu & Anderson, 2005; A. Liu et al., 2005). While cargo adaptors, factors stabilizing cargoes on the trains and projection domains have been implicated in loading of specific cargo (K. F. Lechtreck, 2015), our current understanding of IFT and cargo interactions is incomplete. IFT-cargo interactions need to be studied for each specific cargo given the underlying complexity and heterogeneity of these interactions.

Finally, a number of IFT subunits such as IFT88 and IFT27 have been described for their cilia-independent functions during cell-cycle regulation, mitosis, cell migration, microtubule stability and orientation (Yuan & Sun, 2013). Future studies are required to dissect the molecular mechanism of the nonciliary functions of IFT that will reveal exciting avenues for uncovering the complexity of the IFT.

1.3.3 BBSome Complex

Bardet-Biedl syndrome (BBS) is a ciliopathy characterized by multiple symptoms including retinal degeneration, polydactyly, cystic kidneys, obesity and is linked to mutations affecting 22 reported genes to date. BBSome complex was first purified from bovine retina and identified as an obligate complex of eight different BBS syndrome proteins using tandem affinity purification of BBS4 from mammalian cells (Figure 4) (Maxence V. Nachury et al., 2007). The octameric BBSome complex was later purified from various sources including bovine retina (Jin et al., 2010), *Chlamydomonas* (K.-F. Lechtreck et al., 2009), *Paramecium* (Valentine et al., 2012) and *Trypanosoma* (Langousis et al., 2016), indicating that this complex is highly conserved in ciliated organisms that use their cilia for sensory functions. The eight subunit BBSome complex is very stable as it does not separate into subcomplexes when treated with harsh buffers and disassembles only when any of its subunits is disrupted (Jin et al., 2010).

Mutations in BBSome components in different organisms are all associated with ciliary signaling defects, rather than structural defects in the cilium assembly reported for IFT mutants. In agreement with such sensory function, BBSome is lost in ciliated organisms that use cilia only for motility (Carvalho-Santos et al., 2010). Among the signaling defects of BBSome mutants are the defects in Hedgehog signaling pathway, which is the most studied and understood signaling pathway associated with the cilia to date. For example, mutations in genes encoding BBSome proteins exacerbate the patterning defects like polydactyly and exencephaly of cilia-mutant mice (Goetz et al., 2017; Yee et al., 2015) and result in skeletal patterning abnormalities in zebrafish (Tayeh et al., 2008). Two recent genome-wide CRISPR screens for Hedgehog regulators both identified all BBSome subunits as high scoring hits (Breslow et al., 2018; Pusapati et al., 2018), which supports the previous studies that showed defects in Hedgehog-induced transcriptional response in mammalian cells (Zhang et al., 2012). Finally, disrupted BBSome function results in inability to mount an infection and evade the host immune system in *Trypanosome* and *Leishmania* and swimming defects away from high light dosages in *Chlamydomonas* (Langousis et al., 2016; Price et al., 2013). The regulatory functions of the BBSome complex in Hedgehog signaling and other yet unidentified sensory signaling pathways in part must be the defective mechanisms underlying the developmental symptoms of BBS.

BBSome complex moves both in retrograde and anterograde directions in *Chlamydomonas*, mammalian cells and *C. elegans*, suggesting possible roles in ciliary import and/or exit (Erich A. Nigg et al., 2014). It was initially proposed to function in ciliary import of proteins based on experiments where the Somatostatin Receptor 3 (SSTR3) and Melanocortin concentrating hormone receptor 1 (MCHR1), Polycystin 1 (PC1), and Smoothed (Smo) were lost from cilia in cells defective for the BBSome function or Arl6 function (Nicolas F. Berbari et al., 2008; Jin et al., 2010; Zhang et al., 2011). This function was further supported by the direct interaction between the BBSome complex and these ciliary targets (Klink et al., 2017; Seo et al., 2011; Su et al., 2014). The ciliary import function of the BBSome complex was challenged by further studies that identified its role in the ciliary exit of the membrane-associated phospholipase D (PLD) from cilia in *Chlamydomonas* (K. F. Lechtreck et al., 2013; K.-F. Lechtreck et al., 2009) and dopamine receptor (Drd1), Sstr3, GPr161, Smo and the Hedgehog receptor Patched 1 in mammalian cells (Klink et al., 2017; Seo et al., 2011; Su et al., 2014; Ye et al., 2018; Zhang et al., 2012).

Recent studies have convincingly limited BBSome function to ciliary exit, instead of a dual function in both ciliary entry and exit, and identified IFT-A/TULP3 complex as the major mediator of ciliary entry for membrane proteins (Figure 4). Real-time imaging of the ciliary receptors showed that activated GPCRs were lost as packed in ectosomes from ciliary tips when BBSome-mediated ciliary exit was inhibited. These results suggested that decreased ciliary levels of signaling receptors reported in BBS mutants is likely due to increase in ectocytosis rather than import defects (Ye et al., 2018). Moreover, a wide variety structurally diverse ciliary membrane proteins including sixteen different GPCRs, the polycystin complex PC1/PC2, the cytoprotein pKDH1, Smo was shown to physically interact with IFT-A complex components and to be recruited to cilia by the tubby family protein TULP3 that functions as a general adapter for ciliary trafficking (Badgandi et al., 2017; Fu et al., 2016; Hirano et al., 2017). Given that BBSome moves both in retrograde and anterograde directions, it is proposed to effectively capture and bind to its cargoes only in the anterograde direction either constitutively as in the case of PLD or in a regulated manner as in the case of Gpr161 and Sstr3. Future studies dissecting the molecular machinery and the signals that regulate the affinity of the BBSome to its different cargoes are required to reveal a more complete picture of the intraciliary trafficking.

1.3.4 Centriolar Satellites

Centriolar satellites are an array of 70-100 nm membrane-less granules that localize around the centrosomes and cilia in animal cells (Figure 4) (Barenz et al., 2011). They move along microtubules in a molecular-motor dependent manner and have been implicated in various centrosome and cilium-mediated cellular processes. The best-characterized function of satellites is their role in cilium assembly as many satellite proteins including their scaffolding protein PCM1 were shown to inhibit or interfere ciliogenesis. Despite their well-established role in cilium assembly, the molecular mechanism of action for satellites in this process is not known. Given their movement along microtubules, satellites have been proposed to regulate transport of proteins to or away from the centrosome/cilium complex. While this function has been reported for a wide variety of proteins with functions ranging from cell cycle progression to pericentriolar material recruitment and assembly, such function for satellites has only started to be discovered. The function of satellites in regulation of ciliary targeting has most been studied for the BBSome component BBS4, which localizes to centriolar satellites in unciliated cells and redistributes to the primary cilium in ciliated cells (Figure 4) (Maxence V. Nachury et al., 2007). Cells depleted for satellites components PCM1, CCDC66, Cep72 and Cep290 have significant defects in the ciliary recruitment of BBS4 (Deniz Conkar et al., 2017; Maxence V. Nachury et al., 2007; Timothy R. Stowe et al., 2012). Two models have been proposed to explain the function of satellites in ciliary recruitment of BBS4. The first model is the molecular motor-based trafficking of satellite-BBS4 complex to the cilium. Supporting this model, BBS4 interacts with PCM1 and p150Glued subunit of dynein/dynactin complex, identifying BBS4 as a bridging factor between satellites and dynein (Jun Chul Kim et al., 2004). The second model is possible sequestration function for satellites that stores BBS4 in unciliated cells and releases it to form the BBSome complex in ciliated cells (Timothy R. Stowe et al., 2012). Future studies are required to distinguish between these models and to determine the exact function of satellites in regulation of the ciliary composition.

1.3.5 Vesicular Trafficking

Vesicular transport of lipids and proteins from the trans-Golgi network (TGN) and recycling endosomes to the ciliary base is an important mechanism of assembling and maintaining the cilium (Figure 4). Ciliary membrane biogenesis and ciliary targeting of membrane proteins are both regulated by components of the vesicular trafficking pathways such as Rab GTPases, their cognate GTPase-activating proteins (GAPs) and ADP-ribosylation factor (ARF)-like small GTPases (ARLs) (Sung & Leroux, 2013). The small G protein ARF4 for rhodopsin and fibrocystin trafficking, the GGA1 adapters for PC1/2 trafficking and the Tulp3 and tubby adapters for trafficking of multiple GPCRs are among the well-characterized components of the secretory pathway with functions in ciliary trafficking (Pedersen et al., 2016).

Vesicles budding from the TGN are directed to a specific docking site at the periciliary base where coordinated interactions between the Rab family small GTPases related to the exocyst complex tether the vesicles to the periciliary membrane. The small GTPases Rab11 and Rab8 mediate vesicular transport to the ciliary base where the guanine nucleotide exchange factor Rabin 8 binds to the exocyst complex, which then tethers vesicles to the periciliary membrane. Following tethering, vesicle fusion with the periciliary membrane is likely to be mediated by v-SNARE and t-SNARE interactions. Once the membrane proteins are incorporated to the periciliary membrane, they then cross the ciliary gate and enter the ciliary membrane. In addition, this pathway, lateral diffusion or transport of proteins from the apical plasma membrane into the ciliary membrane were reported for Smo, but since this report, there has not been other proteins shown to be transported through this lateral pathway, indicating that it might be a specific transport pathway for Smo.

1.3.6 Ectosomes

Ciliary proteins are expelled from the tip of the cilium via extracellular vesicles termed ectosomes, which are now recognized as an important pathway for ciliary trafficking and function (Figure 5). While the precise role of ectosomes is not known, recent studies have assigned functions for ectosomes in maintaining ciliary composition by disposing selected proteins from the cilium, mediating intercellular communication through exchange of proteins and genetic material, and remodeling of the extracellular space (Wood & Rosenbaum, 2015). Ectosomes were first described in *Chlamydomonas* by electron microscopy as membrane-bound vesicles budding from the flagella

that function in budding (Bergman et al., 1975). Subsequent studies defined these vesicles as bioactive for they contain lytic enzymes required for digesting the mother cell wall and allowing post-mitotic hatching of the daughters (Wood et al., 2013). In *C. elegans*, ciliary ectosomes are shed and released by ciliated sensory neurons, which play a role in modulating animal-to-animal communication (J. Wang & Barr, 2016). Ectosomes were recently identified for their function in forming the light-sensitive discs of the specialized photoreceptor cilium. In photoreceptor cells, the cilium has an innate ability to produce massive amounts of ectosomes. The release of these ectosomes are blocked by the membrane protein peripherin that retains them at the cilium and shapes them into the photoreceptor discs of the outer segment through elongation and flattening (Molday & Goldberg, 2017; Salinas et al., 2017). In mammalian cilia, activated GPCRs that fail to undergo BBSome-mediated retrieval from the cilia concentrate into membranous bunds at the ciliary tip and then released into ectosomes. Interestingly, actin/myosin 6/drebrin mediates the scission of ectosomes from the cilia tips (Figure 5). Moreover, Hedgehog signaling fails when both ectocytosis and retrieval are blocked by inhibition of the actin-regulated machinery (Andrew R. Nager et al., 2017).

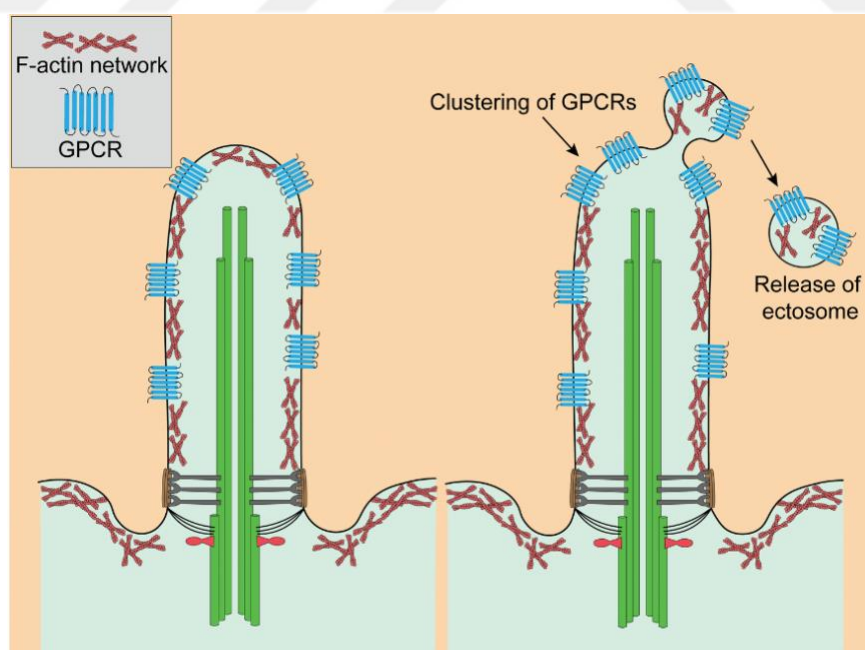


Figure 5. Ectosome release from the ciliary tip.

Ciliary proteins are expelled from the tip of the cilium via extracellular vesicles termed ectosomes. Ectosomes function in maintaining ciliary composition by disposing selected proteins from the

cilium, mediate intercellular communication through exchange of proteins and genetic material and remodel the extracellular space. In mammalian cilia, activated GPCRs that fail to undergo BBSome-mediated retrieval from the cilia concentrate into membranous buds at the ciliary tip, which are released into ectosomes. Scission of ectosomes from the ciliary tips is mediated by an actin-regulated machinery that is composed of actin, myosin 6 and drebrin.

Given the emerging functions of ectosomes in ciliary processes, there has been significant interest in dissecting the underlying molecular mechanism of biogenesis, shedding and release of ectosomes. In addition to the actin-regulated machinery identified in mammalian cells, studies in *C. elegans* and *Chlamydomonas* identified IFT for its role in these processes. The proteome of the ectosomes purified from *Chlamydomonas* flagella is different from the flagellar membrane proteome. Ectosomes comprise a subset of flagellar membrane proteins, the endosomal sorting complex required for transport (ESCRT) proteins, small GTPases and ubiquitinated proteins, corroborating the function of ectosomes in cell signaling and turnover of ciliary proteins (Long et al., 2016). Ciliary ectocytosis is an emerging new field and future work on these structures has potential to reveal their function and mechanism of action in physiological and pathological contexts.

1.4 Primary Cilium as the Sensory Organelle

Primary cilium is a specialized compartment for localization of components linked to sensory perception and multiple signaling pathways; thus, is considered as the cellular antenna. The first signaling pathway associated with cilia is the one that induces gamete activation and cell-cell fusion in *Chlamydomonas* during fertilization through interactions between agglutinin on the cilia of the plus and minus gametes (Q. Wang et al., 2006). Since then, many important signaling pathways have been linked to the primary cilium. About 25 rhodopsin-family GPCRs, proteins linked to polycystic kidney disease including TRP-channel family proteins PC/2, the single-pass transmembrane protein fibrocystic and sonic hedgehog (Shh) family components including Smoothened (Smo) and the orphan GPCR Gpr161 localize to the primary cilium in a dynamic manner (Gabrielle Whewey et al., 2018). In addition to the Hedgehog signaling, a diverse array of other signaling pathways including Hedgehog, Wnt, Notch, Hippo, GPCR, PDGF (and other receptor tyrosine kinases including FGF), mTOR, and transforming growth factor beta have been linked to the cilium. The key functions of the primary cilium in various signaling pathways is

highlighted by the diseases including ciliopathies and cancer that are linked to defects in the structure and function of the cilium (Jeremy F. Reiter & Leroux, 2017).

The best-characterized signaling pathway mediated by the primary cilium is the vertebrate Hedgehog (Hh) pathway (Figure 6) (Bangs & Anderson, 2017). Unlike other developmental signaling pathways linked to the primary cilium, Hh signaling is completely dependent on the primary cilium in mammalian cells, which therefore fail to respond to Hh in the absence of primary cilia. The key discovery that linked the primary cilium to Hedgehog signaling came genetic screens for mouse embryonic patterning mutants that identified IFT proteins among the mutated genes (Huangfu et al., 2003). The regulation of Hh signaling is complex where multiple factors traffic into and out of cilia in response to pathway activation by secreted ligands, which are Sonic hedgehog (Shh), Indian (Ihh) and Desert (Dhh) in mammalian cells (Figure 6). In the absence of ligand, the Shh receptor Patched (Ptch1) localizes to the cilium and it inhibits the activity of the 7-pass transmembrane protein Smoothened (Smo), which are stored in vesicles in the cytoplasm. The main effectors of the Hedgehog signaling Gli2 and Gli3, kinesin-4 motor protein Kif7 and Suppressor of Fused (SuFu) are present in low levels at the ciliary tip, where Gli2/3 are processed to their repressor forms and Hedgehog target gene transcription is turned off (Pedersen & Akhmanova, 2014). When cells receive the Hh signal, Shh binds to Ptch1, causing the Ptch1 to exit the cilium concomitantly with Smo ciliary entry and accumulation of Kif7, Gli2/3 and SuFu at the ciliary tip. Gli2/3 are processed at the ciliary tip by posttranslational modifications to their full-length activator forms, which exit the cilia and enter the nucleus to activate transcription of downstream Hh target genes. The switch between the repressor and activator forms of Gli2/3 has been shown to be tightly regulated by sequential phosphorylation and dephosphorylation events by kinases PKA, GSK-3 β and CK1. Regulation of dynamic ciliary localization of the Hh pathway components is very important for the pathway activity and IFT complex mediates the movement of these components into and out of the cilium.

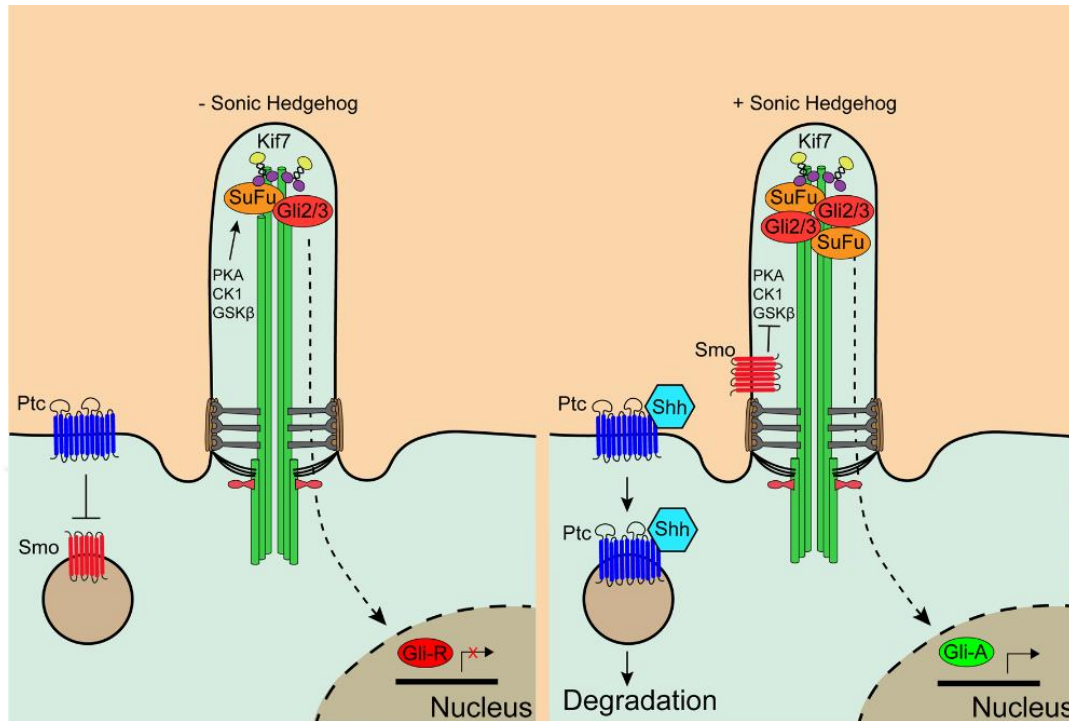


Figure 6. Hedgehog Signaling Pathway.

The core components of the Hedgehog signaling pathway localize to the primary cilium in a tightly controlled and activation-dependent manner. In the absence of Hedgehog ligand Sonic Hedgehog (Shh), the G protein coupled receptor Patched (Ptc1) localizes at the ciliary membrane and inhibits the localization of the transmembrane receptor protein Smoothened (Smo) to the cilium. Transcription factors Gli2 and Gli3 along with Sufu and Kif7 accumulate at the ciliary tip, where they are processed by PKA, CK1 and GSK β into a “repressor” form (Gli-R). Gli-R enters the nucleus and represses transcription of downstream Hedgehog target genes. When cells receive the Shh, Shh binds to Ptc1, which then internalized and is targeted for degradation. Activated Smo can now localize to the cilium, where it inhibits PKA, CK1 and GSK β . This allows Gli2 and Gli3 to be processed into “activator” form (Gli-A), which enters the nucleus and activates transcription of downstream Hedgehog target genes.

Although Hg pathway has been a major focus of many studies, biochemical steps leading from ligand binding to target gene activation in Hg signaling are still far from clear. Identification of Hedgehog regulators in an unbiased way was recently achieved by two different genome-wide functional CRISPR screens, which both identified positive and negative Hedgehog regulators with high sensitivity. The single guide RNA lentiviral library was combined with an engineered Hh-

pathway-sensitive reporter for antibiotic-based selection by Breslow and colleagues (Breslow et al., 2018) and with a fluorescence-based transcriptional reporter for phenotypic enrichment by Pusapati and colleagues (Pusapati et al., 2018). These screens generated datasets that serves as a valuable resource for the discovery of new components for Hh signaling and consequently to the associated diseases such as ciliopathies and other developmental ones. While the function of the primary cilium in Hedgehog signaling has been addressed by many studies, there are still many remaining questions regarding the role of primary cilium in specialized cells and different organisms. Importantly, the function of the primary cilium in signaling pathways other than Hedgehog signaling is poorly understood and future studies are required to address this question.

1.5 Conclusion

Research over the past several decades has elucidated the broad, diverse and complex ways of primary cilium assembly, maintenance and function using a combination of genetics, proteomics, developmental biology and cell biology approaches. The primary cilium has turned out to be fascinating, complex and directly linked to human diseases including the ciliopathies and cancer. It has become clear that almost all major signaling pathways in vertebrates converge on the primary cilium, leading to the adoption of the term “the cell’s antenna” for describing primary cilium. A deeper understanding of the mechanisms that control cilia formation *in vivo* and the dissection of biochemical steps of the Hedgehog pathway in future will provide insight into the development diagnostic and therapeutic tools in both ciliopathies and cancer.

Chapter 2: CCDC66 INTERACTS WITH CEP290 AND FUNCTIONS IN CILIUM FORMATION AND TRAFFICKING

2.1 Introduction

Centriolar satellites are an array of granules that localize and move around the centrosome/cilium complex in a microtubule-dependent manner. They are linked to a variety of centrosome-related processes including centrosome/cilium protein trafficking and ciliogenesis, and implicated in ciliopathies. Although centriolar satellites are essential structures for regulating the structure and function of the centrosome/cilium complex, the molecular mechanism of this regulation remains poorly understood. To elucidate the role of satellites during ciliogenesis, we determined the proximity partners of the satellite protein CEP72. Focusing on one of its proximity partners, we identified the retinal degeneration gene product CCDC66 as a microtubule-associated protein that localizes to the centrosome, centriolar satellites and the primary cilium. Like BBS4, CCDC66 relocates from satellites to the primary cilium during ciliogenesis. Proximity mapping of CCDC66 revealed extensive proximity interactions with centriolar satellite proteins and co-immunoprecipitation experiments identified interactions between CCDC66, CEP290 and PCM1. Loss of CCDC66 causes defects in ciliogenesis, ciliary recruitment of BBS4 and centriolar satellite organization. Our findings identify CCDC66 as a targeting factor for centrosome/cilium proteins.

2.2 Literature Review

Centrosomes are main microtubule-organizing centers of animal cells. They create various microtubule arrays, and thus influence cell shape, polarity and motility, as well as spindle formation, chromosome segregation and cell division (Luders & Stearns, 2007). At the core of a typical centrosome are two cylindrical, microtubule-based structures termed centrioles, which recruit a matrix of associated PCM that nucleates and organizes microtubules. Importantly, centrioles are essential for nucleating the formation of the primary cilium, which functions as a nexus for developmentally important signaling pathways such as Hedgehog, Wnt and platelet-derived growth factor receptor- α signaling (N. F. Berbari et al., 2009; S. Kim & Dynlacht, 2013). In addition to centrioles and PCM, vertebrate cells have an array of non-membranous 70-100 nm granules that localize and move around the centrosome/cilium complex in a microtubule-dependent manner, called “centriolar satellites” (Barenz et al., 2011; M. A. Tollenaere et al., 2015).

Importantly, there are many links between the centrosome/cilium complex and human disease (M. Bettencourt-Dias et al., 2011; E. A. Nigg & Raff, 2009). Abnormalities of centrosome structure and number have long been associated with cancer (Vitre & Cleveland, 2012). Moreover, mutations affecting components of the centrosome/cilium complex and satellites cause a set of disease syndromes termed ciliopathies, which are characterized by a diverse set of phenotypes, including renal disease, retinal degeneration, polydactyly, neurocognitive deficits and obesity (N. A. Adams et al., 2007; Hildebrandt et al., 2011). Although more than 40 genes have been identified as causative genes for ciliopathies, the underlying molecular mechanisms of these disease syndromes remains poorly understood (Waters & Beales, 2011). To determine these mechanisms, it is important to elucidate the biogenesis of the centrosome/cilium complex and the control mechanisms that regulate their structure and function.

Emerging evidence suggests that centriolar satellites are key regulators of the centrosome/cilium complex. Centriolar satellite proteins identified so far play important roles in various processes such as cilium formation (L. Wang et al., 2016), centrosome duplication (Firat-Karalar, Rauniyar, et al., 2014; Kodani et al., 2015), microtubule organization, mitotic spindle formation (K. Kim & Rhee, 2011), chromosome segregation (K. Kim et al., 2012; Staples et al., 2012), stress response (Villumsen et al., 2013), autophagy (Pampliega et al., 2013; Tang et al., 2013) and neurogenesis (M. A. Tollenaere et al., 2015). Interestingly, a significant percentage of

satellite components also localize to the centrosome/cilium complex (M. A. Tollenaere et al., 2015)). Moreover, all satellite-linked diseases including ciliopathies, primary microcephaly and various neurological disorders are also associated with abnormalities of the centrosome/cilium complex (Kodani et al., 2015; M. A. Tollenaere et al., 2015). These lines of evidence together suggest that satellites are ubiquitous and essential structures for the centrosome/cilium complex in vertebrates. However, the molecular mechanism of how the centrosome/cilium complex is regulated by centriolar satellites remains poorly understood.

Centriolar satellites play important roles during cilium assembly and function, most likely by trafficking proteins to or away from the centrosome/cilium complex or sequestering them (M. A. Tollenaere et al., 2015). Among the proteins that localize to centriolar satellites are the ciliopathy proteins CEP290 and BBS4, which function in the highly regulated trafficking of ciliary proteins into and out of the cilium. CEP290 is component of the transition zone that forms a selective barrier at the base of the cilium (Betleja & Cole, 2010). BBS4 is one of the seven highly conserved BBS proteins that together form a coat complex and traffic membrane proteins to cilia (M. V. Nachury et al., 2007; M. V. Nachury, 2008). In addition to the transition zone and the BBSome complex, intraflagellar transport proteins are also critical for ciliary trafficking, as they function in the bidirectional movement of multiprotein complexes along the axoneme (Scholey, 2008). Ciliary protein trafficking is key to the formation and function of the primary cilium and impaired ciliary trafficking is a significant defect associated with ciliopathies.

To elucidate the molecular mechanism of how centriolar satellites regulate cilium assembly and function, it is important to identify the full repertoire of satellite proteins. To this end, we have started building a proximity interaction map for satellite proteins implicated in cilium assembly and function. The proximity interactors of these satellite proteins include both known satellite proteins and previously uncharacterized proteins (Firat-Karalar, Rauniyar, et al., 2014, p.). Among these proteins, we chose *CCDC66*, for further study, based on its previous implication in microtubule-mediated functions including spindle pole and centriolar satellite organization (G. D. Gupta et al., 2015; Sharp et al., 2011) and its link to retinal degeneration (Dekomien et al., 2010; Gerding et al., 2011). A homozygous mutation in the *CCDC66* gene was shown to cause autosomal recessively inherited, generalized progressive retinal atrophy, the canine counterpart of Retinitis pigmentosa (Dekomien et al., 2010). Moreover, *CCDC66* knockout in mouse leads to early

photoreceptor degeneration, with a slow, progressive retinal phenotype and physiological impairment of the retina (Gerding et al., 2011). These studies together suggest an important role of CCDC66 in retinal development and function.

In this study, we functionally and biochemically characterized CCDC66 in ciliated RPE1 cells. We showed that CCDC66 localizes to the centrosome and the centriolar satellites. Strikingly, CCDC66 redistributes between centriolar satellites and the primary cilium in ciliated cells, which identifies CCDC66 as the only protein other than BBS4 with such relocalization pattern. CCDC66 binds to microtubules *in vitro* and *in vivo* and when overexpressed, it bundles microtubules, disrupts the cellular distribution of centriolar satellites and inhibits ciliogenesis. BioID proximity mapping of CCDC66, but not the CCDC66 deletion mutant that mimics the truncated protein described in retinal degeneration, revealed extensive proximity interactions with centrosome and satellite proteins. In agreement with these interactions, CCDC66 colocalizes and interacts with the satellite proteins PCM1 and CEP290 and functions in cilium formation, satellite organization and BBS4 recruitment to the primary cilium. Together, our findings identify a critical function of CCDC66 in cilium formation and a possible function in ciliary trafficking.

2.3 Materials and Methods

2.3.1 Cell Culture and Transfection

hTERT-immortalized retinal pigment epithelial (RPE-1, ATCC, CRL-4000) and LAP-BBS4-hTERT-RPE1 cells were grown in DMEM/F12 50/50 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA). Human embryonic kidney (HEK293T, ATCC, CRL-3216) cells were grown in DMEM supplemented with 10% FBS. All cells were cultured at 37 °C and 5% CO₂. All cell lines were authenticated by Multiplex Cell line Authentication (MCA) and were tested for mycoplasma contamination by MycoAlert Mycoplasma Detection Kit (Lonza).

RPE1 cells were transfected with the plasmids using Lipofectamine LTX (Invitrogen). HEK293T cells were transfected with the plasmids using 1 µg/µl polyethylenimine, MW 25 kDa (PEI, Sigma-Aldrich, St. Louis, MO). For microtubule-depolymerization experiments, cells were treated with 10 µg/ml nocodazole (US Biological, Swampscott, MA) for 3 h at 37 °C. RPE1 cells stably expressing GFP-CCDC66, GFP-CCDC66^{RR} or GFP were generated by infection of cells with GFP-CCDC66, GFP-CCDC66^{RR} and GFP-expressing lentivirus. Lentivirus were generated as described (Mahjoub et al., 2010), using pLVPT2-GFP-N1-CCDC66, pLVPT2-N1-CCDC66^{RR} and pLVPT2-N1 plasmids as transfer vectors.

2.3.2 RNAi and Rescue Experiments

CCDC66 was depleted using an siRNA with the sequence 5'-CAGTGTAATCAGTTCACAATT-3'. Silencer Select Negative Control No. 1 (Thermo Scientific) was used as a control. CEP290 depletion was carried out using ON-TARGET Plus SMARTpool (Thermo Scientific). siRNAs were transfected into RPE1-hTERT cells using Lipofectamine RNAiMax following manufacturer's instructions (Thermo Fisher Scientific). For rescue experiments, RPE-1 cells stably expressing GFP (RPE-1::GFP) or GFP-CCDC66^{RR} (RPE-1::CCDC66^{RR}-GFP) were transfected with control and CCDC66 siRNAs using Lipofectamine RNAiMax (Thermo Fisher Scientific). 72 h post-transfection with the siRNAs, cells were fixed and stained.

2.3.3 Immunoprecipitation and BioID pulldowns

For the co-immunoprecipitation experiments, HEK293T cells were transiently transfected, incubated for 24 h, washed with PBS, and lysed in lysis buffer (50 mM Tris, pH 7.4, 266 mM NaCl,

2.27 mM KCl, 1.25 mM KH₂PO₄, 6.8 mM Na₂HPO₄·7H₂O and 1% NP-40, protease inhibitors). Insoluble material was pelleted, and soluble material was incubated with goat anti-GFP antibodies (Rockland) and then with Protein A beads (Affi-Prep). Beads were washed in lysis buffer, eluted in sample buffer, and run on SDS-PAGE gels. BioID streptavidin pulldown was done as described previously (Firat-Karalar, Sante, et al., 2014).

2.3.4 Microtubule Pelleting Assays

Purified bovine brain tubulin was diluted to a concentration of 2 mg/ml in BRB80 buffer (80 mM PIPES, pH 6.8, 1 mM MgCl₂, 1 mM EGTA including protease inhibitors, 1 mM GTP, 1 mM dithiothreitol and 20 μM taxol), precleared at 90,000 rpm for 5 min at 4°C and brought to a final concentration of 20 μM Taxol in a step-wise manner at 37°C. 0.5 nmol of MBP-CCDC66 fragments affinity purified from *E. coli*, or 1.5 mg cell extracts were diluted in 250 final volume of BRB80, cleared at 90,000 R.P.M. for 5 min at 4°C. Protein samples were mixed with taxol-stabilized microtubules or buffer control and incubated for 30 min at 30°C. Samples were loaded onto 40% glycerol BRB80 cushions and centrifuged at 55,000 rpm for 10 min. Supernatant and pellet fractions were separated by SDS-PAGE for analysis.

2.3.5 Mass Spectrometry and Data Analysis

Mass spectrometry analysis was done as described previously with the following change (Firat-Karalar, Rauniyar, et al., 2014): The MS/MS spectra were searched with the ProLuCID algorithm against the Uniprot human database (downloaded on 03/20/2014). For each BioID experiment data were derived from three biological replicates. Control data were derived from mock-transfected and BirA*-transfected HEK293T cells. To compare data across different runs and assess the abundance of each proximity partner, we applied the normalization of spectral counts (Zybailov et al., 2006). Individual spectral count values were normalized against the sum of all spectral counts for a particular run, resulting in normalized spectral abundance factor (NSAF). For presentation purposes, we multiplied each NSAF value by 100. To distinguish the nonspecific interactors, we calculated the ratio of the NSAF of each identified protein to the NSAF of the corresponding proteins in the control datasets. A protein was considered a contaminant if the ratio was smaller than 2.5. For analysis of the data from three independent BioID experiments, we only accounted for the proteins that were identified in at least 2 independent experiments and that have a spectral count greater than 4.

2.3.6 Immunofluorescence, microscopy and quantitation

For immunofluorescence experiments, cells were grown on coverslips and fixed in either methanol or 4% paraformaldehyde in PBS. After rehydration in PBS, cells were blocked in 3% BSA (Sigma-Aldrich) in PBS + 0.1% Triton X-100. Coverslips were incubated in primary antibodies diluted in blocking solution, and Alexa Fluor 488-, 594-, or 680-conjugated secondary antibodies were diluted in 1:500 in blocking solution (Invitrogen). Biotinylated proteins were detected with Alexa Fluor 594-coupled streptavidin (1:500; Invitrogen). Samples were mounted using Mowiol. Coverslips of cells were imaged using LAS X software (Premium; Leica) on a scanning confocal microscope (SP8; Leica Microsystems) with Plan Apofluar 63X 1.4 NA objective and by spinning disk confocal microscopy using a Zeiss Axiovert with a Yokogawa CSU-10 confocal head using a cooled charge-coupled device camera (Orca ER, Hamamatsu Photonics; Cascade EM-CCD, Photometrics). For deconvolved images, Huygens Deconvolution software was used.

Quantitative immunofluorescence for PCM1 and CEP290 was performed by acquiring a z-stack of control and depleted cells using identical gain and exposure settings. The z-stacks were used to assemble maximum-intensity projections. The centrosome region was defined by γ -tubulin staining in each cell and its fluorescence pixel intensity was measured in a circular 3 μm^2 area centered on the centrosome. The satellite region was defined as the 19 μm^2 -ring around the centrosome. Quantifications and image processing were performed using ImageJ (National Institutes of Health, Bethesda, MD). Background was quantified by measuring fluorescence intensity of a region of equal dimensions in the area neighboring the centrosome and the satellites. Primary cilium formation was assessed by counting the total number of cells and the number of cells with primary cilia.

2.3.7 Statistical Analysis

Statistical significance and p values were assessed by one-way analysis of variance and Student's t tests using Prism software (GraphPad Software, La Jolla, CA). Error bars reflect SEM. Following key is followed for asterisk placeholders for p -values in the figures: *** p <0.001, ** p <0.01, * p <0.05.

2.4 Results

2.4.1 Proximity mapping identified new centriolar satellites proteins

To identify new components of centriolar satellites that function during ciliogenesis, we determined the proximity interactors of CEP72 at the centriolar satellites using the BioID approach (Firat-Karalar & Stearns, 2015; Roux et al., 2013). CEP72 localizes to centriolar satellites and interacts with CEP290 to recruit BBS proteins to the primary cilium (T. R. Stowe et al., 2012). We generated an N-terminal myc-BirA* fusion of CEP72 and showed that myc-BirA*-CEP72 (hereafter BirA*-CEP72) localized to the centriolar satellites, assessed by anti-Myc staining (Figure 7A) and stimulated localized biotinylation of the centriolar satellites, assessed by streptavidin staining (Figure 7B). HEK293T cells were then transfected with BirA*-CEP72, supplemented with 50 μ M biotin 24 h post-transfection, and incubated for 18 h. After cells were solubilized with denaturing lysis buffer, biotinylated proteins were precipitated by streptavidin beads and analysed by mass spectrometry. HEK293T cells transfected with BirA*, or mock-transfected, were processed in parallel as controls. The proximity interactors for CEP72 are listed by normalized spectral abundance factor (NSAF) value in Figure 7C and Table 1. NSAF value is an estimate of relative abundance of proteins in a sample. A significant percentage of proximity interactors of CEP72 is centrosome and/or centriolar satellite components, validating the power of our approach to identify proximity interactors at the centriolar satellites. The CEP72 BioID hits also include proteins that were not previously implicated for functions in ciliogenesis. Among these hits is the retinal degeneration gene CCDC66 (Dekomien et al., 2010; Gerding et al., 2011), which was reported to localize to centriolar satellites (G. D. Gupta et al., 2015) and its mRNA was found to interact with microtubules in a screen for microtubule-binding transcripts in *Xenopus laevis* egg extracts (Sharp et al., 2011). CCDC66 was also previously identified a proximity partner of the canonical satellite protein PCM1 (G. D. Gupta et al., 2015), but not for CCDC14 and KIAA0753, which regulate centriole duplication (Firat-Karalar & Stearns, 2015). Based on its proximity to regulators of ciliogenesis, disease link and cellular localization, we chose CCDC66 for further study.

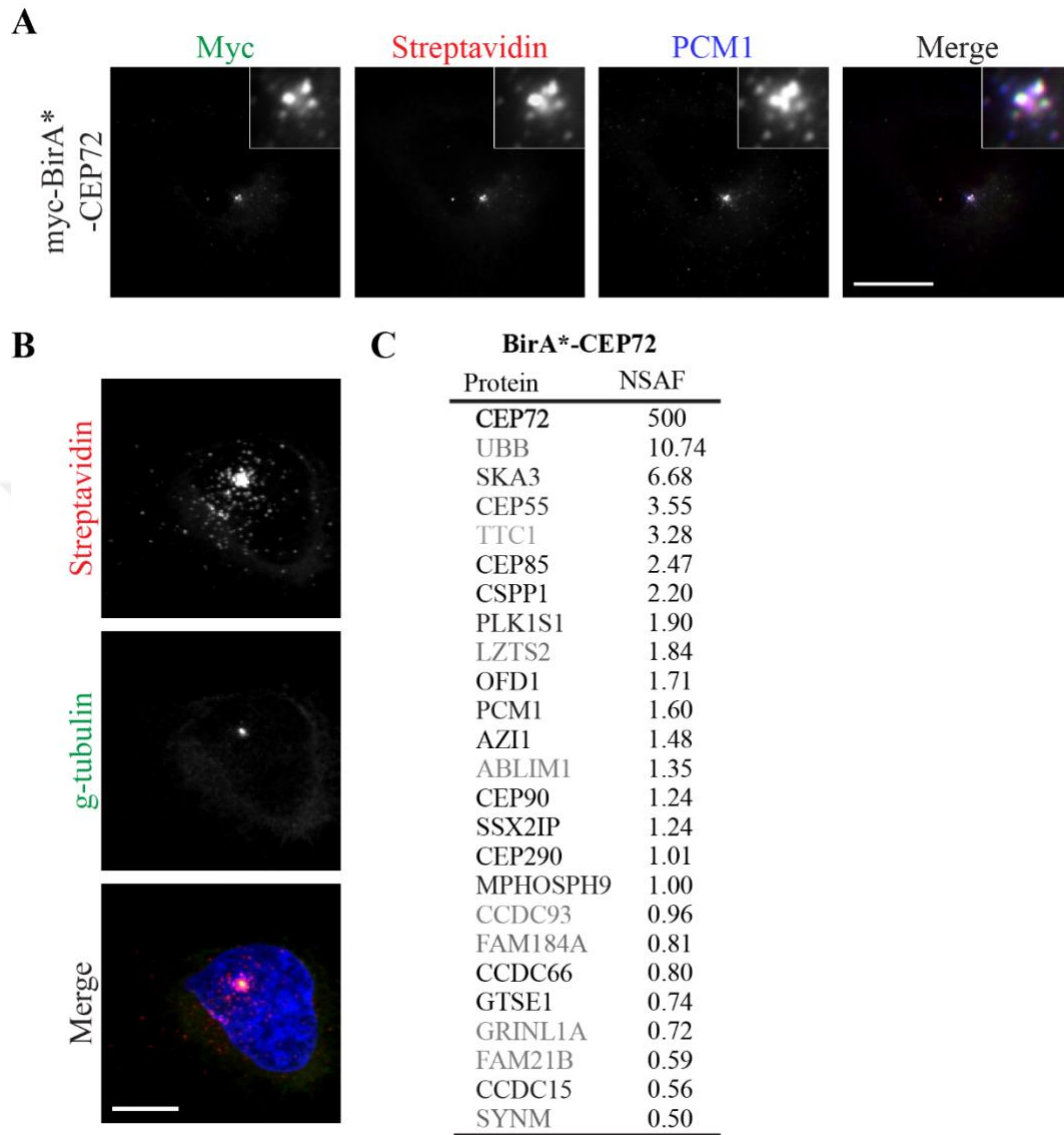


Figure 7. Localization, activity and proximity interactors of BirA*-tagged CEP72

A. BirA*-CEP72 localizes to centriolar satellites and stimulates biotinylation at the centriolar satellites. RPE1 cells were transfected with Myc-BirA*-tagged CEP72. After 18 h incubation with biotin, cells were fixed and stained for Myc-BirA*-CEP72 with anti-Myc antibody, biotinylated proteins with fluorescent streptavidin and centriolar satellites with anti-PCM1 antibody. Scale bar, 10 μ m. **B.** BirA*-CEP72 stimulates biotinylation at the centriolar satellites. RPE1 cells were transfected with Myc-BirA*-tagged CEP72. After 18 h incubation with biotin, cells were fixed and stained for biotinylated proteins with fluorescent streptavidin and centrosomes with anti- γ -tubulin antibody. DNA was stained with DAPI. Scale bar, 10 μ m. **C.** Mass spectrometry analysis of proximity interactors of Myc-BirA*-CEP72. Proximity interactors are ranked in the order of their

normalized spectral abundance factor (NSAF) values (average of three independent experiments). Proteins in black were previously shown to localize to the centrosome/cilium complex, proteins in bold were previously shown to interact physically with CEP72 and proteins in grey were not associated with the centrosome/cilium complex in previous studies.



BirA*-CCDC66		BirA*-CCDC66-RD		BirA*-CEP72	
Protein	NSAF	Protein	NSAF	Protein	NSAF
CCDC66	20	CCDC66-RD	17	CEP72	50
PCM1	1.31	TUBA1B	1.57	UBB	10.74
HSPA1A	1.10	TUBA1A	1.52	SKA3	6.68
TUBA1B	0.88	HSPA1A	1.50	CEP55	3.55
ALMS1	0.65	TUBA4A	1.14	TTC1	3.28
AZI1	0.56	FASN	1.12	CEP85	2.47
TUBA1A	0.52	HSPA8	1.04	CSPP1	2.20
HSPA1L	0.50	PRKDC	0.70	PLK1S1	1.90
HSPA8	0.45	TUBB4B	0.58	LZTS2	1.84
CEP152	0.45	EIF4A1	0.54	OFD1	1.71
DDX3X	0.42	TCP1	0.53	PCM1	1.60
EIF5	0.39	TUBB6	0.41	AZI1	1.48
OFD1	0.33	DYNC1H1	0.39	ABLIM1	1.35
MAP4	0.29	CCT6A	0.34	CEP90	1.24
CEP290	0.26	MCCC2	0.30	SSX2IP	1.24
MPHOSPH9	0.23	RANPE	0.27	CEP290	1.01
HDLBP	0.23	PCCB	0.26	MPHOSPH9	1.00
TJP1	0.21	LDHB	0.24	CCDC93	0.96
CKAP5	0.20	HSBD1	0.24	FAM184A	0.81
CCDC138	0.20	CLTC	0.24	CCDC15	0.80
MRE11A	0.20	LARS	0.24	GTSE1	0.74
NUFIP2	0.18	MYBBP1A	0.23	GRINL1A	0.72
ENO1	0.18	IARS	0.21	FAM21B	0.59
NIN	0.18	SF3B1	0.21	CCDC66	0.56
ARMC9	0.17	CCT3	0.20	SYNM	0.50
CEP192	0.17	NAP1L1	0.19	NIN	0.42
CEP350	0.16	EPRS	0.19	ARPC5	0.42
CEP128	0.16	CCT4	0.19	KIAA1731	0.35
KIF7	0.16	RPL9	0.19	NUP37	0.34
IFT74	0.15	PRPF8	0.19	SQSTM1	0.34
MAP7D3	0.15	ARF1	0.18	SIPA1L1	0.30
CCDC14	0.15	CSE1L	0.17	ITGB3BP	0.29
CEP162	0.15	CKAP5	0.16	RNF114	0.27
TNRC6B	0.15	GART	0.16	ALMS1	0.26
PCNT	0.15	ARF3	0.15	CCNB2	0.16
		HSPA9	0.15	U2AF1	0.16
		EIF4A3	0.15	SMN2	0.13
		RPS3	0.15	TTC31	0.10
		CAND1	0.15	SLAIN2	0.10
		IRS4	0.15		

Table 1. Proximity interactors of CCDC66 and CCDC66-RD

Mass spectrometry analysis of proximity interactors of the indicated Myc-BirA* fusion proteins. Proximity interactors are ranked in the order of their NSAF values (average of three independent experiments). Proteins in black were previously shown to localize to the centrosome and proteins in bold were shown to interact physically with the corresponding centrosome protein in published data or in this work.

2.4.2 CCDC66 Localizes to The Centrosome/Cilium Complex and The Centriolar Satellites

To gain insight into the molecular mechanism of satellite function during ciliogenesis, we characterized CCDC66 in ciliated hTERT-RPE1 (hereafter RPE1) cells. Orthologs of CCDC66 are present in hemichordates and placozoans but absent in arthropods, nematodes and *Chlamydomonas*. The evolutionary conservation pattern of CCDC66 is similar to the core centriolar satellite protein PCM1 (Hodges et al., 2010) but not BBS4 (J. C. Kim et al., 2004) and CEP290 (J. Kim et al., 2008). CCDC66 contains 2 coiled-coil (CC) domains: CC1 (amino acids 252-283) and CC2 (amino acids 466-564) and an evolutionarily conserved CCDC66 domain (Pfam: 15236) (amino acids 408-564), part of which overlaps with CC2 (Figure 8).

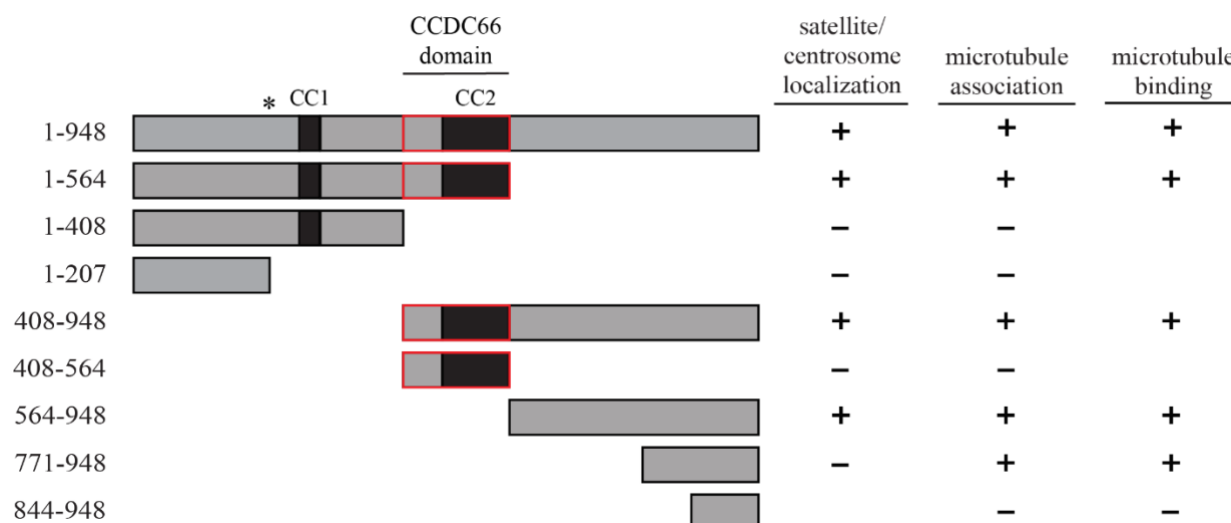


Figure 8. Localization and interaction analysis of CCDC66 deletion mutants

Schematic representation of CCDC66 full length (1-948) and deletion constructs and summary of their localization to centriolar satellites and the centrosome and interaction/association with microtubules. Satellite/centrosome localization and microtubule association were determined by analyzing RPE1 cells transiently transfected with GFP-CCDC66 constructs and staining fixed cells for GFP and PCM1, g-tubulin or microtubules. Microtubule binding was determined by in vitro microtubule pelleting assays of extracts from cells transiently expressing GFP-tagged full-length CCDC66 and its deletion constructs and/or purified proteins. Numbers indicate amino acid positions, CC1 and CC2 indicate coiled coil domains and “CCDC66 domain” indicate conserved domain in CCDC66 orthologs. “*” indicates the early point mutation at amino acid 207 in dogs with retinal degeneration.

Expression studies in dog and mouse tissues via qRT-PCR (Dekomien et al., 2010) and a genome-wide analysis of transcription profiles (Krupp et al., 2012) showed that CCDC66 is not only expressed in retina but most tissues including heart, brain, liver, and blood. Immunoblotting with an antibody against CCDC66 in RPE1, HeLa and HEK293T cell extracts validated the wide expression profile of CCDC66 (Figure 9A). We examined the localization of endogenous CCDC66 and a GFP-CCDC66 fusion protein in RPE1 cells. Antibody against endogenous CCDC66 revealed that it localizes to the centrosome and centriolar satellites in interphase cells, as assessed by co-localization with PCM1, a marker for the centriolar satellites and gamma-tubulin, a marker for the centrosome (Figure 9B). The punctate pericentrosomal distribution of satellite proteins is dependent on PCM1, a centrosomally-focused microtubule array and the microtubule motor dynein (J. Kim et al., 2008; J. C. Kim et al., 2004; Lopes et al., 2011). Upon depolymerization of microtubules, the centriolar satellite pool of CCDC66 dispersed throughout the cytoplasm (Figure 9B), as expected for centriolar satellites. Moreover, depletion of PCM1 caused loss of CCDC66 satellite localization (Figure 9B). In both cases CCDC66 remained associated with the centrosome (Figure 9B). These results show that the CCDC66-containing foci are canonical centriolar satellites and that CCDC66 localizes to the centrosome in addition to the centriolar satellites. In agreement, immunostaining of centrosomes purified from HEK293T cells with CCDC66 revealed co-localization with α -tubulin and centrin (Figure 9C).

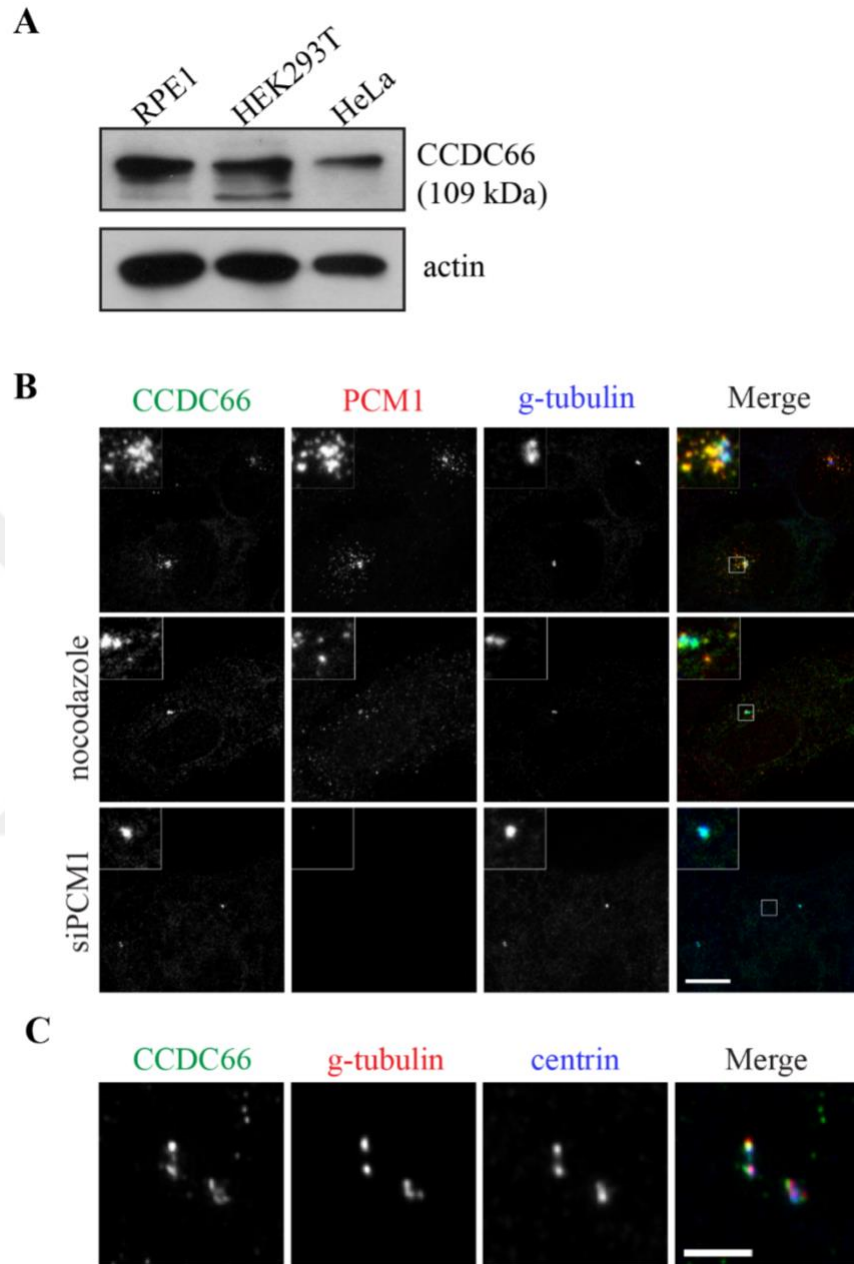


Figure 9. Endogenous CCDC66 expression and localization in cultured RPE1 cell line.

A. CCDC66 is expressed in various mammalian cell lines. Extracts from RPE1, HEK293T and HeLa cells were blotted for CCDC66 and actin (loading control). **B.** Localization of endogenous CCDC66 in interphase, in nocodazole-treated and PCM1-depleted cells. RPE1 cells were fixed and stained for CCDC66, PCM1 and gamma-tubulin. Effect of microtubule depolymerization on CCDC66 localization. RPE1 cells were treated with 10 μ g/ml nocodazole for 3 h, fixed and stained

for GFP, PCM1 and γ -tubulin. Effect of PCM1 depletion on CCDC66 localization. RPE1 cells were fixed 48 h after transfection with control PCM1 siRNA, fixed and stained. **C.** Localization of CCDC66 to purified centrosomes. Purified centrosomes were spun down on coverslips, fixed and stained for CCDC66, gamma-tubulin and centrin.



A similar localization to the centrosome and centriolar satellites was observed in RPE1 cells stably expressing GFPCCDC66 fusion protein at near-endogenous level (Figure 10A and 10B). CCDC66 localizes to the centriolar satellites and the centrosome during centriole duplication and growth in S and G2 phases (Figure 10A). However, its localization becomes restricted to the centrosomes and the spindle microtubules during mitosis (Figure 10A and 10C). Live-imaging of RPE1-[GFPCCDC66] cells revealed dynamic distribution of CCDC66 between the centrosome, centriolar satellites and microtubules throughout the cell cycle, consistent with the localization of endogenous CCDC66 (Figure 10D). Such dynamic behavior is similar to the localization of other satellite proteins including CEP290, CCDC11, FOP and CCDC13 (J. Kim et al., 2008; J. Y. Lee & Stearns, 2013; Silva et al., 2016; Staples et al., 2014).

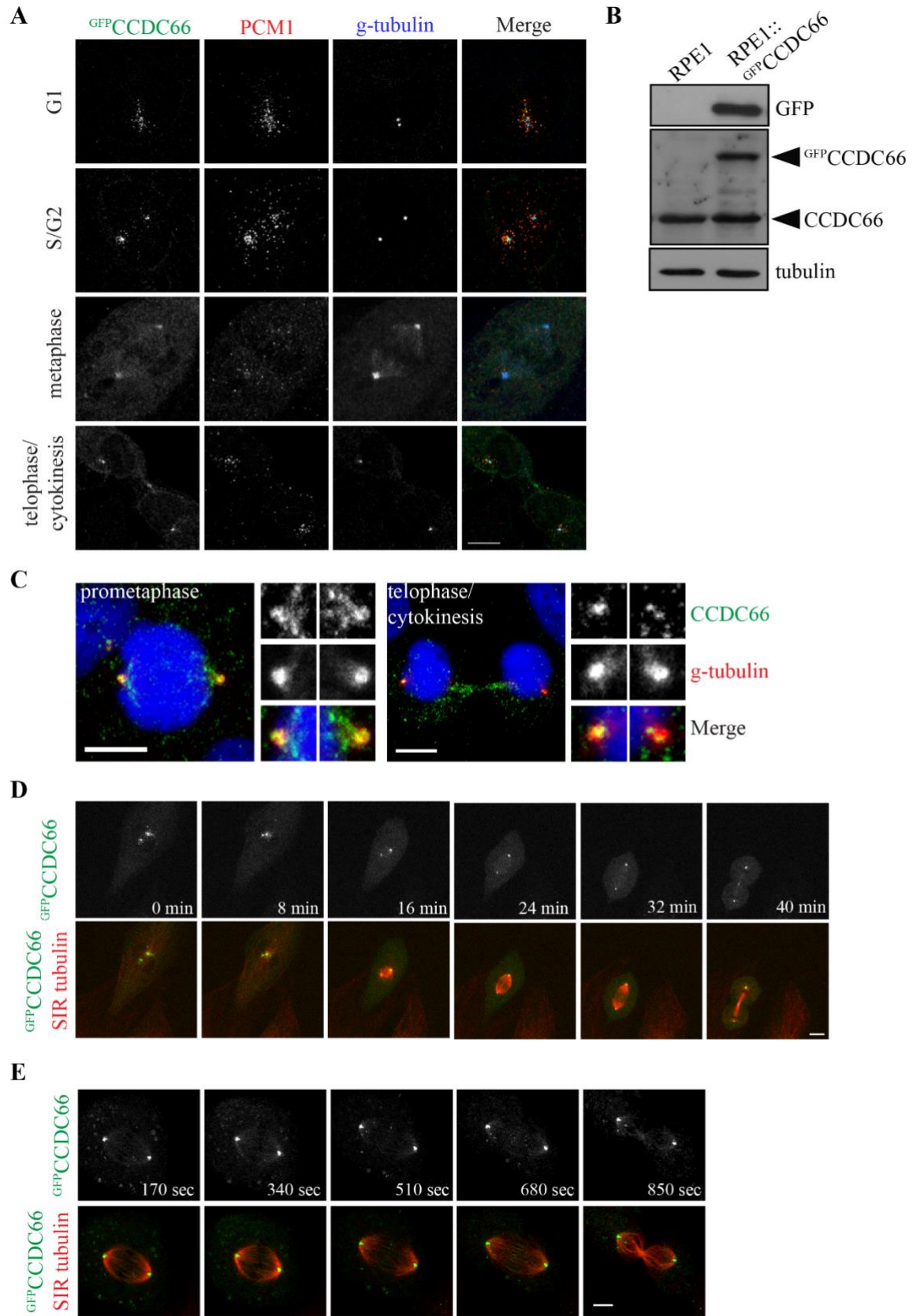


Figure 10. Expression and cell cycle dependent dynamic localization of GFPCCDC66 in RPE1 cells.

A. Asynchronously growing stably expressing RPE1-[GFPCCDC66] cells were fixed and stained for GFP, PCM1, and g-tubulin. DNA was stained with DAPI. **B.** GFPCCDC66 is expressed at near endogenous CCDC66 levels in RPE1-[GFPCCDC66] cells. RPE1 or RPE1-[GFPCCDC66] cell extracts were immunoblotted for GFP, CCDC66, and tubulin (loading control). **C.** Localization of endogenous CCDC66 in prometaphase and telophase/cytokinesis stages of the cell cycle. Asynchronously growing RPE1 cells were fixed and stained for CCDC66 and g-tubulin. DNA was stained with DAPI. Scale bars, 10 μ m, all insets show 4x enlarged centrosomes. **D and E.** Dynamic localization of GFPCCDC66 spanning the whole cell cycle (D) and from metaphase until telophase (E). Images represent a deconvolved maximum projection of sections Scale bar, 5 μ m.

To assess the localization of CCDC66 upon primary cilium assembly, RPE1-[GFPCCDC66] cells were serum-starved for 48 h. In most non-ciliated serum-starved cells, the satellite pool of GFPCCDC66 diminished significantly and GFPCCDC66 localized prominently to the centrosome (Figure 11A). Remarkably, in 95 ± 4.5 percent of ciliated cells ($n=500$), GFPCCDC66 redistributed from centriolar satellites to the primary cilium and basal body, analogous to the BBSome complex component BBS4 (M. V. Nachury et al., 2007) (Figure 11B and 11C). In the remaining cells, GFPCCDC66 localized to the cilium, basal body and centriolar satellites. In ciliated cells, GFPCCDC66 co-localized with PCM1 and CEP290 at the centriolar satellites, with poly-glutamylated tubulin at the primary cilium and the basal body. We did not observe CCDC66 localization at the ciliary transition zone, as assessed by staining with CEP290 (Figure 11C). Of note, we did not detect localization of CCDC66 to the primary cilium of RPE1 cells using the current antibodies, which is likely due to the technical difficulties in the fixation and processing of intraciliary antigens (Follit et al., 2006; M. V. Nachury et al., 2007). These results identify CCDC66 as the only protein other than BBS4 that has so far been identified to redistribute between centriolar satellite and ciliary pools in ciliated cells.

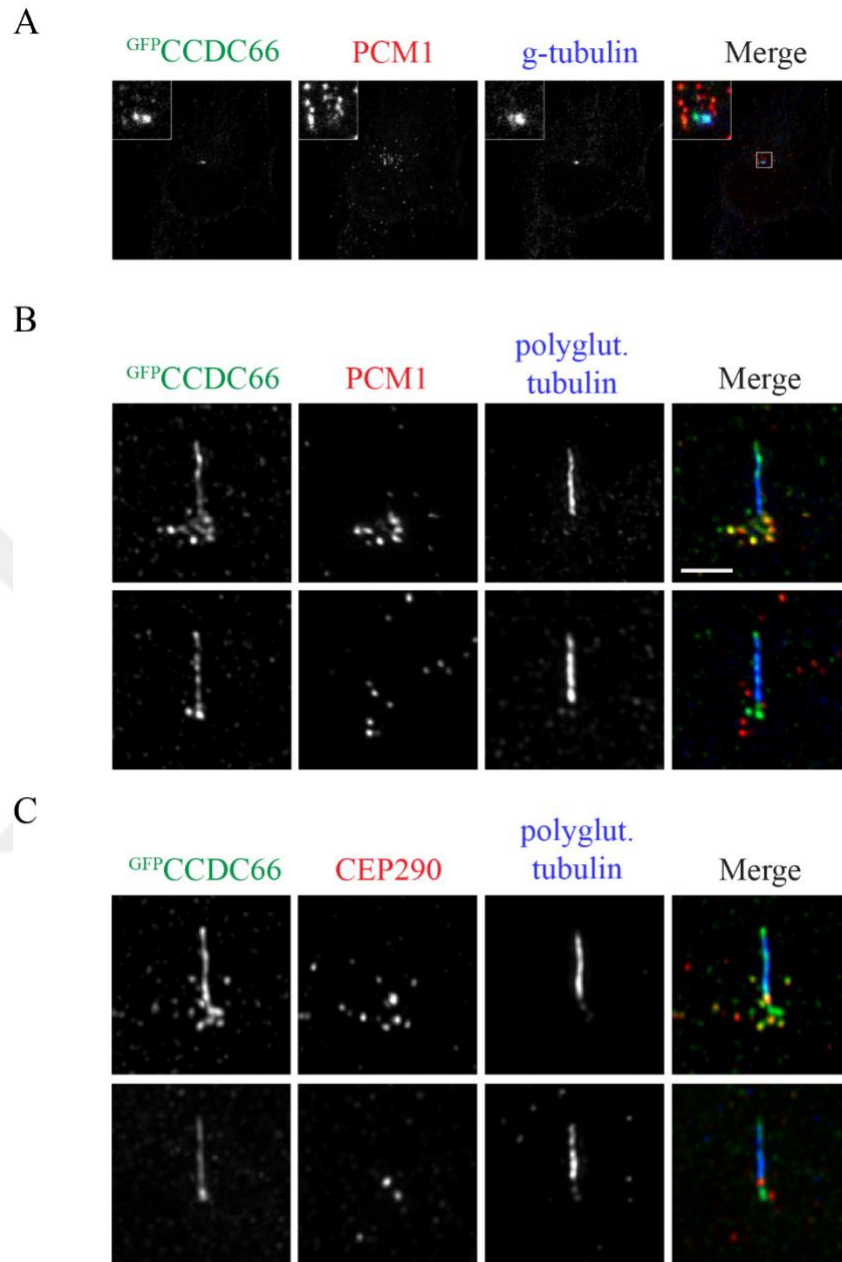


Figure 11. CCDC66 localization in serum starved RPE1 cells.

A. Localization of GFPCCDC66 in serum starved non-ciliated RPE1-[GFPCCDC66] cells, which were fixed and stained for CCDC66, PCM1 and gamma tubulin. **B. and C.** Localization of CCDC66 in ciliated cells. RPE1-[GFPCCDC66] cells were fixed 48 h after serum starvation and stained for GFP, PCM1, polyglutamylated tubulin (B) and GFP, CEP290 and polyglutamylated tubulin (C). Images represent deconvolved maximum projection of sections. Scale bars, 2.5 μ m.

2.4.3 Analysis of CCDC66 Deletion Mutants for Localization To The Centriolar Satellites and The Centrosome

To identify the functionally important domains of CCDC66 for localization to centriolar satellites and the centrosome, we generated a series of GFP-tagged CCDC66 deletion mutants (Figure 8). GFP-CCDC66 truncations lacking the conserved CCDC66 region failed to localize to satellites and the centrosome and instead had diffuse cytoplasmic and/or nuclear distribution, except for GFP-CCDC66 (564-948), which co-localized with γ -tubulin (Figure 12A and 12B). The conserved CCDC66 region did not localize to centriolar satellites and the centrosome by itself (Figure 12A and 12B). These data suggest that more than one region of CCDC66 is required for its localization to the centrosome and the satellites. GFP-CCDC66 (408-948) co-localized with PCM1-positive puncta in transfected cells, in which the distribution of satellites changed possibly due to changes in the organization of microtubules (Figure 12B). The deletion mutant CCDC66-RD (amino acids 1-207) mimics the truncated protein described in the dogs with a point mutation in CCDC66, which causes a frameshift that introduces a premature stop codon at amino acid 207 (Dekomien et al., 2010). Interestingly, CCDC66-RD localizes diffusely throughout the cytoplasm and nucleus (Figure 12), suggesting that retinal degeneration phenotype might result from disruption of the localization and interactions of CCDC66 in cells.

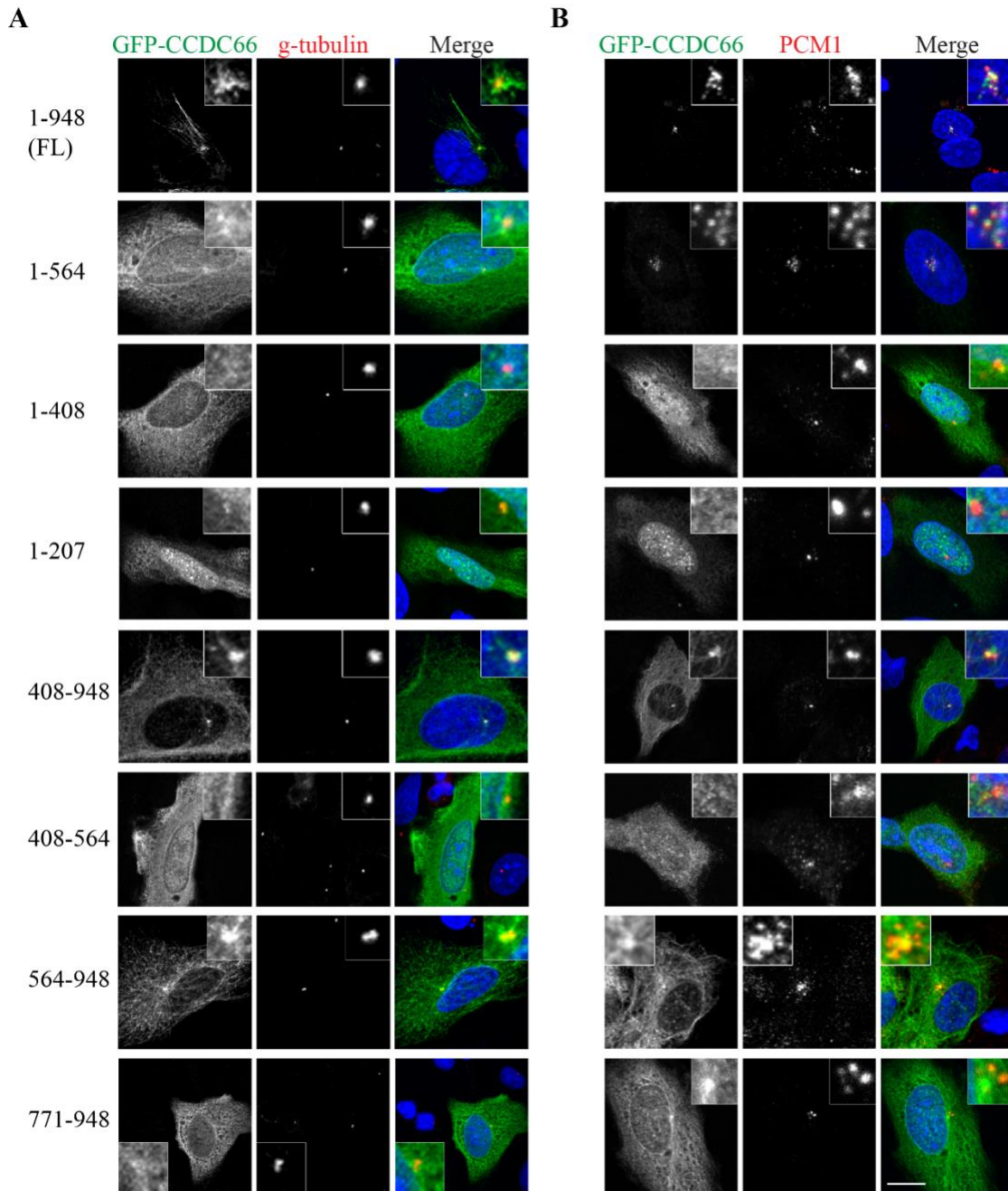


Figure 12. Analysis of CCDC66 and its deletion mutants for colocalization with centrosomes and centriolar satellites

Localization of full-length CCDC66 and its deletion constructs. RPE1 cells transfected with indicated constructs were fixed and stained for GFP and gamma-tubulin (**A**) or GFP and PCM1 (**B**). Scale bars, 10 μ m, all insets show 4x enlarged centrosomes.

2.4.4 CCDC66 is a Microtubule-Associated Protein

CCDC66 mRNA was previously shown to associate with microtubules in a screen for microtubule-binding mRNAs in *Xenopus laevis* eggs (Sharp et al., 2011), suggesting a possible function for CCDC66 in microtubule-based cellular processes. To address this possibility, we examined the affinity of CCDC66 to microtubules using *in vitro* and *in vivo* assays. In transiently transfected high-expressing cells, GFP-CCDC66 localized to microtubules (Figure 13A). Interestingly, we found that 67 ± 6.3 percent of transfected cells ($n=200$) had microtubule bundles, which was not observed in control cells transfected with GFP. In a subset of cells, the microtubule bundles were associated with the centrosome and stained for alpha-tubulin and PCM1 but did not stain for ciliary axoneme marker poly-glutamylated tubulin, suggesting that they are not normal cilia (Figure 13B). Although GFP-CCDC66 overexpression induced microtubule bundles, there was no significant change ($p=0.58$) in the total fluorescence intensity of alpha-tubulin between GFP-CCDC66-expressing cells and control GFP-expressing cells.

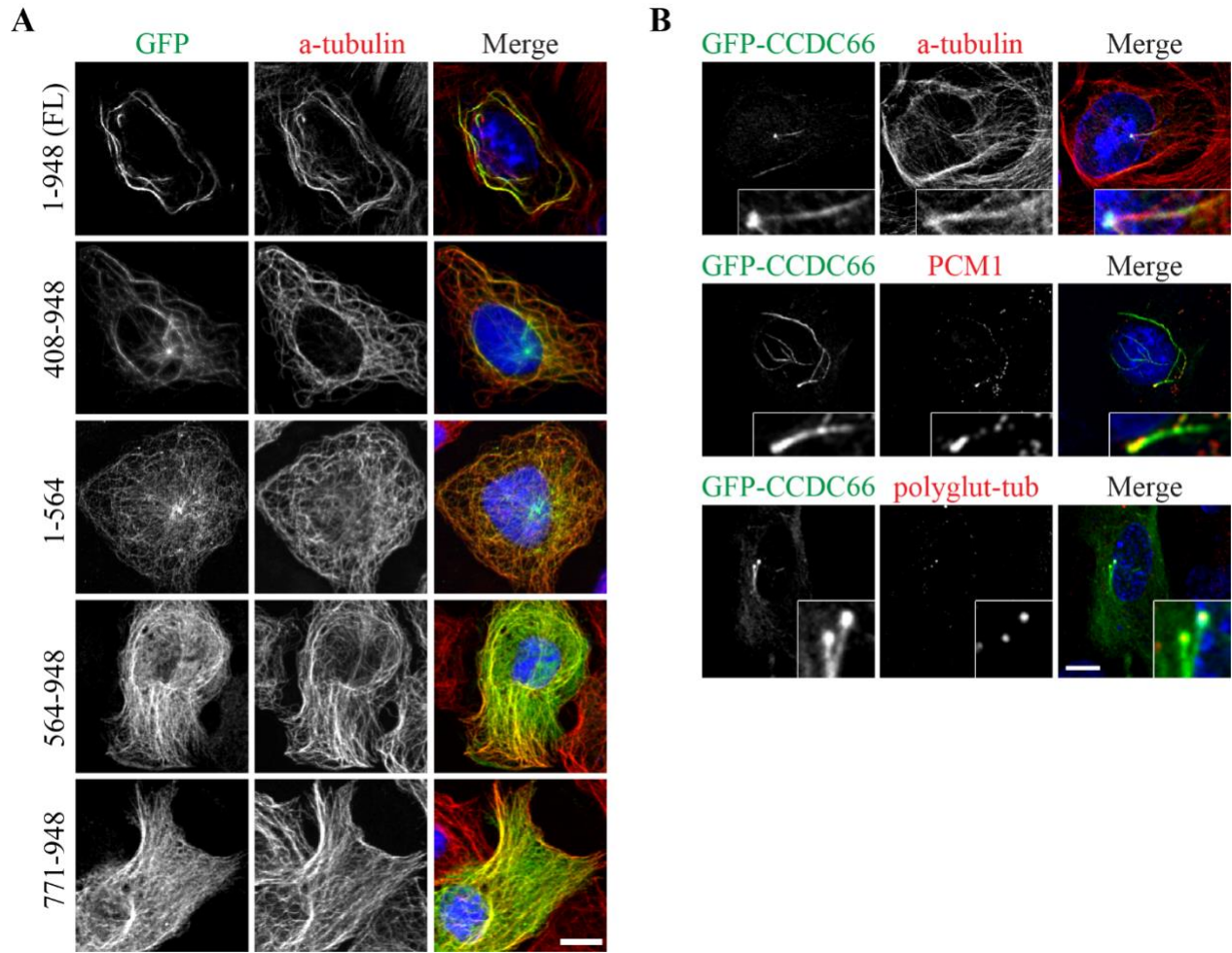


Figure 13. CCDC66 overexpression associates with and results in microtubule bundles

A. GFP-CCDC66 localizes to microtubules in transiently transfected cells. RPE1 cells transfected with GFP-tagged full-length CCDC66 and its deletion constructs were fixed and stained for GFP and alpha-tubulin. **B.** The bundles associated with the centrosome co-localize with alpha-tubulin and PCM1. RPE1 cells transiently transfected with GFP-CCDC66 were fixed and stained for GFP and alpha-tubulin or glutamylated-tubulin or PCM1.

Given the association of CCDC66 with microtubules *in vivo*, we next studied whether CCDC66 interacts with microtubules using *in vitro* microtubule pelleting assays. We first performed the pelleting assays with lysates from cells transfected with GFP-CCDC66 or GFP as a negative control. In agreement with the localization results, GFP-CCDC66, but not GFP, co-pelleted with taxol-stabilized microtubules (Figure 14A). To determine whether endogenous CCDC66 also interacts with microtubules and that the interaction of GFP-CCDC66 to microtubules is not a consequence of overexpression, we performed *in vitro* microtubule pelleting assays with cell lysates and demonstrated that endogenous CCDC66 co-pellets with taxol-stabilized microtubules (Figure 14B). As controls, the cytoplasmic protein GAPDH remained in the supernatant, whereas the microtubule-associated protein PLK1 co-pelleted with microtubules (Figure 14B). Affinity of endogenous CCDC66 to microtubules is consistent with the localization of CCDC66 to the spindle microtubules and mid-zone during mitosis (Figure 10C).

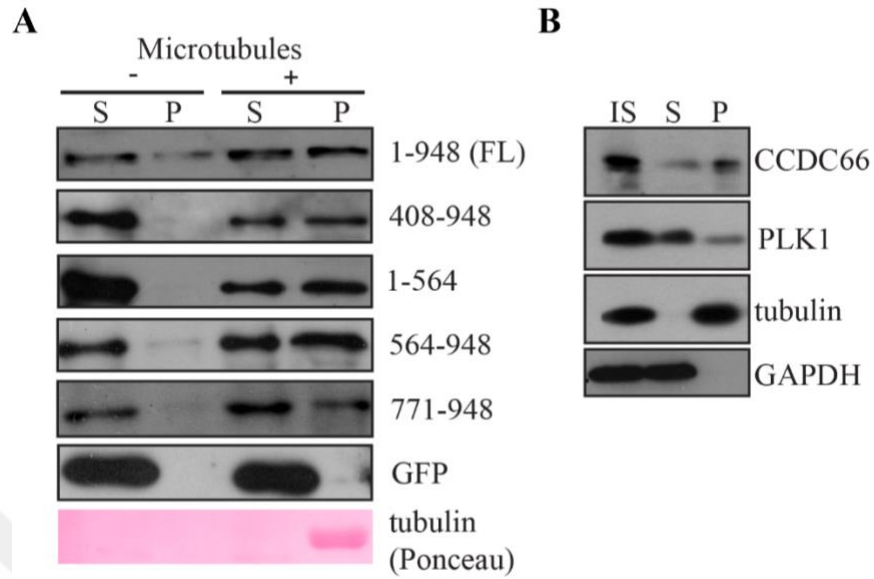


Figure 14. CCDC66 associates with microtubules *in vitro*.

Microtubule pelleting assay were performed using extracts from cells expressing GFP-tagged full-length CCDC66 and its deletion constructs **(A)** or extracts from untransfected cells **(B)**.

To identify the regions of CCDC66 that are important for microtubule association and binding, we determined the localization of these mutants in transfected cells (Figure 13A, Figure 15A) and tested whether the GFP-tagged CCDC66 truncation mutants that are positive for microtubule association bind to microtubules using the *in vitro* microtubule pelleting assays (Figure 14A). The N-terminal 564 amino acids, containing the CCDC66 conserved region and the C-terminal 177 amino acids localized and interacted with microtubules (Figure 14). Interestingly, expression of these fragments did not induce formation of microtubule bundles, instead they decorated the microtubule network in cells (Figure 13A). These data suggest that two different regions mediate CCDC66 interaction with microtubules and that only the full-length protein can change the organization of microtubule network through bundling.

To test whether association of CCDC66 with microtubules reflects direct binding, we purified recombinant MBP-tagged CCDC66 (564-948) and CCDC66 (844-948) and used in *in vitro* microtubule pelleting assays. Consistent of with localization results, the microtubule-binding MBP-CCDC66 (564-948), but not the negative control MBP-CCDC66 (844-948) pelleted with microtubules (Figure 15B and 15C). The fraction of MBP-CCDC66 (564-948) in the pellet increased with increasing concentrations of taxol-stabilized microtubules (Figure 15D). Together, these data indicate a direct interaction of CCDC66 with microtubules and identify CCDC66 as a microtubule-associated protein.

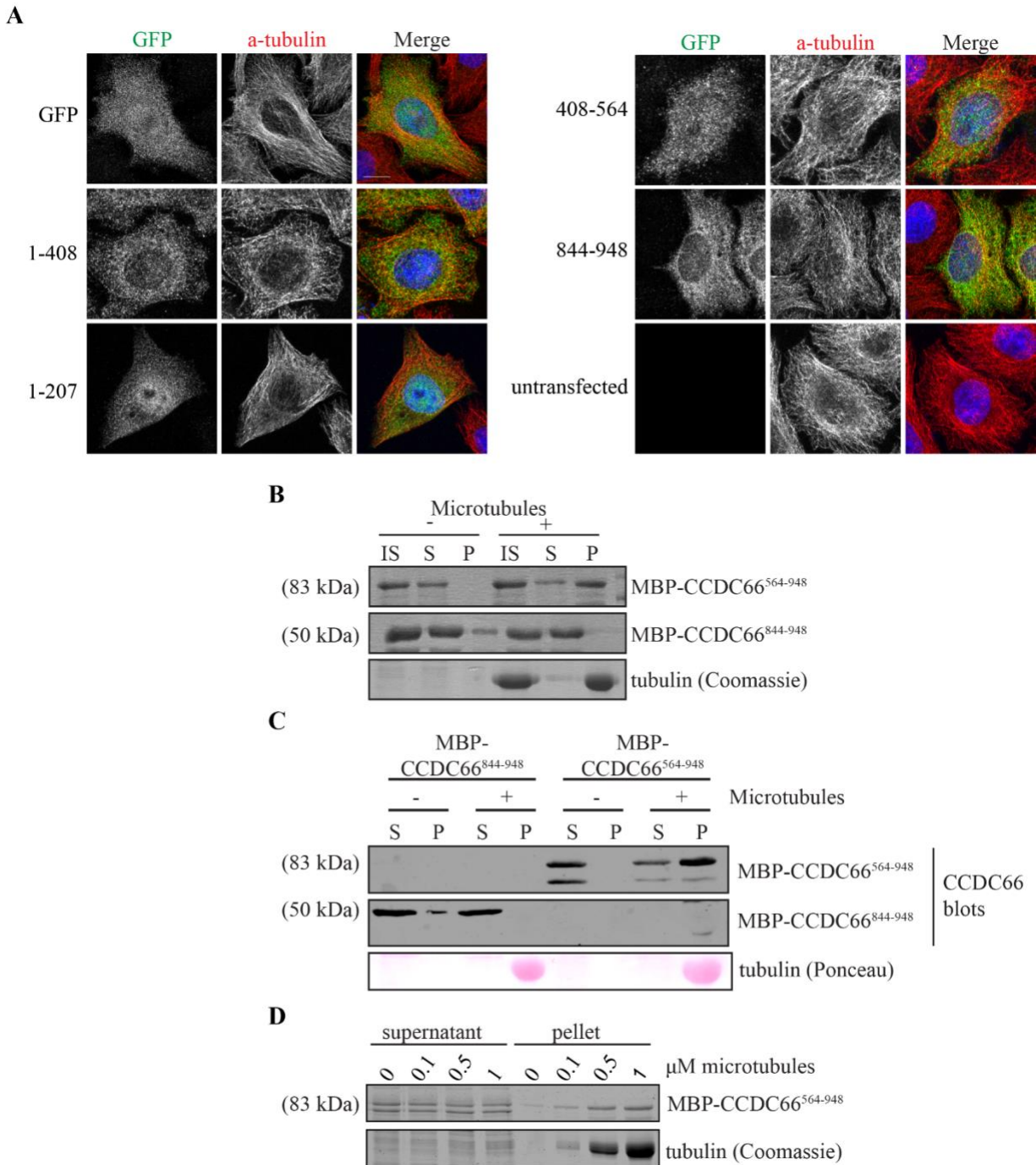


Figure 15. C terminal of CCDC66 binds to microtubules

A. Localization of CCDC66 deletion constructs. Untransfected RPE1 cells or RPE1 cells transfected with indicated constructs were fixed and stained for GFP and alpha-tubulin. DNA was stained with DAPI Scale bars, 10 μ m. **B.** 0.5 nmol purified MBP-tagged CCDC66 fragments.

Indicated cell extracts or purified proteins were incubated with taxol-stabilized microtubules or buffer, loaded onto a 40% glycerol cushion and spun down. Initial sample (IS), Supernatant (S) and pellet (P) fractions were blotted for GFP, CCDC66, PLK1 (positive control), GAPDH (negative control) or tubulin. Microtubules were detected using the Ponceau stain in (C). Proteins were detected using Coomassie blue stain in (B and D). **C.** MBP-CCDC66⁵⁶⁴⁻⁹⁴⁸ binds to microtubules. 0.5 nmol MBP-CCDC66⁵⁶⁴⁻⁹⁴⁸ was incubated with increasing amounts of taxol-stabilized microtubules, loaded onto a 40% glycerol cushion and spun down. Supernatant and pellet fractions were resolved by SDS–PAGE and proteins were detected using Coomassie blue stain. **D.** CCDC66 binds to microtubules *in vitro*. Microtubule pelleting assay were performed using purified MBP-tagged CCDC66 fragments. Indicated proteins were incubated with taxol-stabilized microtubules or buffer, loaded onto a 40% glycerol cushion and spun down. Supernatant (S) and pellet (P) fractions were blotted for CCDC66 and microtubules were detected using the Ponceau stain.

2.4.5 Overexpression of CCDC66 Disrupts Organization of Centriolar Satellites and Inhibits Primary Cilium Formation

Overexpression of centriolar satellite proteins CEP290 and BBS4 disrupts centriolar satellite distribution by forming aggregates that sequester PCM1 (J. Kim et al., 2008; J. C. Kim et al., 2004). Unlike CEP290 and BBS4 and most other centriolar satellites proteins, we did not observe formation of cytoplasmic aggregates upon overexpression of CCDC66 (Figure 13A). In high-expressing cells, GFP-CCDC66 formed cytoplasmic microtubule bundles that sequestered endogenous PCM1, with a corresponding reduction in peri-centrosomally distributed centriolar satellites (Figure 16A). Because the punctate pericentrosomal distribution of centriolar satellites depends on an intact microtubule network, these results suggest that GFP-CCDC66 disrupts centriolar satellite organization through inducing microtubule bundle formation. In serum-starved RPE1 cells, overexpression of GFP-CCDC66 inhibited primary cilium formation, while control untransfected cells ($66 \pm 5.6\%$, $n = 150$) and GFP-expressing cells ($52 \pm 5.6\%$, $n = 150$) efficiently ciliated (Figure 16B).

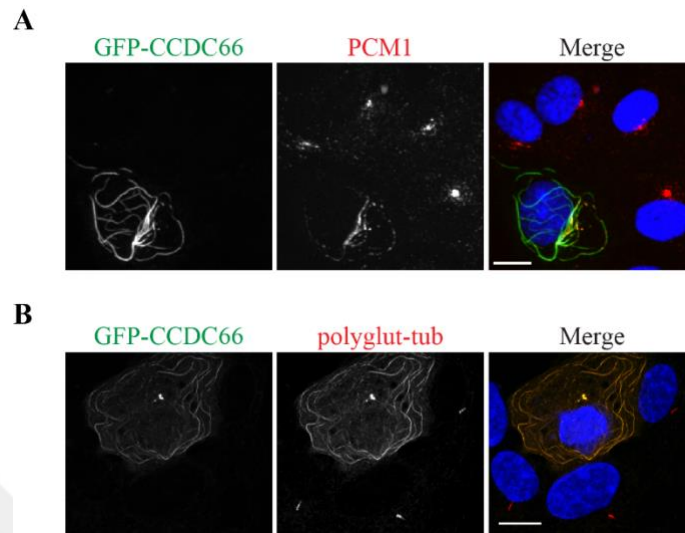


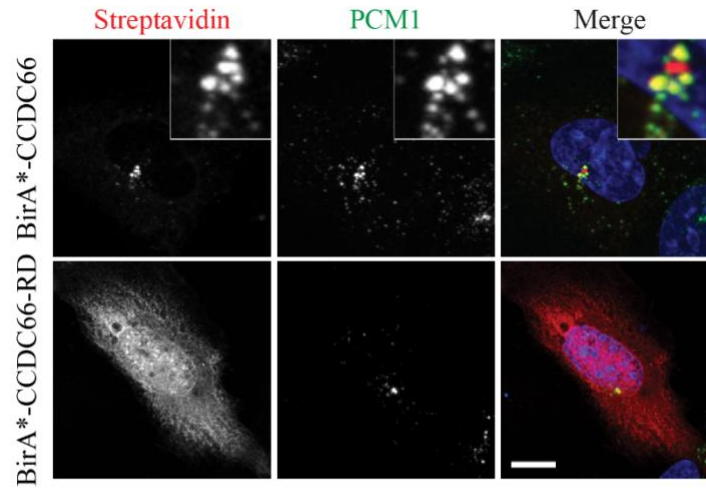
Figure 16. CCDC66 overexpression disrupts organization of centriolar satellites

A. RPE1 cells transiently transfected with GFP-CCDC66 were fixed and stained for GFP and PCM1. **B.** GFP-CCDC66 overexpression inhibits ciliogenesis. Ciliation was induced in GFP-CCDC66-transfected RPE1 cells by serum starvation for 24 h. Cells were fixed and stained for GFP and polyglutamylated tubulin. DNA was stained with DAPI. Scale bar, 10 μ m.

2.4.6 CCDC66 Interacts with The Centriolar Satellite Proteins CEP290 and PCM1

To gain insight into the cellular functions of CCDC66, we identified its proximity interactors at the centriolar satellites using the BioID approach (Firat-Karalar & Stearns, 2015). We generated an N-terminal myc-BirA* fusion of CCDC66 and showed that myc-BirA*-CCDC66 (hereafter BirA*-CCDC66) localized to centriolar satellites and stimulated localized biotinylation of the centriolar satellites, assessed by streptavidin staining (Figure 17). HEK293T cells were then transfected with BirA*-CCDC66 and processed for pulldown of biotinylated proteins as for BirA*-CEP72.

The proximity interactors for CCDC66 are listed by NSAF value in Figure 17B and Table 1. A significant percentage of proximity interactors are centrosome and/or centriolar satellite components and microtubule-associated proteins. Several of the proteins identified as proximity interactors for CCDC66 stand out by their known relationship to satellites and/or their involvement in primary cilium formation. CEP290 localizes to centriolar satellites and functions together during relocalization of BBS4 from centriolar satellites to primary cilia during ciliogenesis (T. R. Stowe et al., 2012). CEP162, a distal appendage protein, like CCDC66, binds to microtubules and functions in ciliogenesis (W. J. Wang et al., 2013). Other proximity interactors include PCM1, which is the scaffolding protein for satellites; AZI1, which functions during ciliogenesis (Hall et al., 2013), and CEP55, which functions during cytokinesis (Martinez-Garay et al., 2006; van der Horst et al., 2009). Although we identified CCDC66 as a proximity partner for CEP72, we failed to identify CEP72 as a proximity partner for CEP72, which could be due to differences in their relative abundance or the amount of time CCDC66 spends in its different cellular pools.

A**B**

BirA*-CCDC66		BirA*-CCDC66-RD	
Protein	NSAF	Protein	NSAF
CCDC66	20	CCDC66-RD	17
PCM1	1.31	TUBA1B	1.57
HSPA1A	1.10	TUBA1A	1.52
TUBA1B	0.88	HSPA1A	1.50
ALMS1	0.65	TUBA4A	1.14
AZI1	0.56	FASN	1.12
TUBA1A	0.52	HSPA8	1.04
HSPA1L	0.50	PRKDC	0.70
HSPA8	0.45	TUBB4B	0.58
CEP152	0.45	EIF4A1	0.54
DDX3X	0.42	TCP1	0.53
EIF5	0.39	TUBB6	0.41
OFD1	0.33	DYNC1H1	0.39
MAP4	0.29	CCT6A	0.34
CEP290	0.26	MCCC2	0.30
MPHOSPH9	0.23	RANPE	0.27
HDLBP	0.23	PCCB	0.26
TJP1	0.21	LDHB	0.24
CKAP5	0.20	HSBD1	0.24
CCDC138	0.20	CLTC	0.24
MRE11A	0.20	LARS	0.24
		MYBBP1A	0.23
		IARS	0.21
		SF3B1	0.21
		CCT3	0.20

Figure 17. Localization and proximity interactors of BirA*-tagged CCDC66 and CCDC66-RD

A. RPE1 cells were transfected with Myc-BirA*-tagged CCDC66 and CCDC66-RD. After 18 h biotin incubation, cells were fixed and stained for biotinylated proteins with fluorescent streptavidin and centriolar satellites with anti-PCM1 antibody. DNA was stained with DAPI. Scale bar, 10 μ m, inset shows 4x enlarged centrosomes. **B.** Mass spectrometry analysis of proximity interactors of Myc-BirA*-CCDC66 and Myc-BirA*-CCDC66-RD. Proximity interactors are ranked in the order of their NSAF values (average of three independent experiments). Proteins in black were previously shown to localize to the centrosome, proteins in bold were previously shown to interact physically with CCDC66 and proteins in grey were not associated with the centrosome/cilium complex in previous studies.

To determine which proximity interactions reflect physical association, we performed co-immunoprecipitation experiments in HEK293T cells. CCDC66 interacted with both CEP290 and PCM1 (Figure 18A). Consistent with its interaction with CEP290, myc-CCDC66 co-localized with GFP-CEP290 at centriolar satellites and microtubules (Figure 18B). Interestingly, like CEP162, the microtubule bundles induced by overexpression of CCDC66 sequestered endogenous CEP290 (Figure 18C), suggesting that CEP290 might associate with microtubules through CCDC66 (W. J. Wang et al., 2013). Although CCDC66 was initially identified as a proximity partner for CEP72, this proximity interaction did not reflect a physical interaction in co-immunoprecipitation experiments (Figure 18D).

An early point mutation in the CCDC66 gene was implicated in retinal degeneration (Dekomien et al., 2010), but its effect on CCDC66 function has not yet been characterized. To gain insight into the molecular defects caused by this mutation, we identified the proximity interactors of the CCDC66-RD mutant and compared them with those of CCDC66. Myc-BirA*-CCDC66-RD localizes to and stimulates biotinylation diffusely at the cytoplasm and nucleus (Figure 17A). Consistent with this localization, the proximity interactors of CCDC66-RD consist of cytoplasmic and nuclear proteins, which is in contrast to the profile of CCDC66 proximity interactors (Figure 17B, Table 1). Together, these results suggest that defects in satellite localization and function contribute to the retinal degeneration phenotype.

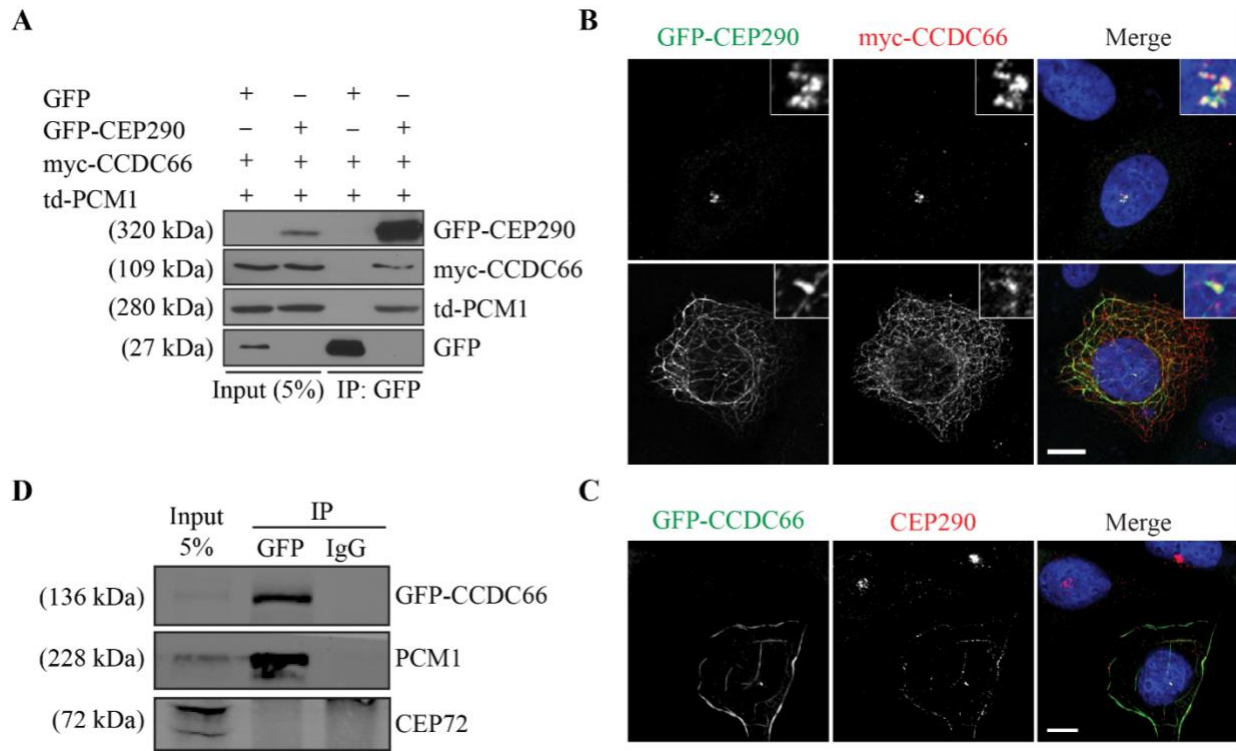


Figure 18. CCDC66 interactions with CEP290 and CEP72

A. Co-immunoprecipitation of GFP-CEP290, tomato-PCM1 and Myc-CCDC66 after co-expression in HEK293T cells. Complexes were immunoprecipitated with anti-GFP antibody and co-precipitated proteins were detected with anti-GFP, anti-Myc and anti-PCM1 antibodies. **B.** CCDC66 and CEP290 co-localize at the centriolar satellites and microtubules. RPE1 cells expressing GFP-CEP290 and Myc-CCDC66 were stained with anti-Myc, and anti-GFP antibodies. DNA was stained with DAPI. Scale bar, 10 μ m, all insets show 4x enlarged centrosomes. **C.** GFP-CCDC66 recruits CEP290 to microtubules. RPE1 cells transiently expressing GFP-CCDC66 were stained with anti-GFP, and anti-CEP290 antibodies. DNA was stained with DAPI. Scale bar, 10 μ m. **D.** CCDC66 does not interact with CEP72. Co-immunoprecipitation of GFPCCDC66 from RPE1-[GFPCCDC66] cells. Complexes were immunoprecipitated with anti-GFP antibody and co-precipitated proteins were detected with anti-GFP, anti-PCM1 and anti-CEP72 antibodies.

2.4.7 CCDC66 Is Required For Efficient Ciliogenesis and Centriolar Satellite Distribution

The cellular functions of CCDC66 are not known. Physical and proximity interactions of CCDC66 to proteins that function in ciliogenesis (i.e CEP72, CEP290, PCM1) suggest a similar function of CCDC66. By depleting CCDC66 in RPE1 cells, we tested whether CCDC66 is required for efficient ciliogenesis. Transfection with small interfering RNA (siRNA) against CCDC66 resulted in effective depletion after 72 h, as verified by Western blot of siRNA-transfected cells (Figure 19A). Depletion of CCDC66 significantly ($p < 0.001$) reduced cilium formation in serum-starved RPE1 cells (Figure 19B), and this phenotype was rescued by expression of RNAi-resistant GFPCCDC66RR, which was verified Western blot of siRNA-transfected RPE1-[GFP] and RPE1-[GFPCCDC66RR] cells (Figure 19C and 19D). In cells where ciliogenesis phenotype was rescued, GFPCCDC66RR prominently localized to the cilium and to satellites in a subset of cells suggesting that ciliary and satellite localization of CCDC66 is important for its function during ciliogenesis (Figure 19E). The ciliogenesis phenotype was not due to a defect in cell cycle progression, since both control and CCDC66-depleted cells had similar cell cycle profiles as analyzed by flow cytometry (Figure 19F).

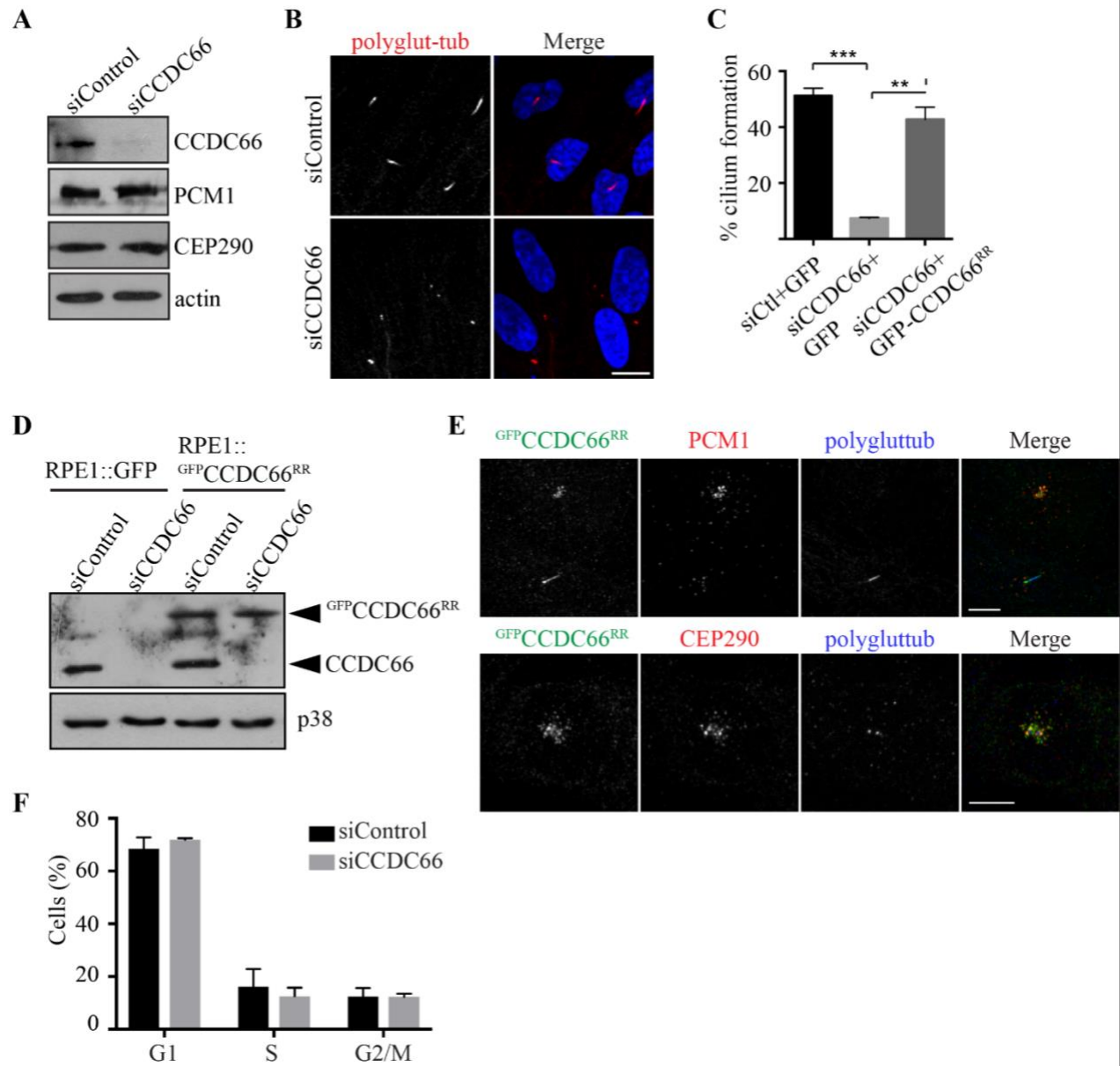


Figure 19. CCDC66 regulates cilium formation

A. CCDC66 siRNA effectively depletes CCDC66. RPE1 cells were transfected with control siRNA or CCDC66 siRNA, and 72 h after transfection extracts from cells were immunoblotted for CCDC66, CEP290, PCM1 and actin (loading control). **B.** Effect of CCDC66 depletion on cilium formation. RPE1 cells were fixed 72 h after transfection with control siRNA or CCDC66 siRNA, and percentage of ciliated cells was determined by staining for glutamylated-tubulin and DAPI. **C.** Quantification of rescue experiments. For rescue experiments, RPE-1 cells stably expressing GFP

(RPE1-[GFP]) or GFP-CCDC66RR (RPE1-[GFPCCDC66RR]) were transfected with control and CCDC66 siRNAs. 72 h post-transfection cells were fixed, stained and quantified. $n = 300$ cells for each sample, from three independent experiments. ANOVA test was used for statistical analysis.

D. Localization of GFPCCDC66RR in CCDC66-depleted cells. For rescue experiments, RPE-1 cells stably expressing GFPCCDC66RR were transfected with CCDC66 siRNA. 72 h post-transfection cells were fixed and stained for GFP, polyglutamylated tubulin and PCM1 or CEP290. DNA was stained with DAPI. **E.** Extracts from RPE1-[GFP] and RPE1-[GFPCCDC66RR] cells treated with control siRNA or CCDC66 siRNA for 72 h, were resolved by SDS-PAGE and immunoblotted with anti-CCDC66 antibodies and anti-p38 antibodies. **F.** Cell cycle progression is not disrupted in CCDC66-depleted cells. Flow cytometry analysis of control or CCDC66-depleted cells. Control and depleted RPE-1 cells were harvested and fixed in 70% ethanol, washed with PBS and stained with 40 $\mu\text{g/ml}$ propidium iodide and 10 $\mu\text{g/ml}$ RNaseA. DNA content was assessed by analyzing cells using a Becton Dickinson Accuri C6 Flow Cytometer running BD Sampler software. Data represent mean value from two experiments per condition, $\pm$ SEM.

Because centriolar satellite organization and function is essential for ciliogenesis (L. Wang et al., 2016), we assessed the effects of CCDC66 depletion on these processes. Depletion of CCDC66 resulted in a significant ($p < 0.001$) decrease in the amount of pericentrosomal PCM1 and a corresponding increase in cytoplasmic centriolar satellites, as determined by quantitative immunofluorescence (Figure 20A and 20B). While CCDC66 depletion resulted in a significant ($p < 0.01$) reduction in the amount of centriolar satellite-associated CEP290 (Figure 20C and 20D), CEP290 depletion resulted in a significant ($p < 0.01$) increase in the amount of pericentrosomal GFP-CCDC66 (Figure 20E and 20F) and PCM1 (Figure 20G and 20H). Both PCM1 and CEP290 redistribution phenotypes were rescued by expression of RNAi-resistant GFP-CCDC66^{RR} (Figure 20A and 20C). The redistribution of centriolar satellites and altered localization of PCM1, CEP290 or CCDC66 was not due to reduction in the protein levels of these proteins (Figure 19A and Figure 20I).

Given the interaction between CCDC66 and CEP290, we next determined the effect of CCDC66 and CEP290 co-depletion on centriolar satellite organization. Interestingly, co-depletion of CCDC66 and CEP290 rescued the satellite redistribution phenotypes caused by individual CCDC66 and CEP290 depletions (Figure 20G and 20H), suggesting that CEP290 and CCDC66 have opposing roles in satellite distribution in RPE1 cells.

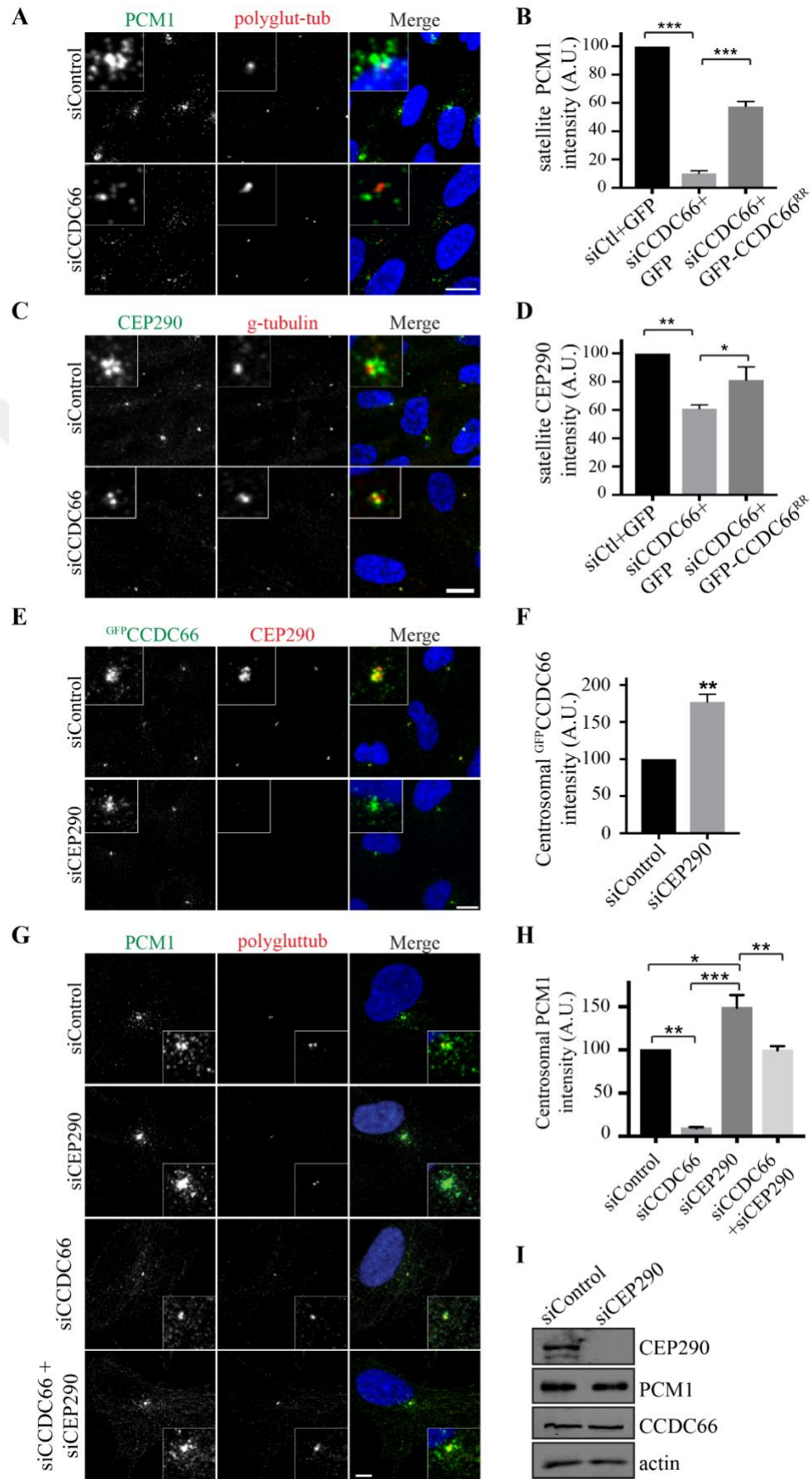


Figure 20. CCDC66 regulates centriolar satellite distribution

A. Effect of CCDC66 depletion on centriolar satellite organization. RPE1 cells were fixed 72 h after transfection with control siRNA or CCDC66 siRNA, and satellite organization was determined by staining with PCM1, polyglutamylated tubulin and DAPI. Images represent cells from the same coverslip taken with the same camera settings. **B.** Quantification of rescue experiments. $n = 50$ cells for each sample, from two independent experiments. ANOVA test was used for statistical analysis. **C.** Effect of CCDC66 depletion on the pericentrosomal level of CEP290. RPE1 cells were fixed 72 h after transfection with control siRNA or CCDC66 siRNA, and stained for endogenous CEP290, γ -tubulin and DAPI. **D.** Quantification of rescue experiments. $n = 50$ cells for each sample, from two independent experiments. ANOVA test was used for statistical analysis. **E.** Effect of CEP290 depletion on the pericentrosomal level of GFPCCDC66. RPE1 cells were fixed 72 h after transfection with control siRNA or CEP290 siRNA, and satellite organization was determined by staining with GFP, CEP290 and DAPI. **F.** Quantification of E. Pericentrosomal GFPCCDC66 fluorescence intensity was measured for control and CEP290-depleted cells and levels are normalized to 100. $n = 50$ cells for each sample, from 3 independent experiments. t-test was used for statistical analysis. Data in B, D, and F represent mean value from two or three experiments per condition, $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Scale bars = 10 μ m, all insets show 4x enlarged centrosomes. **G.** RPE1 cells were transfected with control or CCDC66 siRNA or CEP290 siRNA or CCDC66 and CEP290 siRNAs together and fixed after 72 h. Cells were stained for PCM1 and glutamylated tubulin. DNA was stained with DAPI. Scale bars = 10 μ m, all insets show 4x enlarged centrosomes. **H.** Quantification of G. Pericentrosomal PCM1 fluorescence intensities was measured for control and depleted cells and levels are normalized to 100. $n = 50$ cells for each sample, from two independent experiments. ANOVA test was used for statistical analysis. Data in represent mean value from two experiments per condition, $\pm$ SEM. Following key is followed for asterisk placeholders for p-values in the figures: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. **I.** CEP290 siRNA effectively depletes CEP290. RPE1 cells were transfected with control siRNA or CEP290 siRNA, and 72 h after transfection extracts from cells were immunoblotted for GFPCCDC66, CEP290, PCM1 and actin.

Given that CCDC66 binds to microtubules, we assessed whether it is required for the maintenance of a radial microtubule array in control and CCDC66-depleted cells by staining for microtubules (Figure 21A). Control cells ($72 \pm 2.9\%$, $n = 150$) had significantly ($p < 0.05$) higher percentage of centrosomally-organized radial arrays than CCDC66-depleted cells ($45 \pm 5.2\%$, $n = 150$) (Figure 21B). This result indicates that CCDC66 plays a role in maintaining a stable microtubule array and that satellite redistribution phenotypes could be due to these organization changes.



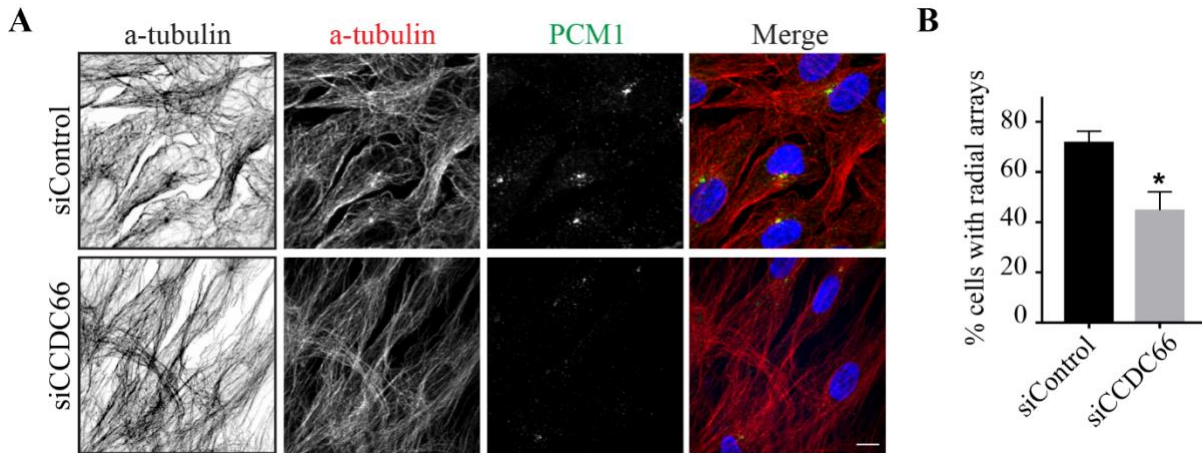


Figure 21. CCDC66 regulates organization of interphase microtubules.

A. RPE1 cells were transfected with control or CCDC66 siRNAs and fixed after 72 h. Cells were stained for alpha-tubulin and PCM1. DNA was stained with DAPI. CCDC66 depletion caused redistribution of PCM1 throughout the cytosol and reduced the percentage of cells with radial arrays emanating from the centrosome. Scale bars, 10 μ m. **B.** Quantification of B. t-test was used for statistical analysis.

2.4.8 CCDC66 is Required for Relocalization of BBS4 From Centriolar Satellites to Primary Cilia During Ciliogenesis

During ciliogenesis, the BBSome-associated protein BBS4 relocates from centriolar satellites to primary cilia (J. Kim et al., 2008; J. C. Kim et al., 2004; M. V. Nachury et al., 2007) and CCDC66 also has a similar localization pattern. PCM1, CEP290 and CEP72 were suggested to cooperate with BBS4 to promote ciliogenesis (J. Kim et al., 2008; Lopes et al., 2011; T. R. Stowe et al., 2012). Given the relationship of CCDC66 to these proteins, we assessed the effect of its depletion on the localization of BBS4 using the RPE1 cell line that stably expresses LAP-BBS4 (M. V. Nachury, 2008). Since ciliogenesis was decreased in CCDC66-depleted cells, we assayed BBS4 relocalization in the cells that formed cilia. A significant reduction ($p < 0.01$) in the fraction of cilia containing LAP-BBS4 relative to control ciliated cells ($73 \pm 3.7\%$ of total) was observed for cells depleted of CCDC66 ($37 \pm 2.5\%$ of total) (Figure 22). In these cells, LAP-BBS4 remained associated with centriolar satellites rather than localizing to the cilium (Figure 22A). Thus, disruption of centriolar satellite function by depletion of CCDC66 results in a failure of BBS4 recruitment to the cilium. Interestingly, we have not detected any interaction between CCDC66 and BBS4, neither in the BioID-pulldown of CCDC66 (Table 1) nor in the co-immunoprecipitation experiments (data not shown), suggesting that CCDC66 indirectly functions in relocalization of BBS4 to the cilium.

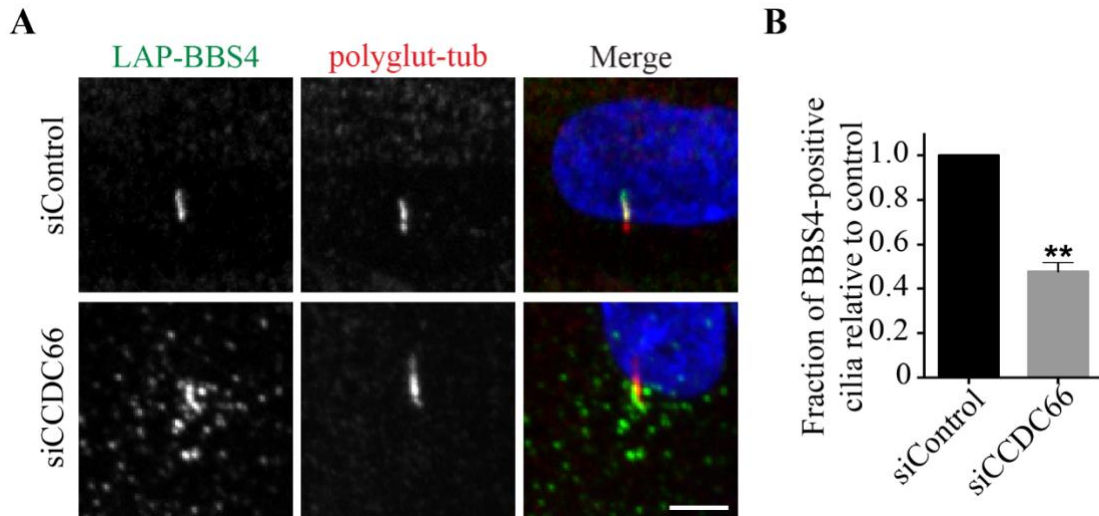


Figure 22. CCDC66 regulates LAP-BBS4 recruitment to the cilium

A. Effect of CCDC66 depletion on BBS4 relocalization from centriolar satellites to the primary cilium. RPE1 cells were fixed 72 h after transfection with control siRNA or CCDC66 siRNA and stained for LAP-BBS4 with anti-GFP antibody, polyglutamylated tubulin and DAPI. **B.** Quantification of A. Control siRNA-transfected levels were normalized to 1.0; n = 50 cells for each sample, from 3 independent experiments. t-test was used for statistical analysis. Data in B represent mean value from three experiments per condition, +/- SEM. **p<0.01. Scale bars = 10 μ m, all insets show 4x enlarged centrosomes.

2.5 Discussion

Centriolar satellites play important roles in a multitude of centrosome/cilium-mediated cellular processes and are implicated in ciliopathies (M. A. Tollenaere et al., 2015). Interestingly, a significant number of centrosome/cilium complex proteins, including the ciliopathy-associated proteins CEP290, BBS4 and OFD1, localize to centriolar satellites. While these findings identify satellites as ubiquitous and essential structures for regulating the vertebrate centrosome/cilium complex, the molecular and functional relationship of the satellites to the centrosome/cilium-complex and ciliopathies remain poorly understood. In this study, we characterized the retinal degeneration gene product CCDC66 in ciliated retinal epithelial cells.

CCDC66 point mutation in dogs and null mutations in mouse were previously implicated in retinal degeneration, a phenotype that is common to ciliopathies (Dekomien et al., 2010; Gerding et al., 2011). We first identified *CCDC66* as a proximity partner for known centriolar satellite proteins including CEP72, which interacts with CEP290 and functions in cilium formation and ciliary targeting of the BBSome complex (T. R. Stowe et al., 2012). Supporting this proximity relationship, *CCDC66* co-localizes with PCM1 in cells, the canonical component of centriolar satellites and a significant percentage of the proximity partners for *CCDC66* are components of the centriolar satellites and the centrosome/cilium complex. Using co-immunoprecipitation experiments, we showed that *CCDC66* interacts with CEP290 and PCM1, but not with CEP72, suggesting that proximity relationship of *CCDC66* to CEP72 does not reflect physical interaction. Like *CCDC66*, CEP290 was previously implicated in retinal degeneration in human ciliopathies and mouse models (Chang et al., 2006).

Remarkably, *CCDC66* relocates from centriolar satellites to the primary cilium in ciliated cells and thus acts like BBS4, the BBSome component that localizes to satellites and added last during BBSome assembly (M. V. Nachury et al., 2007). This finding identifies *CCDC66* as the only reported PCM1-associated protein other than BBS4 with such localization pattern, suggesting that redistribution of proteins between the centriolar satellites and primary cilium as a common theme in ciliary trafficking. Interestingly, we did not identify *CCDC66* as a component of the BBSome complex in BioID experiments. By analogy to BBS4 functions in cells (J. C. Kim et al., 2004; M. V. Nachury et al., 2007), it is possible that *CCDC66* is part of a new ciliary complex that

functions in ciliary trafficking. Future studies are required to address the functional significance of CCDC66 localization in ciliated cells.

We identified CCDC66 as a microtubule-associated protein using *in vitro* and *in vivo* assays and this is in agreement with a previous microtubule transcriptome screen in *Xenopus tropicalis* eggs (Sharp et al., 2011). Overexpression of CCDC66 in cells induced formation of microtubule bundles, disrupted centriolar satellite organization and inhibited ciliogenesis. Interestingly, endogenous CEP290 was recruited to these microtubule bundles, suggesting that CCDC66 might be required for association of CEP290 with microtubules. These results support the function of CCDC66 in localization and/or trafficking of proteins like CEP290 to and along microtubules.

A significant number of centriolar satellite protein identified to date have been implicated in cilium formation and function (M. A. Tollenaere et al., 2015). In cells depleted for CCDC66, there was a significant defect in ciliogenesis and PCM1- and CEP290-positive puncta did not cluster around the centrosome, instead were distributed throughout the cytosol. Defects in ciliogenesis could be a result of the defects in satellite function due to their redistribution in cells. Given that satellite organization and function depends on an intact microtubule network, it is possible that CCDC66 mediates the interaction between satellites and the cytoplasmic microtubule array, analogous to CEP290 (J. Kim et al., 2008), BBS4 (J. C. Kim et al., 2004) and PAR6a (Kodani et al., 2010). Together, our results support the role of centriolar satellites in cilium formation.

Centriolar satellites were shown to be important for efficient ciliogenesis by controlling the entry of proteins to the cilium either by sequestering them or by trafficking them to or away from the cilium. For example, centriolar satellites sequester BBS4 and regulate recruitment of BBSome complex to the cilium by limiting the amount of BBS4 available for incorporation into ciliary BBSomes (T. R. Stowe et al., 2012). Consistent with this model, we demonstrated that ciliary recruitment of BBS4 is compromised when CCDC66 is depleted. Our results support the role of centriolar satellites in the assembly of BBS4 into the BBSome complex and its subsequent ciliary recruitment. It is likely that CCDC66 regulates ciliary entry of the BBSome complex by interacting with microtubules and forming a complex with CEP290 at the satellites.

To determine how mutations in the *CCDC66* gene affect its function, we determined the localization and the proximity partners of the truncated form of CCDC66 (CCDC66-RD), which mimics the mutated protein found in dogs affected with retinal degeneration (Dekomien et al.,

2010). CCDC66-RD localizes diffusely to the nucleus and cytosol and its proximity partners are almost all nuclear and cytoplasmic proteins. Moreover, CCDC66-RD does not interact with microtubules. These results suggest that defects in satellite localization and microtubule-binding contribute to the retinal degeneration phenotype.

In photoreceptor cells, outer segment represents highly modified cilium that contains visual transduction cascade components. The outer segment is connected to the inner segment by the connecting cilium, which resembles the ciliary transition zone. Phototransduction proteins are synthesized in the inner segment and transported to the outer segment through the connecting cilium (G. Wheway et al., 2014). Almost one-quarter of known retinal degeneration genes are associated with ciliary structure or function, including the BBSome complex (Wright et al., 2010) and the transition zone component CEP290 (Chang et al., 2006). Given the possible role of CCDC66 in ciliary trafficking, perturbation of this function can lead to photoreceptor degeneration through mislocalization of proteins required for phototransduction. Previous studies reported localization of CCDC66 to the inner segments of photoreceptor cells (Dekomien et al., 2010) and to the outer segment of rod cells (Gerding et al., 2011). Higher resolution localization studies in photoreceptor cells are required to determine whether the localization of to the centrosome/cilium complex and/or microtubules in RPE1 cells reflects localization in photoreceptor cells.

While *CCDC66* point and null mutations cause retinal degeneration in dogs and mouse, mutations have not yet been mapped to the *CCDC66* gene in human ciliopathies. There have been discrepancies between human ciliopathies and their respective mouse models, which are likely due to differences in species and/or functional nature of the mutation (Hall et al., 2013; S. T. Jiang et al., 2008; Mykytyn et al., 2004; Won et al., 2011). For most ciliopathies, mouse models fail to completely recapitulate the human phenotype. Therefore; the loss-of-function phenotype of *CCDC66 in vivo* in humans is not known.

Chapter 3: CENTROSOMAL AND CILIARY TARGETING OF CCDC66 REQUIRES COOPERATIVE ACTION OF CENTRIOLAR SATELLITES, MICROTUBULES, AND MOLECULAR MOTORS

3.1 Introduction

Mammalian centrosomes and cilia play key roles in many cellular processes and their deregulation is linked to cancer and ciliopathies. Spatiotemporal regulation of their biogenesis and function in response to physiological stimuli requires timely protein targeting. This can occur by different pathways, including microtubule-dependent active transport and via centriolar satellites, which are key regulators of cilia assembly and signaling. How satellites mediate their functions and their relationship with other targeting pathways is currently unclear. To address this, we studied retinal degeneration gene product CCDC66, which localizes to centrosomes, cilia, satellites and microtubules and functions in ciliogenesis. FRAP experiments showed that its centrosomal pool was dynamic and the ciliary pool associates with the ciliary axoneme and was stable. Centrosomal CCDC66 abundance and dynamics required microtubule-dependent active transport and tethering, and was inhibited by sequestration at satellites. Systematic quantitation of satellite dynamics identified only a small fraction to display microtubule-based bimodal motility, consistent with trafficking function. Majority displayed diffusive motility with unimodal persistence, supporting sequestration function. Together, our findings reveal new mechanisms of communication between membrane-less compartments.

3.2 Literature Review

The mammalian centrosome/cilium complex is composed of the centrosome, the cilium and the centriolar satellites, which together function in a diverse set of cellular processes ranging from cell division to cellular signaling. Centrosomes and cilia are both microtubule-based structures that are organized into structurally distinct domains. Their biogenesis, maintenance and function are tightly regulated in response to physiological stimuli including cell cycle cues (Heydeck et al., 2018; E. A. Nigg & Holland, 2018). In G₀, most animal cells have one centrosome that is composed of a pair of centrioles. During the G₁/S-phase transition, centrioles duplicate to form procentrioles, which elongate throughout S and G₂ phases. The mitotic spindle then segregates the duplicated centrioles to ensure that each daughter cell receives a pair of centrioles. In quiescent cells, the older of the centrioles, the mother centriole, docks to the plasma membrane to form the primary cilium, which is a nexus for developmentally important signaling pathways including Hedgehog signaling. Defects in the structure and function of the centrosome/cilium complex are linked to various human diseases including ciliopathies and cancer (Braun & Hildebrandt, 2017; E. A. Nigg & Holland, 2018; J. F. Reiter & Leroux, 2017).

Bioinformatic, genomic, transcriptomic and proteomic studies have identified over 100 proteins that localize to the centrosomes and cilia (Andersen et al., 2003; T. Avidor-Reiss et al., 2004; Firat-Karalar, Sante, et al., 2014; Hamel et al., 2017; H. Ishikawa et al., 2012; Li et al., 2004; McClintock et al., 2008; D. U. Mick et al., 2015; M. A. Sigg et al., 2017). Dynamic regulation of the biogenesis, maintenance and function of the centrosome/cilium complex in response to physiological stimuli requires timely and efficient targeting of proteins (T. Avidor-Reiss & Gopalakrishnan, 2013; G. D. Gupta et al., 2015; Werner et al., 2017). What makes such spatiotemporal regulation complex is the membrane-less nature of the centrosome and the semi-closed nature of the primary cilium. Although they are not membrane-bound compartments, both centrosomes and cilia are organized into structurally distinct domains (O. E. Blacque & Sanders, 2014; Fry et al., 2017; Winey & O'Toole, 2014). Moreover, some proteins, like gamma-tubulin, are less abundant at the centrosome/cilium than in the cytoplasm (Moudjou et al., 1996). A major unresolved question that pertains to our understanding of centrosomal and ciliary regulation relates to dissecting the pathways that target proteins to centrosomes and cilia at the right time and place, as well as that limit their recruitment from cytoplasmic pools.

The abundance and dynamic localization of proteins at the centrosomes and cilia can be regulated by delivery, retention, and/or removal. For cilia, key regulators of protein content are the vesicular and intraciliary trafficking pathways as well as gating mechanisms such as the transition zone and the septin-based diffusion barrier (M. V. Nachury, 2008; M. V. Nachury & Mick, 2019). For centrosomal protein targeting, among the pathways reported are those dependent on the microtubule cytoskeleton, molecular motors, centriolar satellites, diffusion and mRNA localization (M. Bornens, 2002; C. T. Chen et al., 2014; Fogeron et al., 2013; Gillingham & Munro, 2000; Hehnly & Doxsey, 2014; Hori & Toda, 2017; Sepulveda et al., 2018; Zimmerman & Doxsey, 2000). Passive diffusion by itself is sufficient for targeting all or a fraction of some proteins to the centrosome. For example, the dynamic exchange of centrosomal gamma-tubulin with the cytoplasm does not require microtubules (Khodjakov & Rieder, 1999). However, passive diffusion is limited for the directional and long-range targeting of proteins, which is essential for the timely and rapid changes in composition that occurs during the cell cycle and development. This limitation is overcome in part by microtubule and molecular motor-dependent active transport, which was reported for centrosomal targeting of CDK5RAP2 (Jia et al., 2013; S. Lee & Rhee, 2010), Par6alpha (Kodani et al., 2010), pericentrin, and NUMA (Gaglio et al., 1996; Young et al., 2000; Zimmerman & Doxsey, 2000). The underlying mechanisms of this microtubule-based active transport, as well as its relationship with other transport pathways, in particular how they cooperate or compete with each other in specific cellular contexts and in response to different signals, is not known.

Centriolar satellites are an array of granules that cluster around the centrosome/cilium-complex in a microtubule- and dynein-dependent manner (Hori & Toda, 2017; Kubo et al., 1999). They are scaffolded by Pericentriolar Material-1 (PCM1), which is essential for their integrity and mediates their interaction with a wide range of centrosome and cilium proteins (G. D. Gupta et al., 2015; L. Wang et al., 2016). Satellites are ubiquitous in vertebrate cells and have emerged as key regulators of the biogenesis and function of the centrosome/cilium complex. Although they are widely accepted to mediate their functions through regulating protein targeting, the underlying mechanisms of this regulation remains unresolved. The motility of satellites towards and away from the centrosome along microtubules, and the decrease in the centrosomal levels of several proteins such as pericentrin, centrin and ninein in PCM1-depleted satellite-less cells have suggested a possible active transport function for satellites (Dammermann & Merdes, 2002; Hori et al., 2016; Kubo et al., 1999). According to this model, satellites move along microtubules in a molecular

motor-dependent manner to deliver and remove proteins at the centrosome. However, the absence of quantitative analysis of satellite dynamics and direct evidence showing their role in protein targeting makes this model incomplete. Moreover, recent studies showed that satellites regulate cilium formation by limiting the incorporation of the ubiquitin ligase Mib1 and BBS4 at the centrosome and cellular stress response by sequestration of Cep131 and other satellite proteins (M. A. X. Tollenaere et al., 2015). This indicates a possible sequestration function (T. R. Stowe et al., 2012; L. Wang et al., 2016). To elucidate the molecular mechanism of how satellites regulate protein targeting and their relationship to microtubules and molecular motors, we focused on studying the dynamic compartmentalization of CCDC66 within the centrosome/cilium complex.

CCDC66 is a component of the centrosome/cilium complex and its mutations in dogs and mouse are linked to retinal degeneration (D. Conkar et al., 2017; Dekomien et al., 2010; Gerding et al., 2011). In interphase cells, it localizes to the centrosome and centriolar satellites. As cells enter mitosis, it accumulates at spindle poles and spindle microtubules with concomitant dissolution of its satellite pool that reassembles at the end of mitosis. Upon serum starvation, CCDC66 also localizes to the primary cilium, analogous to BBS4 of the BBSome, which regulates ciliary protein trafficking (D. Conkar et al., 2017). The multitude of cellular pools of CCDC66 is paralleled by its various functions in cilium assembly, ciliary recruitment of the BBSome complex, and spindle pole and centriolar satellite organization (D. Conkar et al., 2017; Sharp et al., 2011). Importantly, the retinal degeneration mutation disrupts the centrosomal and ciliary localization of CCDC66 and its interactions (D. Conkar et al., 2017).

How CCDC66 dynamically compartmentalizes between its different cellular pools is not known. Understanding the mechanism underlying this compartmentalization is required to gain insight into regulation of its various functions and the cellular defects that lead to CCDC66-linked retinal degeneration. To address these questions, we developed imaging assays to quantify the dynamic behavior of CCDC66 at the centrosomes, satellites and cilia and showed that CCDC66 has different dynamics at these compartments. We then elucidated the interplay between satellites, microtubules and molecular motors in CCDC66 centrosomal and ciliary targeting by employing these assays in cells selectively disrupted for satellite integrity and distribution, or microtubule-dependent active transport, organization and stability. While satellites inhibited the centrosomal and ciliary recruitment of CCDC66, microtubules and motors facilitated its dynamic localization

at the centrosome. In addition, systematic quantification of the dynamic behavior of satellites showed that the majority displayed random diffusive motility, while the rest were persistently motile towards or away from the centrosome. This suggests that only a small percentage of satellites might mediate trafficking at a given time. Together, our results identify “sequestration” as another layer of regulation of protein targeting by satellites, in addition to trafficking-based mechanisms, and provide insight into cellular compartmentalization through membrane-less structures.



3.3 Materials and Methods

3.3.1 Immunofluorescence, antibodies and microscopy

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 MgCl₂, 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 ⁷⁶. After rehydration in PBS, 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 rabbit anti Arl13b (17711-1-AP, Proteintech) at 1:100, 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 (3E6) 1:750, mouse anti alpha tubulin (Sigma, DM1A) at 1:1000 and mouse anti-PCM1 1:1000, rabbit anti CEP152 (Bethyl, A302-480A) at 1:500, mouse anti p150^{glued} (BD Biosciences, 610473) at 1:1000. Rabbit anti-PCM1 and anti-GFP antibodies were generated and used for immunofluorescence as previously described ⁶⁵. Secondary antibodies used for immunofluorescence experiments were AlexaFluor 488-, 568- or 633-coupled (Life Technologies) and they were used at 1:2000.

Time lapse live imaging was performed with Leica SP8 confocal microscope equipped with an incubation chamber. Asynchronous cells were imaged at 37°C with 5% CO₂ with a frequency of 0.375 seconds at a specific position for 100 frames in 512x512 pixel format using HC PL APO CS2 40x 1.3 NA oil objective. For ciliogenesis videos, cells were incubated with 0.5% FBS in DMEM-12 just before imaging and imaged overnight with a frequency of 10 minutes per frame with 0.3 µm step size and 3.5 µm stack size in 1024x1024 pixel format. For centrosomal protein level quantifications, images were acquired with Leica DMi8 fluorescent microscope with a stack size of 8 µm and step size of 0.3 µm in 1024x1024 format 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. For STED analysis of ciliary GFP-CCDC66, images were acquired with Leica TCS SP8 STED 3X super resolution microscope, using HC PL APO 100x1.40

NA oil STED WHITE objective. All images were taken from single sections and deconvolved in Huygens software.

All centrosomal and ciliary level quantifications were done in Image J ⁷⁷. 2.5 μm^2 or 17 μm^2 region of interest (ROI) around the centrosome was determined using a centrosomal marker and the corresponding centrosomal signal was quantified. Cytoplasmic signal was subtracted from this value for every single cell. Statistical analysis was done by normalizing these values to their mean. Statistical significance was determined by Student's t-test using Prism (GraphPad, La Jolla, CA). Ciliary length was measured using acetylated tubulin as the ciliary length marker. Ciliary protein concentration was determined by dividing ciliary protein signal by ciliary length. All values were normalized relative to the mean of the overall quantification (=1).

3.3.2 Fluorescence Recovery After Photobleaching

FRAP experiments were performed with Leica SP8 confocal microscope using FRAP module. Cells were incubated with 10% FBS in DMEM-12 and kept at 37°C with 5% CO₂. ROI was set to 2.5 μm^2 for centrosomal FRAP experiments. Since primary cilium length varied from cell to cell, a specific ROI was defined for each primary cilium. A z-stack of 4 μm with 0.5 μm step size was taken during pre and post bleaching for both centrosome and cilium FRAP experiments. Bleaching was done 2 iterations with 488 Argon laser with 100% power. Maximal projection of the files was performed in Leica LAS X software and analysis was done in ImageJ. Recovery graph quantifications, t-half and mobile pool quantifications were done using the equations as described ⁷⁸.

3.3.3 Cell lysis and Immunoblotting

Cells were lysed in 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100 and protease inhibitors for 30 min at 4°C followed by centrifugation at 15,000 g for 15 min. The protein concentration of the resulting supernatants was determined with the Bradford solution (Bio-Rad Laboratories, CA, USA). For immunoblotting, equal quantities of cell extracts were resolved on SDS-PAGE gels, transferred onto nitrocellulose membranes, blocked with TBST in 5% milk for 1 hour at room temperature. Blots were incubated with primary antibodies diluted in 3% BSA in TBST overnight at 4°C, washed with TBST three times for 10 minutes and blotted with secondary antibodies for 1 hour at room temperature. After washing blots with TBST three times for 10 minutes, they were visualized with the LI-COR Odyssey® Infrared Imaging System and software

at 169 μm (LI-COR Biosciences). Primary antibodies used for immunoblotting were mouse anti-p150^{glued} (BD Biosciences, 610473) at 1:1000, rabbit anti BBS4 (Proteintech, 12766-1-AP) at 1:500, rabbit anti CCDC66 (Bethyl, A303-339A) at 1:2500, rabbit anti PCM1 (Proteintech, 19856-1-AP) at 1:500, rabbit anti CEP290 (Proteintech, 16268-1-AP) at 1:500, rabbit anti CEP72 (Proteintech, 22490-1-AP) at 1:500, mouse anti beta-actin (Proteintech, 60008-1-Ig) at 1:10000, mouse anti alpha-tubulin (Sigma, DM1A) at 1:5000. anti-PCM1 and anti-GFP antibodies were generated and used for immunoblotting as previously described⁶⁵. Secondary antibodies used for western blotting experiments were IRDye680- and IRDye 800-coupled and were used at 1:15000 (LI-COR Biosciences).

3.3.4 Immunoprecipitation

HEK293T cells transfected with GFP or GFP-CCDC66 were resuspended in IP buffer (50 mM Tris-HCl pH 7.4, 260 mM NaCl, 2.27 mM KCl, 1.25 mM KH_2PO_4 , 6.8 mM Na_2HPO_4 , 1% NP-40) freshly supplemented with protease inhibitors (10 $\mu\text{g}/\text{ml}$ Leupeptin, Pepstatin and Chymostatin, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ Aprotinin), tumbled at 4°C for 45 min, and centrifuged at 13,000 R.P.M. for 15 minutes. Supernatants were added with 2 μg of control goat IgG or goat anti GFP antibody and tumbled at 4°C for 2 hours. Meanwhile, SureBeads™ Protein G Magnetic agarose beads (Bio-Rad) were washed with IP buffer three times and 30 μl (50 % slurry) beads were added to supernatant and antibody mixes. Mixes were tumbled overnight. Next day, mixes were centrifuged at 1000 R.P.M for 1 minute and supernatants were saved as unbound fractions. Beads were washed with IP buffer two times and saved as bound fraction.

3.3.5 In vitro Microtubule Pelleting Assay

HEK293T cells transfected with GFP-CCDC66 were lysed in in BRB80 buffer (80 mM PIPES pH 6.8, 1 mM EGTA, 1m M MgCl_2) supplemented with protease inhibitors (10 $\mu\text{g}/\text{ml}$ LPC, 1 mM PMSF and 10 $\mu\text{g}/\text{ml}$ aprotinin) at 4°C for 30 minutes and centrifuged at 13,000 R.P.M. for 15 minutes. Lysate was further cleared by centrifugation at 90,000 R.P.M. for 5 minutes at 4°C with TLA100 rotor (Beckman). 2.5 μg cell lysate was brought to a final volume of 250 μl BRB80 supplemented with 1 mM GTP. Lysate was incubated with 25 μM taxol stepwise at 30°C and with or without 0.5 mM AMP-PNP at 30°C for another 30 minutes. Lysate was centrifuged at 55,000 R.P.M. for 10 minutes at 30°C in a TLA100 rotor (Beckman) through a 125 μl 40% glycerol cushion prepared from BRB90 buffer supplemented with 1 mM GTP. Pellets were resuspended in

the same final volume as the input and equivalent volumes of pellet and supernatant were separated by SDS-PAGE and processed by immunoblotting.

3.3.6 Statistical Tests

Statistical results, average and standard deviation values were computed and plotted by using Matlab and Prism. Two-tailed t-tests and one-way ANOVA (Tukey) tests were applied to compare measurements. Unless otherwise stated, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ were chosen to determine the significance levels.



3.4 Results

3.4.1 Centriolar Satellites Negatively Regulate CCDC66 Centrosomal Recruitment and Kinetics

The localization of CCDC66 to centriolar satellites and its physical and proximity interactions with satellite proteins suggest that satellites play important roles in CCDC66 function and regulation. Centriolar satellites have been implicated in both active transport and in sequestration of centrosome and cilium proteins (Hori & Toda, 2017; Kubo et al., 1999; M. A. X. Tollenaere et al., 2015). To distinguish between these models, we quantified the centrosomal levels and dynamics of CCDC66 in control and PCM1-depleted satellite-less cells. In these experiments, we used the human retinal pigmented epithelial (RPE1) cell line stably expressing GFP-CCDC66 at near-endogenous levels (D. Conkar et al., 2017). Centrosomal levels of GFP-CCDC66 in PCM1-depleted cells (1.32 ± 0.07 , $p=0.0162$) significantly increased relative to control cells (siControl= 1.00 ± 0.05) (Figure 23A and 23B). Depletion of PCM1 did not change the total levels of CCDC66 (Figure 23C), suggesting that satellites regulate CCDC66 targeting to centrosomes. To determine the consequences of satellite loss on the dynamics of centrosomal recruitment, we performed fluorescence recovery after photobleaching (FRAP) experiments in control ($n=16$ in total) and PCM1-depleted cells ($n=16$ in total) (Figure 23D, Figure 24A and 24B). In control cells, $50.4\% \pm 2.49$ of centrosomal GFP-CCDC66 recovered rapidly with a half time of $40 \text{ s} \pm 4.00$, identifying this as a dynamic pool with the rest being stably incorporated at the centrosome (Figure 23E-G). In PCM1-depleted cells, the percentage of recovery significantly increased ($73.3\% \pm 3.10$, $p<0.0001$) but half time ($45.2 \text{ s} \pm 2.40$) did not change (Figure 23E-G). Of note, depletion of PCM1 resulted in a decrease in the percentage of cells with radial microtubule arrays (Figure 24C) (Dammermann & Merdes, 2002). Together, these results identify an inhibitory role for satellites in centrosomal recruitment of CCDC66. However, they do not distinguish whether this regulation is mediated by removal of CCDC66 from the centrosome or its sequestration at satellites.

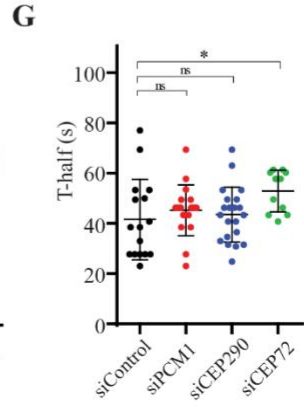
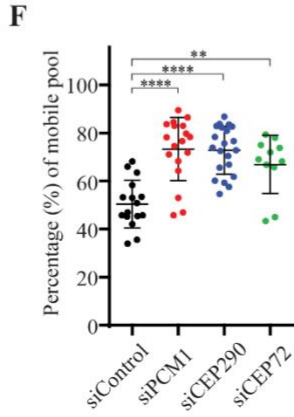
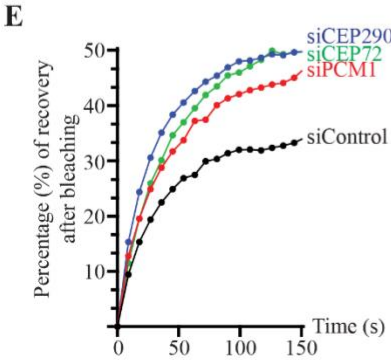
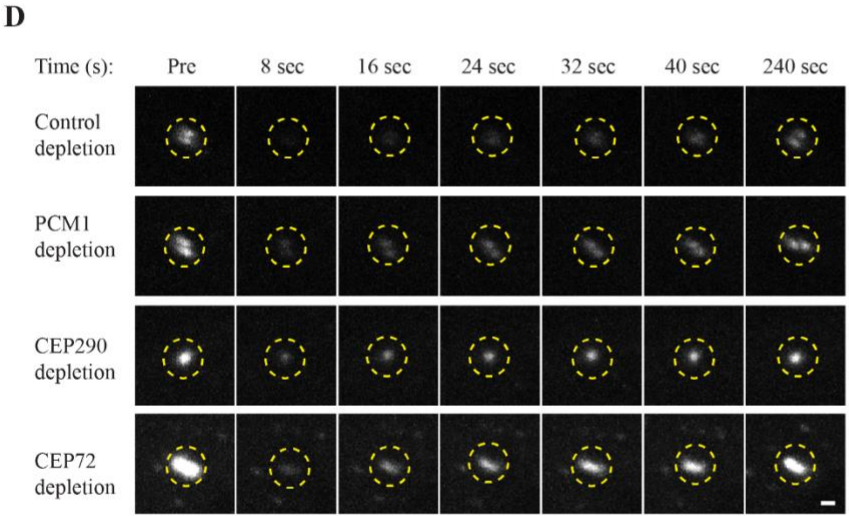
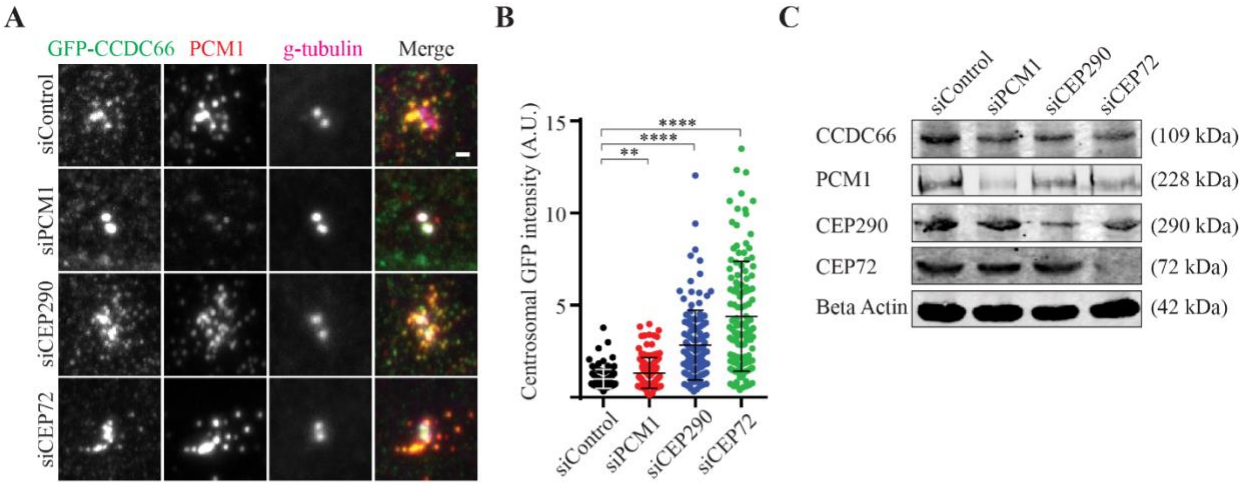


Figure 23. Centriolar satellites inhibit CCDC66 dynamic localization at the centrosome.

A. Effect of PCM1, CEP72 and CEP290 depletion on CCDC66 level at the centrosome. RPE1::GFP-CCDC66 cells were transfected with control, PCM1, CEP290 or CEP72 siRNAs for 48 h. Cells were then fixed and stained for GFP, PCM1 and gamma tubulin. Images represent centrosomes in cells from the same coverslip taken with the same camera settings. Scale bar, 1 μ m.

B. Quantification of (a). GFP-CCDC66 fluorescence intensities were measured in a 2.5 μ m² circular area around the centrosome from two independent experiments. Levels are normalized to the mean of the control group (=1). n=50 cells for each group. t-test was used for statistical analysis. Standard error of mean (SEM): siControl=0.046, siPCM1=0.075, siCEP290=0.17, siCEP72=0.27.

C. Effect of PCM1, CEP72 and CEP290 depletion on total cellular CCDC66 levels. Cells were transfected with control, PCM1, CEP290 or CEP72 siRNAs for 48 h. Cell extracts were cells were immunoblotted for CCDC66, PCM1, CEP290 and CEP72. Beta-actin was used as a loading control.

D. Effect of PCM1, CEP72 and CEP290 depletion on CCDC66 dynamics at the centrosome. RPE1::GFP-CCDC66 cells were transfected with control, PCM1, CEP290 or CEP72 siRNAs for 48 h. 2.5 μ m² circular area around the centrosome marked by yellow dashed circle was photobleached and imaged for 250 seconds after photobleaching. Still images represent centrosomal GFP-CCDC66 signal at the indicated times. Scale bar, 1 μ m.

E. Percentage of recovery graph from (d). Individual FRAP experiments from two independent experiments were fitted into one phase association curves. n=8 for control and PCM1, n=8 for CEP290 and n=6 for CEP72 depleted cells per group. Half-time of and mobile pool were calculated using recovery data.

F. mobile pool of D. Error bars, SEM: siControl=2.49, siPCM1=3.10, siCEP290=2.16, siCEP72=3.64 and **G.** half-time analysis of D. Error bars, SEM: siControl=4.00, siPCM1=2.39, siCEP290=2.89, siCEP72=2.50.

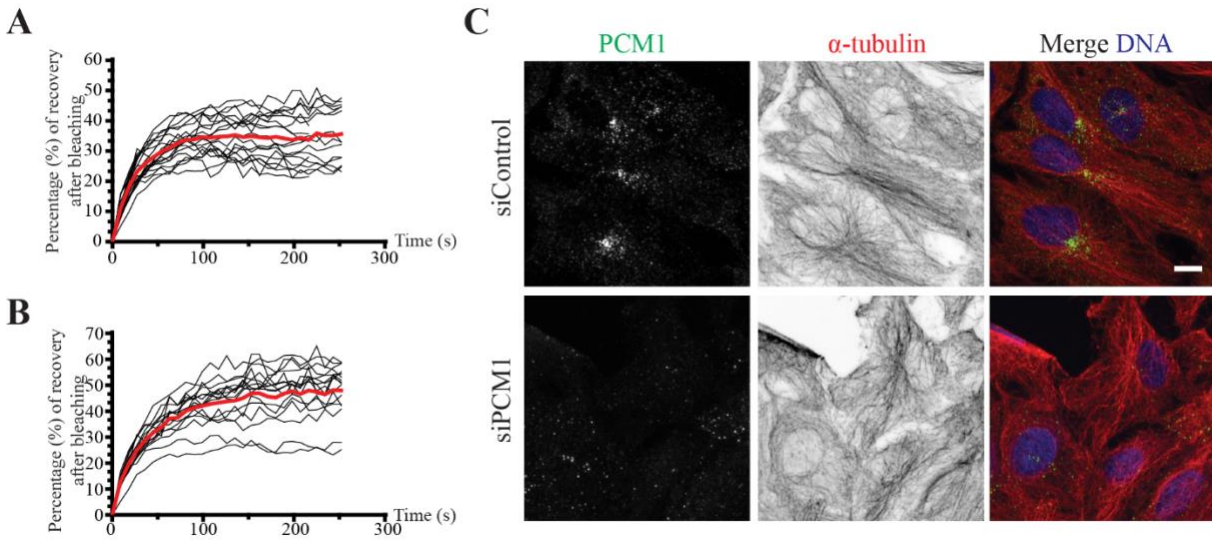


Figure 24. FRAP in PCM1 depleted cells

A and B. Percentage of recovery graphs of individual FRAP experiments in control cells (A) and cells depleted for PCM1 (B). Individual FRAP experiments from two independent experiments were fitted into one phase association curves. $n=8$ for control and PCM1, depleted cells per group. Red curve represents the mean of all experiments. **C.** Effect of PCM1 depletion on microtubule network. RPE1 cells were transfected with control or PCM1 siRNAs. 48 h after transfection, cells were fixed and stained for PCM1 and α -tubulin. Images were taken with the same camera settings. Scale bar, 10 μ m.

In previous work, we identified interactions between CCDC66 and several centriolar satellite proteins: a physical interaction with PCM1 and CEP290 was identified using co-immunoprecipitation experiments, and a proximity interaction with CEP72 using Biotin Identification (BioID) proximity labeling experiments (D. Conkar et al., 2017). The satellite proteins CEP72 and CEP290 are required for cilium assembly and ciliary content regulation, and their depletion results in satellite accumulation around the centrosome (J. Kim et al., 2008; M. V. Nachury et al., 2007; T. R. Stowe et al., 2012). To investigate the role of these interactions and the consequences of satellite clustering on CCDC66 targeting, we quantified the abundance and dynamics of centrosomal CCDC66 in CEP290- and CEP72-depleted cells (Figure 25A and 25B). A significant increase in the centrosomal abundance of CCDC66 relative to control cells (siControl=1.00 $\pm$ 0.05) was observed for cells depleted of either CEP290 (2.79 $\pm$ 0.17, $p < 0.0001$) or CEP72 (4.38 $\pm$ 0.27, $p < 0.0001$, $n = 120$ in total) (Figure 23A and 23B). Likewise, a significant increase in the percentage of recovery of CCDC66 at centrosomes relative to control cells (50.4% $\pm$ 2.49) was observed for cells depleted of either CEP290 (72.8% $\pm$ 2.16, $p < 0.0001$, $n = 17$) or CEP72 (66.9% $\pm$ 3.64, $p = 0.0038$, $n = 12$ in total) (Figure 23D-F, Figure 25C and 25D). While there was no change in the rate of recovery for cells depleted of CEP290 (43.5 s $\pm$ 2.39), a significant increase in halftime was observed in cells depleted of CEP72 (52.9 s $\pm$ 2.50, $p = 0.0323$) compared to control cells (41.5 s $\pm$ 4.00) (Figure 25D). Notably, the changes in the recovery percentages were comparable to the ones in PCM1-depleted cells. Collectively, these results demonstrate that disruption of satellite function and distribution by depleting CEP72 and CEP290, as well as loss of satellites by depleting PCM1, facilitates centrosomal recruitment and dynamics of CCDC66. This suggests that these proteins cooperate at satellites to regulate CCDC66 centrosomal targeting.

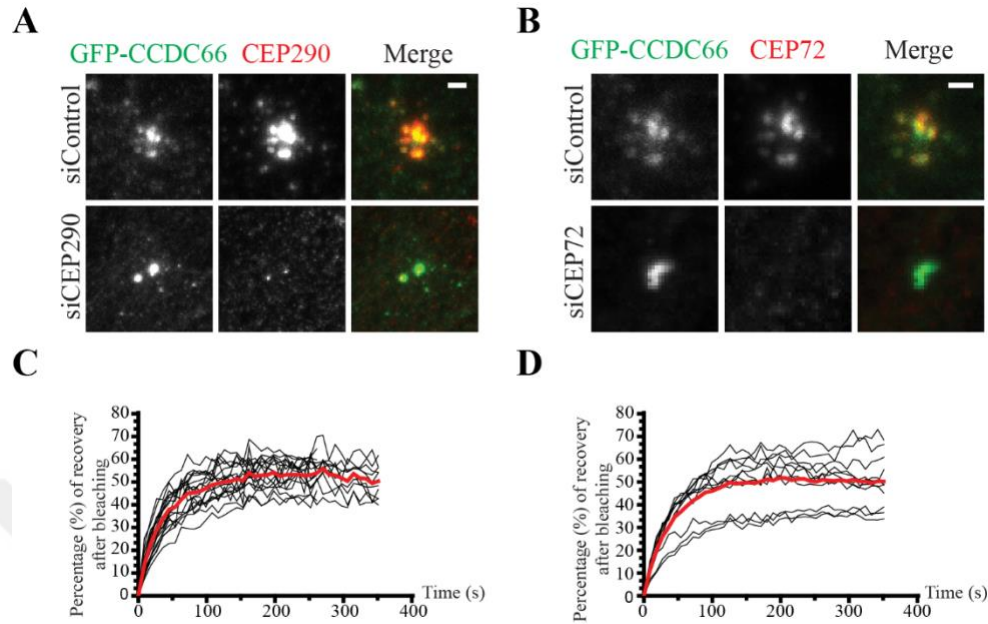


Figure 25. CCDC66 accumulates at centriolar satellites in CEP290 and CEP72 depleted cells

A. RNAi-mediated depletion of CEP290 in RPE1::GFP-CCDC66 cells. RPE1-CCDC66 cells were transfected with control or CEP290 siRNA for 48 h. Cells were fixed and stained for GFP and CEP290. Scale bar, 1 μm. **B.** RNAi-mediated depletion of CEP72 in RPE1::GFP-CCDC66 cells. RPE1-CCDC66 cells were transfected with control or CEP72 siRNA for 48 h. Cells were fixed and stained for GFP and CEP72. Scale bar, 1 μm. **C and D.** Percentage of recovery graphs of individual FRAP experiments in control cells CEP290 (C) and Cep72 (D). Individual FRAP experiments from two independent experiments were fitted into one phase association curves. n=8 for CEP290 and n=6 for CEP72 depleted cells per group. Red curve represents the mean of all experiments.

3.4.2 Centrosomal CCDC66 Recruitment Requires an Intact and Dynamic Microtubule Network

The clustered localization of satellites around the centrosomes requires an intact microtubule network (Kubo et al., 1999). Using *in vitro* and *in vivo* studies, we previously showed that CCDC66 localizes to microtubules and directly interacts with them (D. Conkar et al., 2017). Based on these lines of evidence, we hypothesized that microtubules might regulate CCDC66 targeting either by maintaining satellite proximity to the centrosome for fast exchange of material, tethering CCDC66 at the centrosome by generating binding sites, and/or actively transporting CCDC66 to centrosomes. To test these models, we quantified centrosomal CCDC66 abundance and dynamics in RPE1::GFP-CCDC66 cells treated with either nocodazole to depolymerize microtubules, or taxol to stabilize microtubules. Both drug treatments result in loss of centrosome-nucleated microtubules (De Brabander et al., 1986; Fry et al., 1998) and a consequent declustering of satellites throughout the cytoplasm (Figure 26). In contrast to the phenotypes of satellite-less cells, both treatments resulted in a significant decrease in the centrosomal levels of CCDC66 (DMSO control = 1 ± 0.05 ; nocodazole = 0.17 ± 0.02 , $p < 0.0001$; taxol = 0.34 ± 0.03 , $p < 0.0001$, $n = 70$ in total) (Figure 26D and 26E). FRAP analysis of centrosomal GFP-CCDC66 showed both nocodazole and taxol treated cells had significantly faster recovery rates (DMSO control = $39 \text{ s} \pm 2.67$, $n = 20$; nocodazole = $27.3 \text{ s} \pm 1.95$, $p = 0.0090$, $n = 18$; taxol = $25 \text{ s} \pm 1.52$, $p = 0.0090$, $n = 10$ in total) (Figure 27A, 27B, 27D, 27E-G). There was a significant decrease in the percentage of recovery in nocodazole-treated cells but not in taxol-treated cells (DMSO control = $54.2\% \pm 1.77$; nocodazole = $41.3\% \pm 1.57$, $p < 0.0001$; taxol = $53.2\% \pm 2.13$, $p = 0.9431$) (Figure 27A-C). To examine the relationship between satellites and microtubules for protein targeting to centrosomes, FRAP experiments on centrosomal GFP-CCDC66 were performed in cells depleted for PCM1 and treated with nocodazole. In PCM1-depleted cells, depolymerization of microtubules resulted in a significant decrease in the centrosomal abundance of CCDC66 and its percentage of recovery in FRAP experiments (Control depleted cells: mobile pool = $55.4\% \pm 2.19$, halftime = $34.9 \text{ s} \pm 2.20$, PCM1-depleted cells: mobile pool = $80.8\% \pm 1.48$, halftime = $45.2 \text{ s} \pm 2.39$, $p < 0.0001$, nocodazole-treated PCM1-depleted cells: mobile pool = $69.50\% \pm 1.63$, halftime = $49.1 \text{ s} \pm 6.79$, $p < 0.0001$) (Figure 27H-M). These results reveal that microtubules are required for CCDC66 centrosomal abundance and dynamic localization, and that satellites have an inhibitory role in this process.

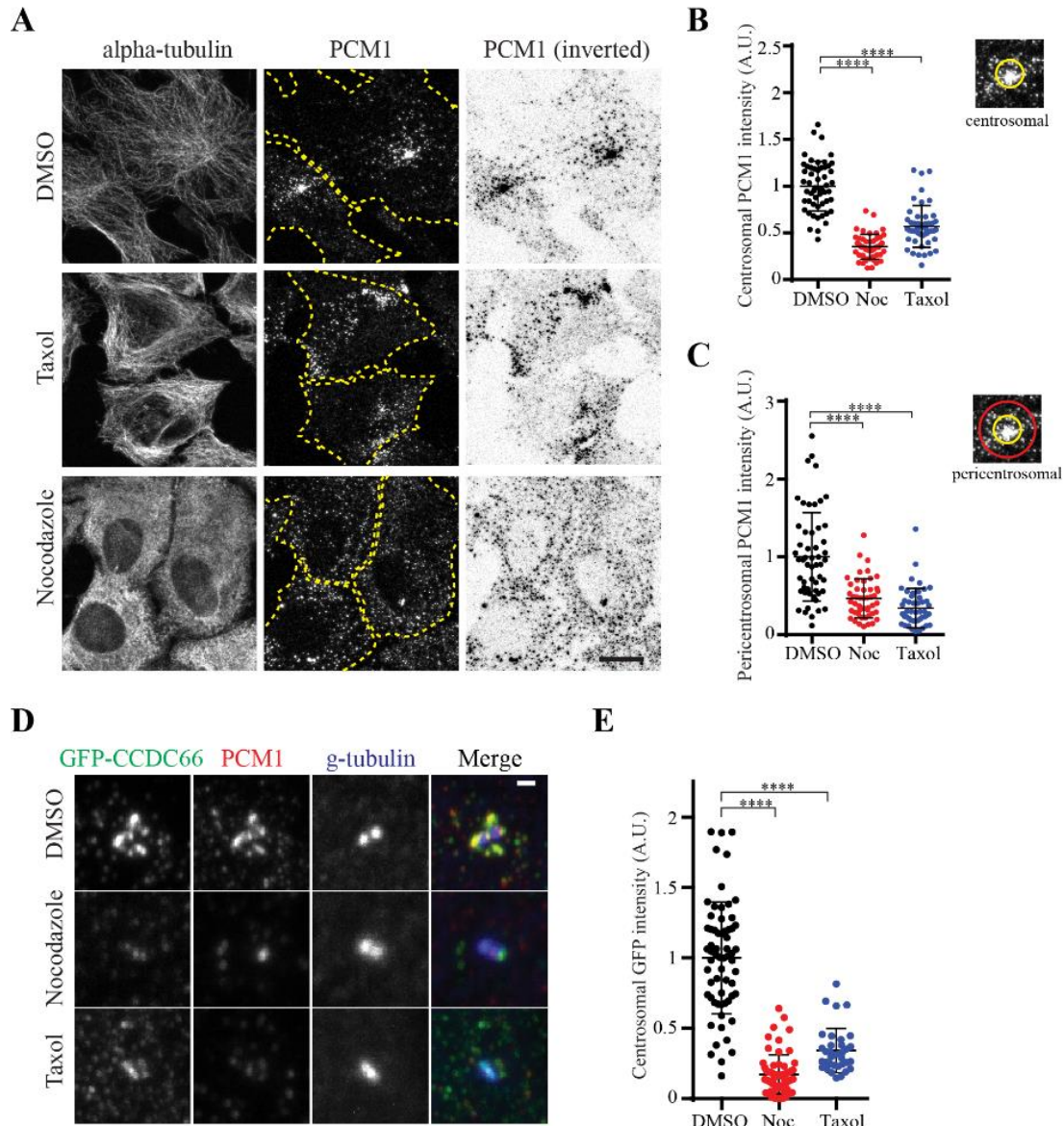


Figure 26. Steady state CCDC66 levels are regulated by microtubule network.

A. Effect of microtubule depolymerization and stabilization on PCM1 distribution. RPE1 cells were treated with 0.1% DMSO, 5 μ g/ml nocodazole or 5 μ M taxol for 1 h. Cells were the fixed with and stained for PCM1 and alpha-tubulin. Scale bar, 5 μ m. **B.** Quantification of A. PCM1 centrosomal fluorescence intensities were measured in a 2.5 μ m² circular area around the centrosome from two independent experiments. Levels are normalized to the mean of the control group (=1). n=50 cells for each group. t-test was used for statistical analysis. Error bars, SEM: DMSO= 0.04, nocodazole=0.02, taxol=0.03. **C.** Quantification of A. PCM1 pericentrosomal fluorescence intensities were measured by subtracting the fluorescence intensity of 17 μ m² circular

area from the $2.5\ \mu\text{m}^2$ circular area around the centrosome. Levels are normalized to the mean of the control group (=1). $n=50$ cells for each group. t-test was used for statistical analysis. Error bars, SEM: DMSO= 0.08, nocodazole=0.04, taxol=0.04. **D.** Effect of microtubule depolymerization and stabilization on CCDC66 level at the centrosome. RPE1::GFP-CCDC66 cells were treated with 0.1% DMSO, $5\ \mu\text{g/ml}$ nocodazole or $5\ \mu\text{M}$ taxol for 1 h. Cells were then fixed and stained for GFP, PCM1 and gamma tubulin. Images represent centrosomes in cells from the same coverslip taken with the same camera settings. Scale bar, $1\ \mu\text{m}$. **E.** Quantification of D. GFP-CCDC66 fluorescence intensities were measured in a $2.5\ \mu\text{m}^2$ circular area around the centrosome from two independent experiments. Levels are normalized to the mean of the control group (=1). $n=50$ cells for each group. t-test was used for statistical analysis. Error bars, SEM: DMSO control= 0.05, nocodazole= 0.02, taxol= 0.03. **<0.05, ***0.0005.

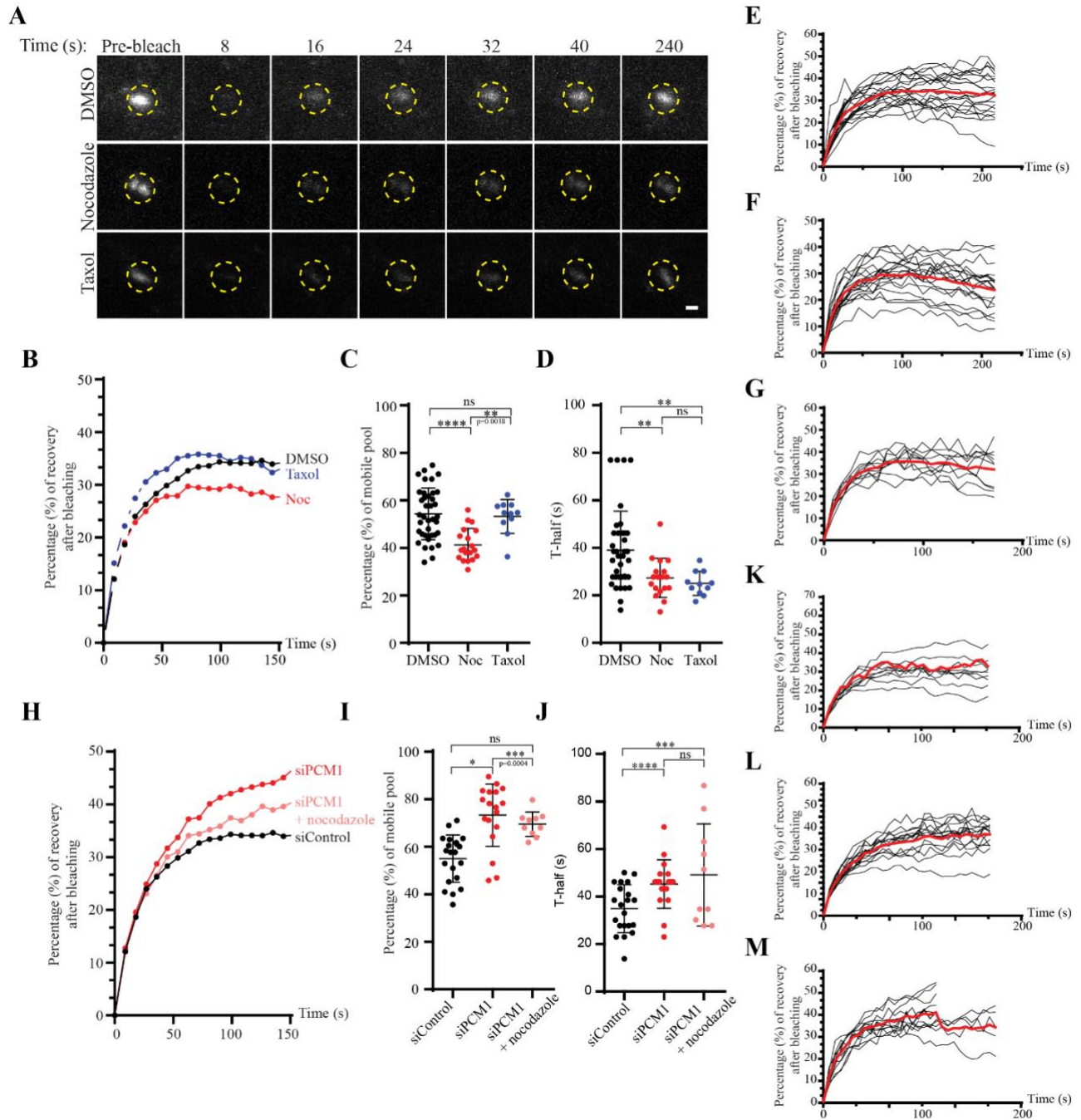


Figure 27. An intact and dynamic microtubule network is required for CCDC66 dynamic localization at the centrosome.

A. Effect of microtubule depolymerization and stabilization on CCDC66 dynamics at the centrosome. RPE1::GFP-CCDC66 cells were treated with 0.1% DMSO, 5 μ g/ml nocodazole or 5 μ M taxol for 1 h. 2.5 μ m² circular area around the centrosome marked by yellow dashed circle was

photobleached and imaged for 250 seconds after photobleaching. Still images represent centrosomal GFP-CCDC66 signal at the indicated times. Scale bar, 1 μ m. **B.** Percentage of recovery graph from A. Individual FRAP experiments from two independent experiments were fitted into one phase association curves. n=10 for DMSO, n=9 for nocodazole and n=5 for taxol treated cells per group. Half-time of and mobile pool were calculated using recovery data. **C.** mobile pool of B. Error bars, SEM: DMSO= 1.77, nocodazole=1.57, taxol=2.13. **D.** half-time analysis of B. Error bars, SEM: DMSO=2.67, nocodazole=1.95, taxol=1.52. **E to G.** Percentage of recovery graphs of individual FRAP experiments in cells treated with 0.1% DMSO (E), 5 μ g/ml nocodazole (F) or 5 μ M taxol (G) for 1 h. FRAP experiments were performed after treatment. Individual FRAP experiments from two independent experiments were fitted into one phase association curves. n=10 for DMSO, n=9 for nocodazole and n=5 for taxol treated cells per group. Red curve represents the mean of all experiments. **H.** Combinatorial effect of microtubule depolymerization and PCM1 depletion on CCDC66 dynamics at the centrosome. RPE1::GFP-CCDC66 cells were transfected with control and PCM1 siRNAs for 48 h and then treated with 5 μ g/ml nocodazole for 1 h. Individual FRAP experiments from two independent experiments were fitted into one phase association curves and percentage of recovery graphs were generated. n=12 for control depleted, n=12 for PCM1 depleted and n=10 for PCM1 depleted and nocodazole treated cells per group. Half-time of and mobile pool were calculated using recovery data. **I.** mobile pool of H. Error bars, SEM: siControl=2.19, siPCM1=1.48, siPCM1+nocodazole=1.63. **J.** half-time analysis of H. Error bars, SEM: siControl=2.2, siPCM1=2.4, siPCM1+nocodazole=6.80. **K to M.** Percentage of recovery graphs of individual FRAP experiments in control depleted cells (K), cells depleted for PCM1 for 48 h (L) and in cells depleted for PCM1 for 48 h followed by nocodazole treatment for 1 h (M). Individual FRAP experiments from two independent experiments were fitted into one phase association curves. n=12 for control depleted, n=12 for PCM1 depleted and n=10 for PCM1 depleted and nocodazole treated cells per group. Red curve represents the mean of all experiments.

3.4.3 The Dynein Complex Interacts with CCDC66 and Mediates Centrosomal Recruitment and Dynamics of CCDC66

The cytoplasmic dynein motor transports its cargo along microtubules by associating with the dynactin complex. Among its cargoes are many centrosome proteins (Reck-Peterson et al., 2018, p.). A recent BioID-based human cytoplasmic dynein complex interactome identified CCDC66 as a proximity partner of the dynein adaptors BICD2 and NINL (Redwine et al., 2017). To examine whether the interaction between microtubules and CCDC66 requires ATPase-dependent motor activity, cells expressing GFP-CCDC66 were treated with the ATP analog AMP-PNP, which enhances the affinity of molecular motors to microtubules at low concentrations (Schroer et al., 1989; Shimizu & Johnson, 1983). *In vitro* microtubule pelleting experiments from control and 0.5 mM AMP-PNP-treated cells revealed that the fraction of GFP-CCDC66 that co-pelleted with microtubules was significantly higher in the presence of AMP-PNP relative to control ($p=0.0038$), suggesting that molecular motors directly or indirectly mediate the association of CCDC66 with microtubules (Figure 28A and 28B). We then tested whether dynein mediates this interaction by performing immunoprecipitation experiments in HEK293T cells expressing GFP-DYNC1IC1. CCDC66 along with BBS4, the dynactin complex subunit p150^{glued} and PCM1 co-pelleted with GFP-DYNC1IC1, but not with GFP (Figure 28C). In agreement with this interaction, GFP-CCDC66 co-localized with p150^{glued} at the centrosome (Figure 28D) and CCDC66 depletion resulted in a significant decrease in the centrosomal levels of p150^{glued} (siControl= 1.00 ± 0.04 ; siCCDC66= 0.6 ± 0.03 , $p<0.0001$, $n=100$ in total) relative to control (Figure 28E and 28F). Likewise, overexpression of the dynein-binding fragment of the dynactin subunit, DsRed-p150^{glued} 217-548 aa (p150^{glued}-CC1) (Quintyne et al., 1999), disrupted the centriolar satellite organization and resulted in formation of cytoplasmic aggregates that sequestered GFP-CCDC66 (Figure 28G). Together, these results reveal a cellular complex between CCDC66, dynein/dynactin, satellites and microtubules.

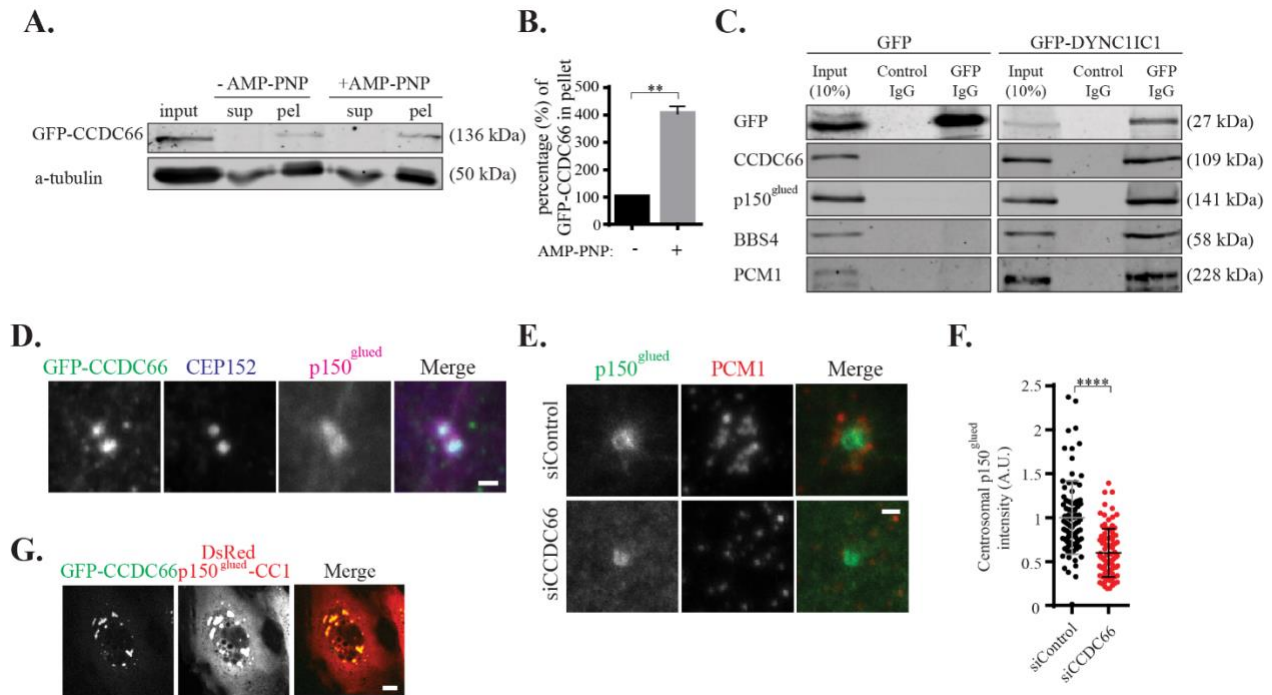


Figure 28. The dynein complex interacts and co-localizes with CCDC66

A. Analysis of CCDC66 microtubule association in cells treated with unhydrolyzable ATP analog AMP-PNP. HEK293T cells were transfected with GFP-CCDC66, treated with 0.5 mM unhydrolyzable ATP analog AMP-PNP and in vitro microtubule pelleting experiments were performed with extracts from control and AMP-PNP-treated cells. Equal volumes of input, supernatant and pellet fractions were immunoblotted for GFP and alpha-tubulin. Graph represents the mean values from two independently performed pelleting experiments, p=0.0038. **B.** Quantification of GFP-CCDC66 amount in pellet from A. Percentage of GFP-CCDC66 in the pellet relative to input was quantified by measuring GFP band intensities of pellet and input fractions and normalizing them to alpha tubulin levels. **C.** Immunoprecipitation of the dynein complex. HEK293T cells were transfected with GFP or GFP-DYNC1IC1 for 48 h. Complexes were immunoprecipitated (IP) with control IgG or anti-GFP antibody, and co-precipitated proteins were detected with GFP, CCDC66, p150glued, BBS4 and PCM1. **D.** Localization of CCDC66 and p150glued at the centrosome. RPE1::GFP-CCDC66 cells were fixed with methanol and stained with GFP, p150glued and CEP152 (centrosome marker). Scale bar, 1 μm. **E.** Effect of CCDC66

depletion on p150glued level at the centrosome. RPE1 cells were transfected with control and CCDC66 siRNAs for 48 h. Cells were then fixed and stained for p150glued and PCM1. Images represent centrosomes in cells from the same coverslip taken with the same camera settings. Scale bar, 1 μm . **F.** Quantification of **E.** p150glued fluorescence intensities were measured in a 2.5 μm^2 circular area around the centrosome from two independent experiments. Levels are normalized to the mean of the control group (=1). n=50 cells for each group. t-test was used for statistical analysis. Error bars, SEM: siControl= 0.04, siCCDC66=0.03. **G.** Effects of p150glued-CC overexpression on localization of CCDC66. RPE1::GFP-CCDC66 were transfected with DsRed-p150glued 217-548 for 24 h, fixed and stained for GFP. Scale bar, 10 μm .

The association of CCDC66 with the dynein/dynactin complex might be required for its centrosomal targeting and dynamics. To test this, we performed quantitative immunofluorescence and FRAP experiments in RPE1::GFP-CCDC66 cells inhibited for dynein activity by expressing p150^{glued}-CC1, which prevents dynein-dynactin binding (Schroer et al., 1989). Analogous to the perturbation of the microtubule cytoskeleton, there was a significant decrease (DsRed-p150^{glued}-CC1 = 0.38 ± 0.07 , $p < 0.0001$, $n = 50$ in total) in the centrosomal abundance of CCDC66 in dynein-inhibited cells relative to control cells (DsRed = 0.93 ± 0.11 , $n = 50$ in total) (Figure 29A and 29B). FRAP analysis of centrosomal GFP-CCDC66 in dynein-inhibited cells showed a significant but small decrease in the percentage of recovery (DsRed = $60.5\% \pm 1.35$, $n = 20$, DsRed-p150^{glued}-CC1 = $54.4\% \pm 1.38$, $p = 0.0024$, $n = 16$ in total) and halftime (DsRed = $38.4 \text{ s} \pm 1.41$, DsRed-p150^{glued}-CC1 = $33.5 \text{ s} \pm 1.95$, $p = 0.030$, $n = 12$ in total) (Figure 29E-H, 29L and 29M). Likewise, centrosomal abundance of CCDC66 significantly decreased upon 2 mM AMP-PNP treatment (Control = 1.00 ± 0.08 ; AMP-PNP = 0.26 ± 0.01 , $p < 0.0001$, $n = 50$ in total) (Figure 29C, 29D, 29N and 29O), which at high concentrations inhibits the activity of both kinesin and dynein motors (Kubo et al., 1999; Schroer et al., 1988). The halftime for centrosomal GFP-CCDC66 also decreased significantly in 2 mM AMP-PNP-treated cells (Control = $40.25\% \pm 2.31$, $n = 20$; AMP-PNP = $23.5\% \pm 1.52$, $p = 0.0006$, $n = 14$ in total), (Figure 29I-K). These results indicate that molecular motors, particularly the activity of the dynein/dynactin-complex, is required for the dynamic localization of CCDC66 to the centrosome, likely through cooperation with microtubules.

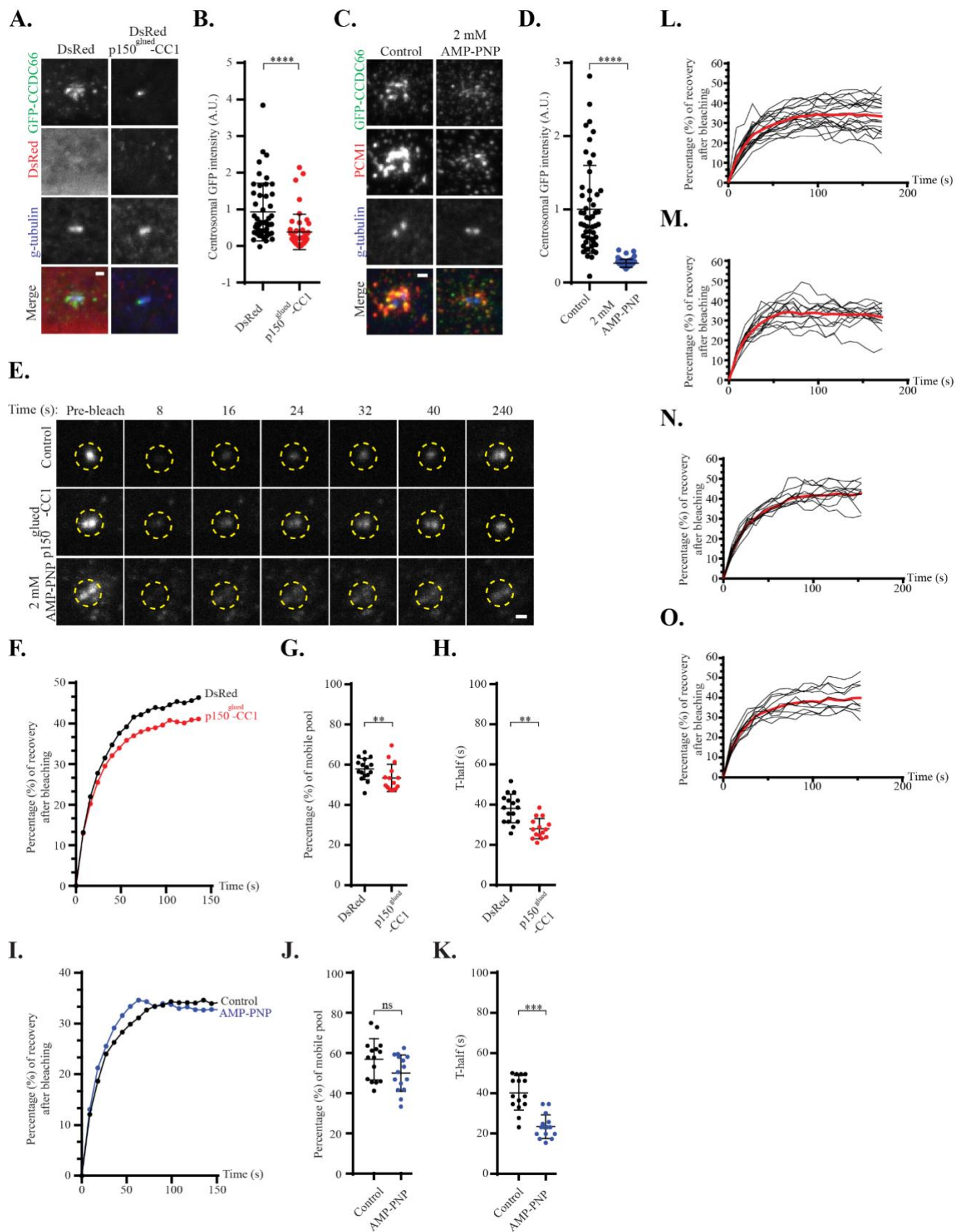


Figure 29. Activity of molecular motors are required for CCDC66 dynamic localization at the centrosome.

A. Effect of inhibiting dynein complex activity on CCDC66 level at the centrosome. RPE1::GFP-CCDC66 cells were transfected with DsRed (control) or DsRed p150glued 217-548 for 24 h. Cells were then fixed with PFA and stained for gamma tubulin. Images represent centrosomes in cells from the same coverslip taken with the same camera settings. Scale bar, 1 μ m. **B.** Quantification of A. GFP-CCDC66 fluorescence intensities were measured in a 2.5 μ m² circular area around the centrosome from two independent experiments. Levels are normalized to the mean of the control group (=1). n=50 cells for each group. t-test was used for statistical analysis. Error bars, SEM: DsRed=0.12, p150glued CC1=0.07. **C.** Effect of inhibiting molecular motor activity on CCDC66 level at the centrosome. RPE1-CCDC66 cells were treated with 2 mM AMP-PNP for 10 min. Cells were then fixed and stained for GFP, PCM1 and gamma tubulin. Scale bar, 1 μ m. **D.** Quantification of A. GFP-CCDC66 fluorescence intensities were measured in a 2.5 μ m² circular area around the centrosome from two independent experiments. Levels are normalized to the mean of the control group (=1). n=50 cells for each group. t-test was used for statistical analysis. Error bars, SEM: control=0.84, AMP-PNP=0.01. **E.** Effect of inhibiting molecular motor activity on CCDC66 dynamics at the centrosome. RPE1::GFP-CCDC66 cells were transfected with DsRed (control) or DsRed p150glued 217-548 for 24 h and in parallel they cells were treated with 2 mM AMP-PNP for 10 min. 2.5 μ m² circular area around the centrosome marked by yellow dashed circle was photobleached and imaged for 250 seconds after photobleaching. Still images represent centrosomal GFP-CCDC66 signal at the indicated times. Scale bar, 1 μ m. **F and I.** Percentage of recovery graph from C. Individual FRAP experiments from two independent experiments were fitted into one phase association curves. n=8 for DsRed and n=6 for p150glued CC1 transfected cells per group. n=10 for control and n=7 for AMP-PNP treated cells per group. Half-time of and mobile pool were calculated using recovery data. **G and J.** mobile pool of D. Error bars, SEM: DsRed=1.35, p150glued CC1=1.38, control=2.65, AMP-PNP=2.34. **H and K.** half-time analysis of D. Error bars, SEM: DsRed=1.41, p150glued CC1=1.95, control=2.23, AMP-PNP=1.52. **L and M.** Percentage of recovery graphs of individual FRAP experiments in control cells transfected with DsRed (L) and in cells inhibited for dynein activity after transfection with DsRed p150glued 217-548 for 24 h (M). Individual FRAP experiments from two independent experiments were fitted into one phase association curves. n=8 for DsRed and n=6 for p150glued CC1 cells per group. Red

curve represents the mean of all experiments. **N and O.** Percentage of recovery graphs of individual FRAP experiments in control cells (N) and in cells treated with 2 mM AMP-PNP for 10 minutes (O). Individual FRAP experiments from two independent experiments were fitted into one phase association curves. n=10 for control and n=7 for AMP-PNP treated cells per group. Red curve represents the mean of all experiments.



3.4.4 Ciliary CCDC66 Is Not Dynamic and Its Ciliary Recruitment Is Regulated By Satellites

In ciliated cells, CCDC66 localizes to the primary cilium in addition to centriolar satellites and the basal body. Time-lapse imaging of RPE1::GFP-CCDC66 confirmed its dynamic localization to the cilium upon serum starvation (Movie 1). To determine whether CCDC66 localizes to the ciliary axoneme or the membrane, we performed super-resolution microscopy using stimulated emission depletion (STED) analysis of CCDC66 relative to the axonemal marker acetylated tubulin and the ciliary membrane marker Arl13b in RPE1::GFP-CCDC66 cells. CCDC66 had a punctate localization pattern along the ciliary axoneme, but not at the ciliary membrane (Figure 30A).

Through its localization at the axoneme, CCDC66 might function either as a structural component or as part of ciliary trafficking and signaling machineries, which would be reflected as static or dynamic ciliary pools, respectively. To test this, we quantified the dynamics of ciliary CCDC66 by performing FRAP analysis in RPE1::GFP-CCDC66 cells serum starved for 48 h. When the whole cilium was photobleached, the GFP-CCDC66 signal at the cilium did not recover even during 45 min of imaging after photobleaching ($n=7$ in total) (Figure 30B and 30C, Figure 31A). This indicates that there is no exchange between the cytoplasmic and the ciliary pools of CCDC66. When we photobleached the basal body and the cilium together, we observed a fast fluorescence recovery of $55.5\% \pm 2.20$ with a half time of $67.2\text{ s} \pm 5.41$ at the basal body and again almost no recovery at the cilium ($n=16$) (Figure 30B, 30D, Figure 31D). To examine whether the low diffusional mobility of ciliary CCDC66 is due to its immobilization within the primary cilium, we performed half-cilium FRAP analysis in ciliated cells photobleached for the distal, middle or proximal parts of the cilium. Surprisingly, like whole-cilium FRAP experiments, there was almost no recovery in half cilium FRAP experiments ($n=7$ in total) (Figure 30B, 30C, Figure 31B and 31C). As a control, the same experiments were performed in RPE1 cells stably expressing GFP-fusion of the BBSome component BBS4. Analogous to CCDC66, BBS4 redistributes from centriolar satellites to the primary cilium upon serum starvation and functions in the ciliary exit of the G protein-coupled receptors (GPCRs) (M. V. Nachury et al., 2007; A. R. Nager et al., 2017). While most GFP-BBS4 did not recover in whole-cilium FRAP experiments, half cilium FRAPs revealed $41.6\% \pm 4.40$ recovery of the bleached region with a half time of $48\text{ s} \pm 7.88$ from its unbleached ciliary pool ($n=8$ in total) (Figure 31E-L). This result shows that BBS4 is retained in the cilium

once it is recruited and it is only dynamic within the primary cilium, analogous to the dynamic behavior of ciliary receptors such as the GPCR somatostatin receptor SSTR3 and serotonin receptor 6 HTR6 (Q. Hu et al., 2010).



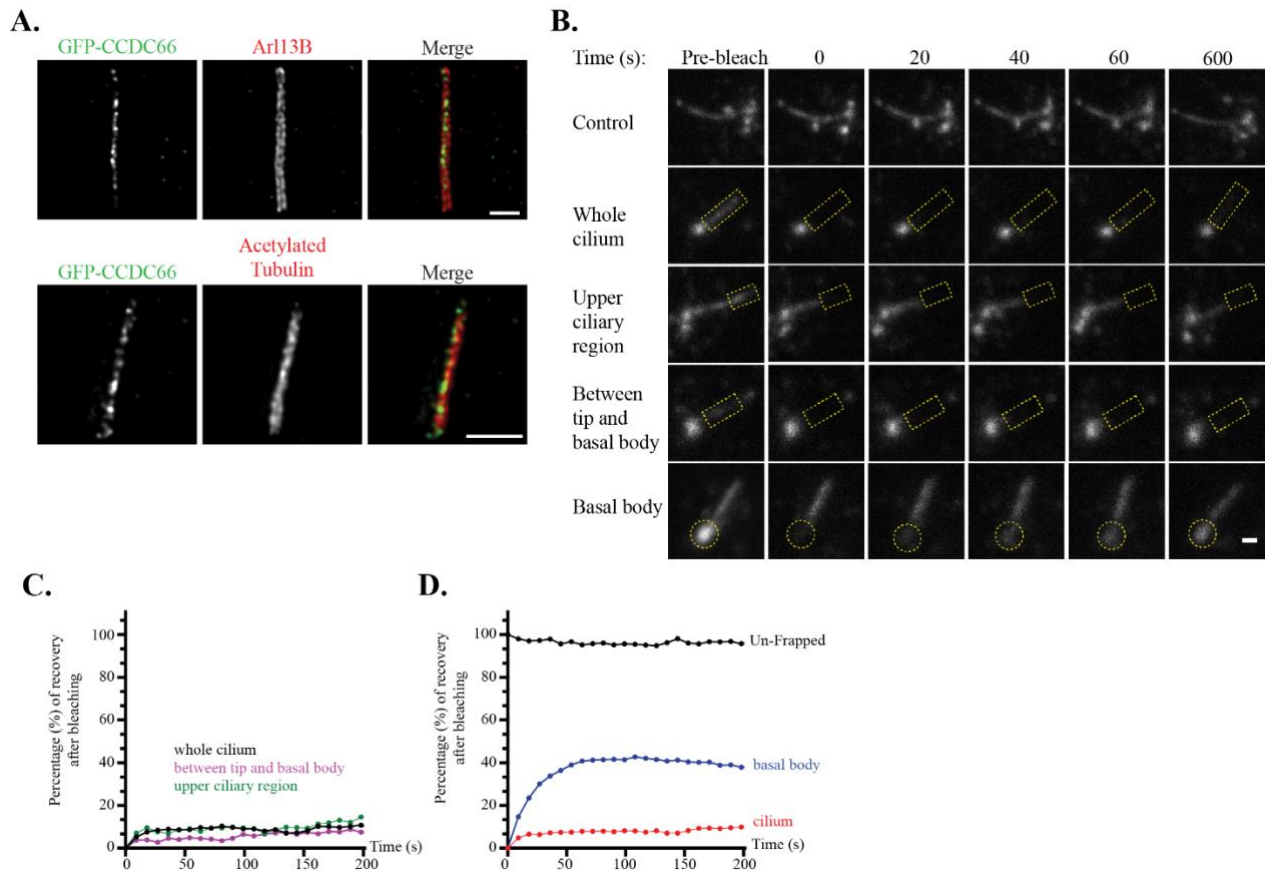


Figure 30. Ciliary CCDC66 is not dynamic.

A. STED analysis of ciliary CCDC66 localization. RPE1::GFP-CCDC66 cells were serum starved for 48 h, fixed with 4% PFA and stained for GFP, Arl13B (ciliary membrane marker) or acetylated tubulin (ciliary axoneme marker). Cells were imaged on a Leica TCS SP8 STED 3X confocal laser scanning microscope. Scale bar, 1 μ m. **B.** FRAP analysis of ciliary GFP CCDC66. RPE1::GFP-CCDC66 cells were serum starved for 48 h. Whole cilium, upper ciliary region or area between the tip and basal body indicated by yellow dashed rectangles was photobleached and cells were imaged for 250 seconds after photobleaching. Still images represent ciliary GFP-CCDC66 signal at indicated time points. Scale bar: 1 μ m. **C and D.** Percentage of recovery graph from B. Individual FRAP experiments from two independent experiments (n=7 in total) were fitted into one phase association curves.

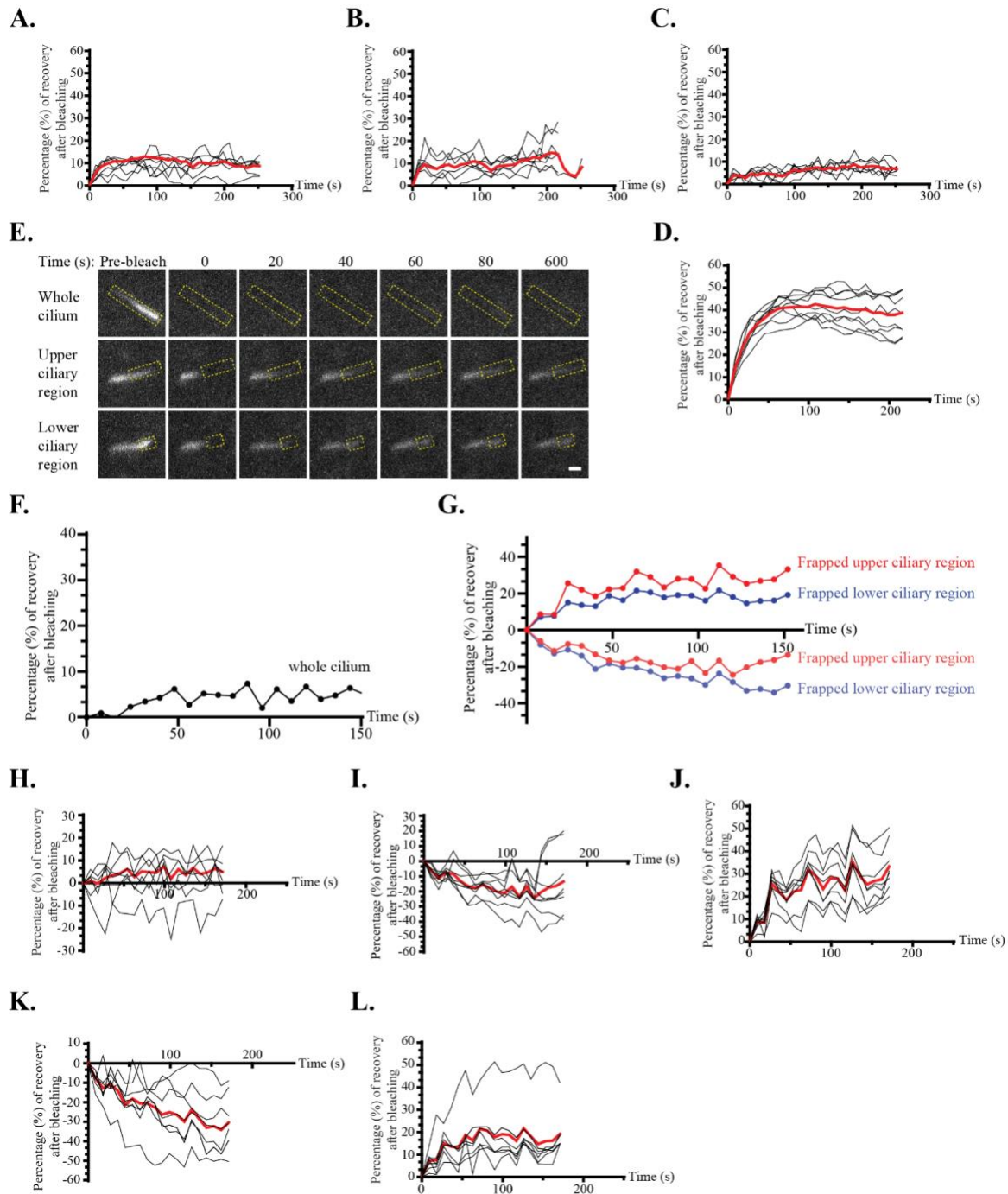


Figure 31. Whole and partial FRAPs of ciliary GFP-CCDC66 and GFP-BBS4

A to D. Percentage of recovery graphs of individual ciliary GFP-CCDC66 FRAP experiments of whole cilium (A), upper ciliary region (B), between tip and basal body (C) and on basal body (D).

Cells were imaged for 250 seconds post-bleaching. Red curve represents the mean of all experiments. **E.** FRAP analysis of ciliary GFP-BBS4. RPE1::GFP-BBS4 cells were serum starved for 48 h. Whole cilium, upper and lower ciliary regions indicated by yellow dashed rectangles were photobleached and cells were imaged for 150 seconds post-bleaching. Still images represent the ciliary GFP-BBS4 signal in indicated time points. Scale bar: 1 μ m. **F.** Percentage of recovery graph of the whole cilium FRAP from (a). Percentage of recovery graphs from two independent experiments were fitted into one phase association curves. n=4 per group. **G.** Percentage of recovery graph of the upper and lower ciliary FRAPs from (A). Percentage of recovery graphs from two independent experiments were fitted into one phase association curves. n=4 cells per group. Curves in the positive y axis represent frapped upper (red) and ciliary (blue) regions. Curves in the negative y axis represent unbleached (un-frapped) upper (red) and ciliary (blue) regions. **H to L.** Percentage of recovery graphs of individual ciliary GFP-BBS4 FRAP experiments from whole cilium (H), upper ciliary region (J) and lower ciliary region (L). Signal from unbleached half of the cilium for upper ciliary (I) and lower ciliary (K) was quantified and plotted as well. Cells were imaged up to 200 seconds post-bleaching. Red curve represents the mean of all experiments.

To test whether satellites regulate ciliary targeting of CCDC66 analogous to their function in its centrosomal targeting, we examined the ciliary levels and dynamics of GFP-CCDC66 in ciliated cells depleted for satellite components. The ciliary abundance of GFP-CCDC66 significantly increased in cells depleted for PCM1 ($\text{siPCM1}=1.16 \pm 0.21$, $p=0.0018$), CEP290 (1.77 ± 0.14 , $p<0.0001$) and CEP72 (2.1 ± 0.27 , $p<0.0001$) relative to control cells ($\text{siControl}=1.00 \pm 0.06$) ($n=100$) (Figure 32A and 32B). Because Cep72, Cep290 and PCM1 depletion compromises ciliogenesis efficiency, we only quantified CCDC66 levels in cells that ciliated (Figure 32C). Loss of CEP72, CEP290 or PCM1 did not affect the percentage and halftime of recovery of ciliary CCDC66 in full-cilium FRAP experiments ($n=10$ in total), supporting that the ciliary pool of CCDC66 does not exchange with its cytoplasmic pool after its ciliary recruitment (Figure 32D-F). Together, our results identify an inhibitory role for satellites in ciliary CCDC66 targeting, which is analogous to how they regulate its centrosomal targeting.

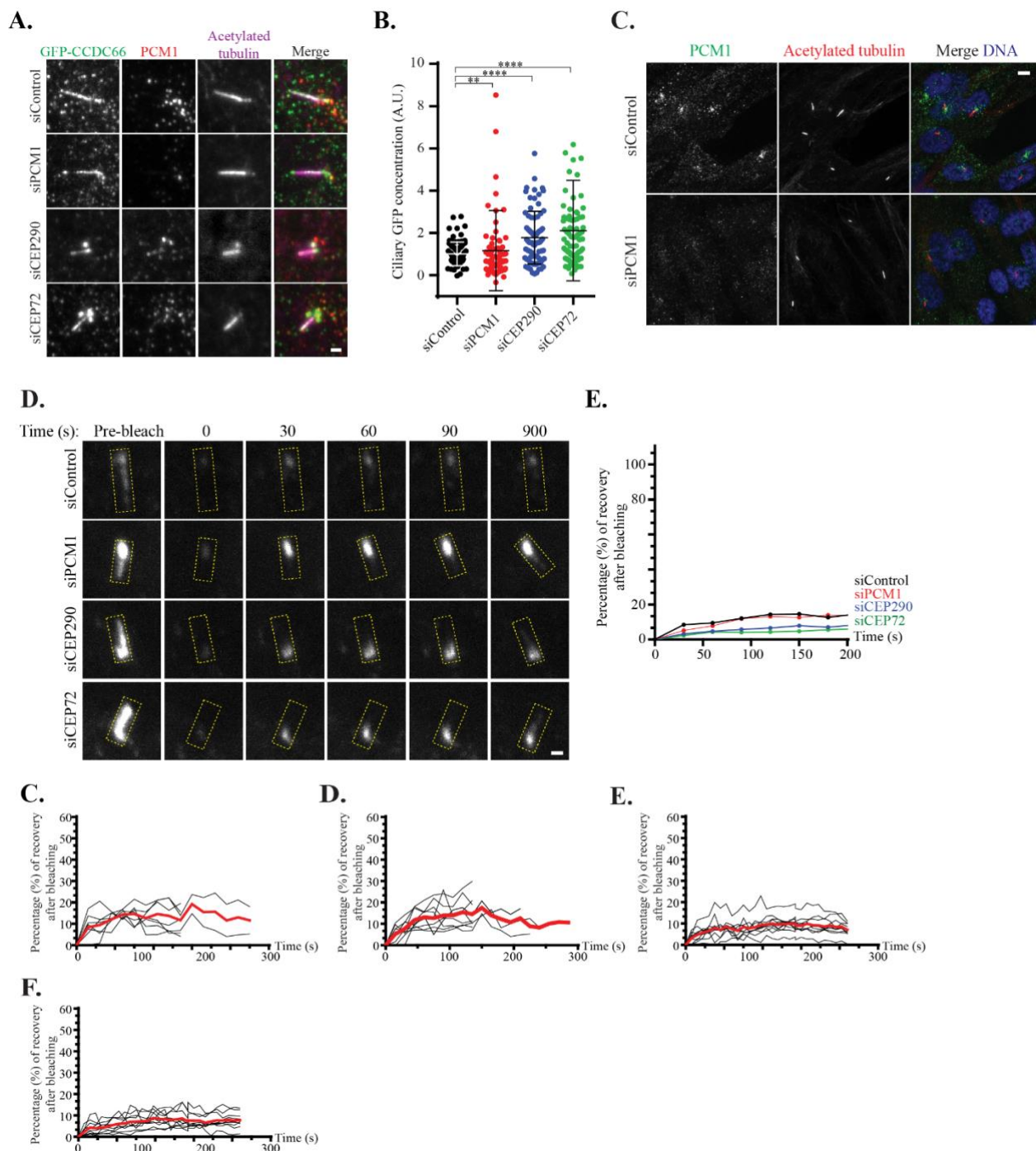


Figure 32. CCDC66 recruitment to primary cilium is regulated by satellites.

A. Effect of PCM1, CEP72 and CEP290 depletion on CCDC66 level at the cilia. RPE1::GFP-CCDC66 cells were transfected with control, PCM1, CEP290 or CEP72 siRNAs. 24 h after transfection, they were serum starved for 48 h. Cells were then fixed and stained for GFP, PCM1 and acetylated tubulin (cilia marker). Images represent cilia in cells from the same coverslip taken

with the same camera settings. Scale bar, 1 μm . **B.** Quantification of GFP-CCDC66 ciliary concentration from (A). GFP-CCDC66 fluorescence intensities were measured in the ciliary area defined by the acetylated tubulin ciliary marker from two independent experiments. Ciliary protein concentrations were determined by dividing fluorescence signal of the protein to the cilium length, which was quantified using acetylated tubulin staining. Levels are normalized to the mean of the control group (=1). $n=50$ cells for each group. t-test was used for statistical analysis. Error bars, SEM: siControl=0.06, siPCM1=0.21, siCEP290=0.14, siCCEP72=0.27. **C.** Effect of PCM1 depletion on cilium formation. RPE1 cells were transfected with control or PCM1, CEP290 or CEP72 siRNAs. 24 h after transfection, they were serum starved for 48 h. Cells were then fixed and stained for PCM1 and acetylated tubulin (cilia marker). Images represent cilia in cells taken with the same camera settings. Scale bar, 5 μm . **D.** FRAP analysis of ciliary GFP-CCDC66 in cells depleted for PCM1, CEP290 or CEP72. RPE1::GFP-CCDC66 cells were transfected with control, PCM1, CEP290 or CEP72 siRNAs. 24 h after transfection, they were serum starved for 48 h. Whole cilium and basal body regions indicated by yellow dashed rectangles were photobleached and cells were imaged for 250 seconds post-bleaching. Still images represent ciliary GFP-CCDC66 signal at indicated time points. Scale bar: 1 μm . **E.** Percentage of recovery graph of different ciliary regions from (D). Ciliary signals were quantified excluding the basal body area. Percentage of recovery graphs from two independent experiments were fitted into one phase association curves. $n=3$ for control, $n=5$ for PCM1, CEP290 and CEP72 depleted cells per group. **F to I.** Percentage of recovery graphs of individual ciliary GFP-CCDC66 FRAP experiments from Control (F), PCM1 (G), CEP290 (H), and siCEP72 (I) depleted and serum starved cells. Cells were imaged up to 840 seconds post-bleaching. Red curve represents the mean of all experiments.

3.4.5 Centriolar Satellites Exhibit Both Diffusive and Directional Motility

The directed motility of a small fraction of satellites towards and away from the centrosome *in vitro* and *in vivo* has been used as evidence to suggest that satellites regulate protein targeting by physically trafficking proteins along microtubules (Kubo et al., 1999). This trafficking model predicts directional movement to or from centrosomes at a rate similar to that of motor-mediated transport along microtubules. To determine whether satellite dynamics is consistent with this model, we quantitatively assayed their dynamic behavior by combining time-lapse confocal imaging of interphase RPE1::GFP-CCDC66 cells with single particle tracking algorithms that we developed based on previous studies (described in Materials and Methods) (Aydin et al., 2016; Crocker & Grier, 1996). We tracked a total of 144 satellites (n=4) and a representative cell indicating the satellites with their associated trajectories is shown in Figure 33A (Movie 2). This revealed different motility characteristics of satellites with varying degrees of persistence ratios (direct distance/total distance) where high persistence represents directional motility while low persistence represents random diffusion. We classified satellites into two groups - color-coded magenta and green - based on their average persistence ratio with a cutoff value of 0.5 (Figure 33A), and quantified the average velocity and instant speeds of each group, as well as analyzed their directionality towards or from the centrosome.

The first group of satellites represented only about 14% of the total population (20 of 144 magenta trajectories in Figure 33A). The histogram distribution of the persistence ratio of satellites showed two peaks with mean values of 0.35 and 0.79. This indicates that this group has bimodal motility, alternating between long range, directional motility (persistence>0.5) and non-directional Brownian-type diffusion (persistence<0.5) (Figure 33B). The cutoff value for this classification was determined based on simulations of different motility modes ranging from low to high persistence (Figure 34). Moreover, the calculation of their persistence ratio with a time difference of 8 ΔT and 12 ΔT both yielded similar distributions (Figure 34). Their direct displacement value was $2.86 \mu\text{m} \pm 0.55$ and mean average velocity was $0.39 \mu\text{m}/\text{sec} \pm 0.07$ (mean $\pm$ SEM) (Figure 33C). Given that the directional motility of satellites was interrupted with stationary pauses, the average speed values underestimated the speeds of long-range satellite movements. To determine whether speeds of satellites with long-range motility are compatible with the ones reported for molecular motors, we determined the instant speeds of satellites in consecutive frames. Only the persistent

satellites (magenta-colored) had broad distribution at instant speeds higher than $0.5 \mu\text{m}/\text{sec}$ (Figure 33D insets), which were consistent with the *in vivo* speeds reported for dynein and kinesins (Cai et al., 2009; Flores-Rodriguez et al., 2011). The rest of satellites had instant speeds slower than $0.5 \mu\text{m}/\text{sec}$, representing the diffusive phases (Figure 33D). One of the key predictions of the trafficking model is the directional motility of satellites towards or away from the centrosome. To test this, we quantified the difference between the distance of each satellite from the centrosome in consecutive frames (Figure 33E). The occurrence of the negative and positive values supports directional movement towards the centrosome and away from the centrosome, respectively.



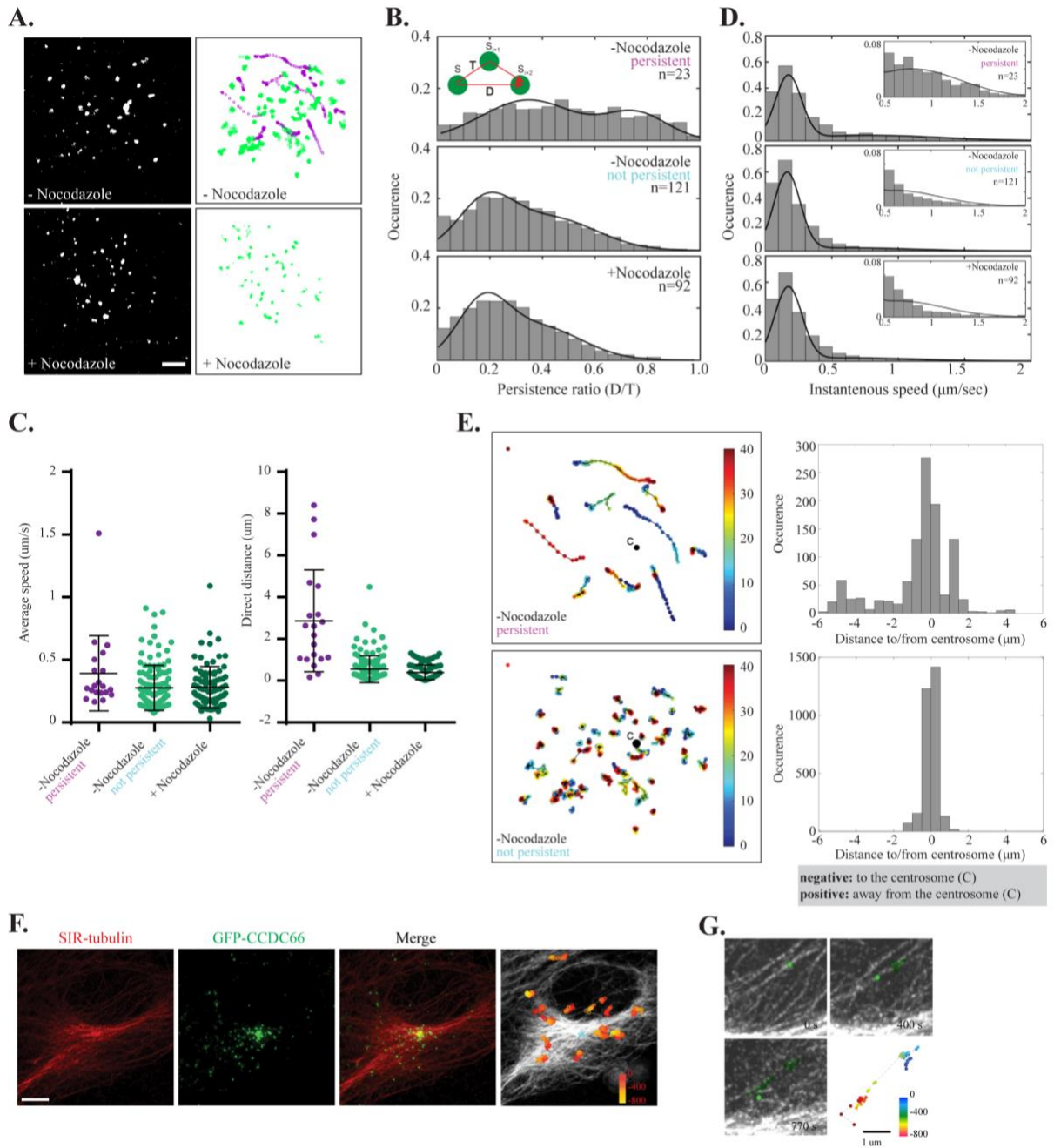


Figure 33. CCDC66-positive centriolar satellites exhibit both diffusive and microtubule-mediated directional motility

A. Representative fluorescence images of satellites from time-lapse videos of RPE1::GFP-CCDC66 cells (top-left and bottom-left panels) and corresponding trajectories of satellites as a function of time (top-right and bottom-right panels). CCDC66-positive satellites were identified and tracked using the single-particle tracking algorithms detailed in Materials and Methods. Satellites were classified into persistent (magenta) and diffusive (green) motility groups using a

persistence ratio cutoff value of 0.5. **B.** The distribution of the persistence ratio (direct distance(D)/total distance(T)) was used to determine the different motility groups. Persistence histogram (gray bars) were fitted with a single or double Gaussian function (black line). **C.** Average speed and direct distance of satellites. **D.** The distribution of satellite instant speed. The changes at higher instant speed values were shown in the inset. **E.** Time-colored trajectories of persistent (top-left) and diffusive (bottom-left) satellites and analysis of their directionality to the centrosome (right-hand graphs). Corresponding distribution of distance from/to centroids were plotted to determine the directed motility of satellites. Centroids were marked as C, indicating the localization of the centrosome. Negative values indicate movement towards the centrosome and positive values indicate movement away from the centrosome. **F.** A representative fluorescence image of RPE1::GFP-CCDC66 cells stained with SIR-Tubulin. Images were overlaid to determine microtubule mediated movement of satellite that move persistently. The right-hand panel indicates the time dependent movement of satellites. **G.** Representative satellite (green) exhibits bimodal motility by alternating between persistent and diffusive movements. Still images from Movie 6 at the indicated time points were shown. The bottom right-hand panel indicates the time dependent movement of the satellite.

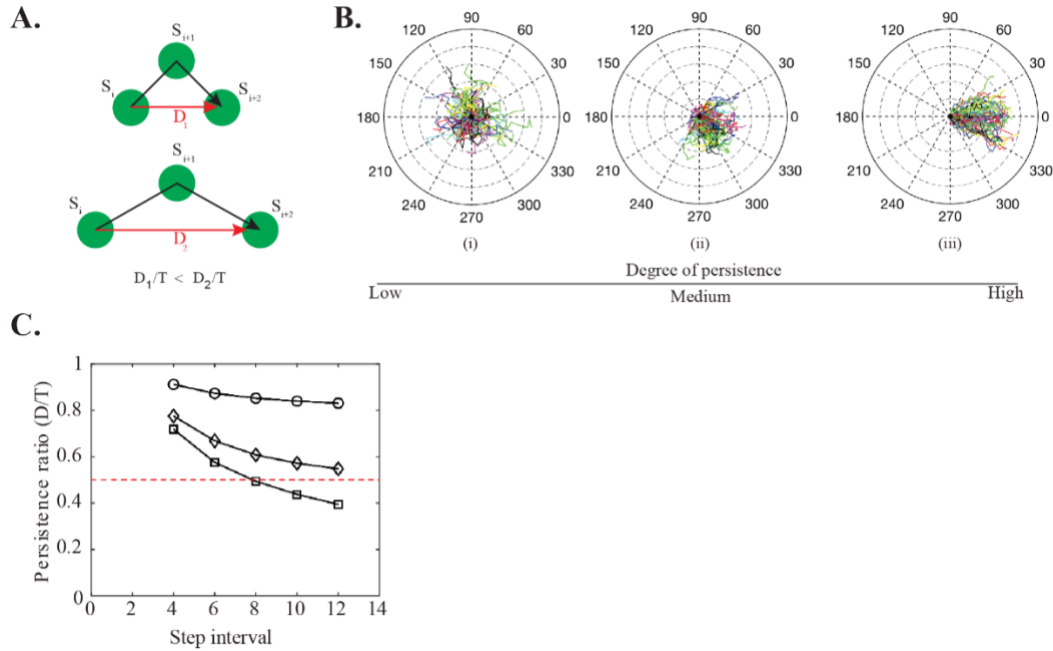


Figure 34. Simulation of motility patterns observed for CCDC66 and PCM1 satellites.

A. Schematic depiction of persistent movement by measuring direct and total distance of satellites. Higher values of direct distance results in persistent movement. **B.** Motility models that describe low, medium and high degree of persistent were shown in polar plots. All simulated trajectories were originated from origin. **C.** Computation of persistence ratio as a function of step interval for direct distance. A red horizontal line marks the cutoff value for persistent and diffusive motility.

However, the majority of the satellites (124 of 144 or $\approx 86\%$ of the total population, green trajectories in Figure 33A) instead had a low persistent ratio with a single peak at 0.2 (Figure 33B). This is indicative of short-range diffusive motility, and is inconsistent with a trafficking function for this population of satellites. They had a direct displacement value of $0.54 \mu\text{m} \pm 0.06$ and mean average velocity of $0.27 \mu\text{m}/\text{sec} \pm 0.02$ (mean $\pm$ SEM) (Figure 33C). In contrast to the persistent satellites, they did not have a group of satellites with instant speeds higher than $0.5 \mu\text{m}/\text{sec}$ (Figure 33D). This high occurrence of diffusive motility suggests that only a small fraction of satellites might mediate trafficking of cargo and the remaining population might mediate sequestration. In addition to satellite with diffusive and long-range motility, we observed fusion and splitting events between satellites and the centrosome, as well as between individual satellites (Figure 35, Movies 3-5). This behavior is reminiscent of liquid-like structures (Banani et al., 2017).

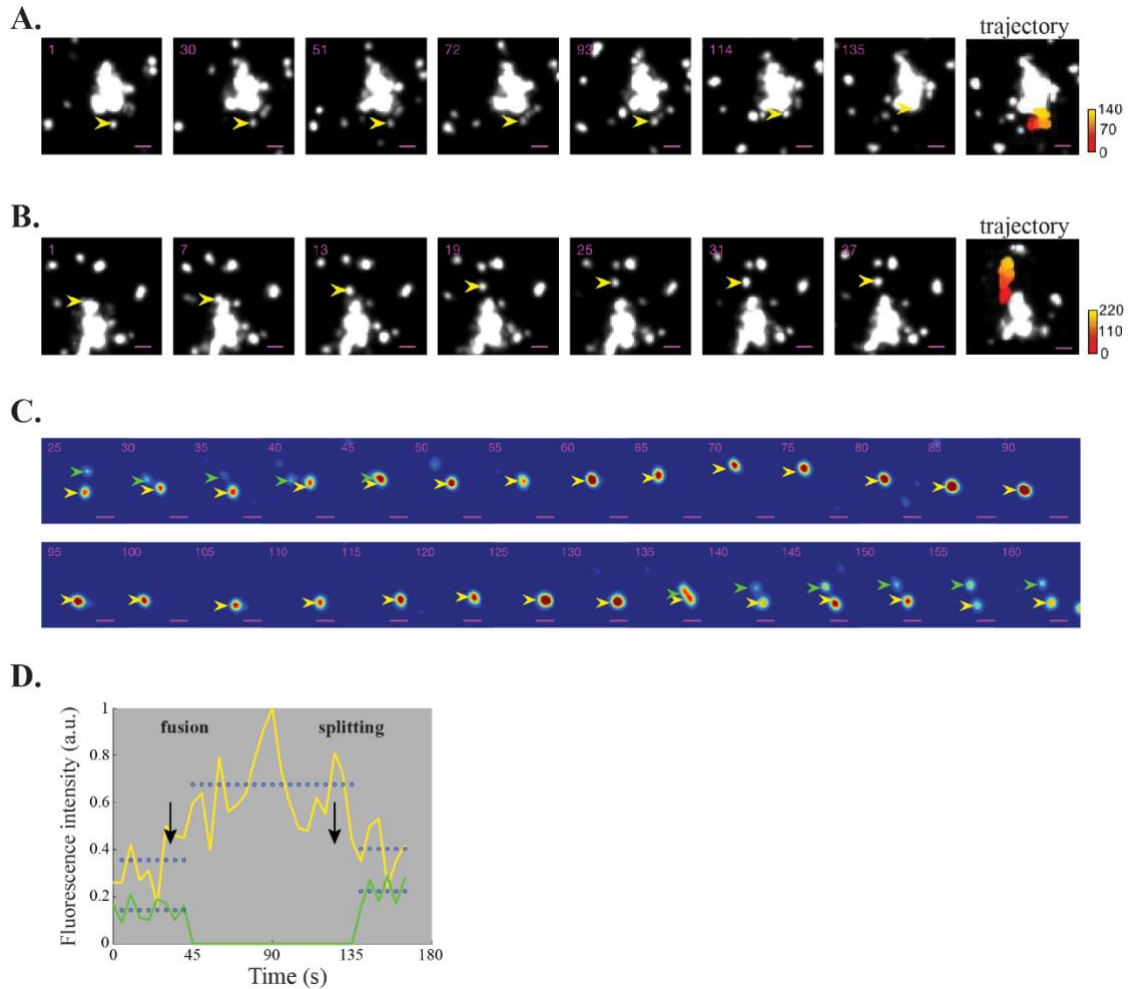


Figure 35. GFP-CCDC66 exhibits fusion and splitting with centrosome and other satellites.

A. Time-lapse fluorescence images (1.43 s/frame) showing GFP-CCDC66 fused with the centrosome. Frame time (seconds) is shown at the top of each image. Time-colored trajectory of a satellite that approaches to the centrosome (right panel). Scale bar= 0.75 μ m. **B.** Time-lapse fluorescence images (1.43 s/frame) showing GFP-CCDC66 splitted from the centrosome. Frame time (seconds) is shown at the top of each image. Time-colored trajectory of a satellite that moves away from the centrosome (right panel). Scale bar= 0.75 μ m. **C.** Representative time-lapse images (5.0 s/frame) showing the fusion and splitting events between GFP-CCDC66-positive satellites (arrowheads). Colors denote the changes of their fluorescence intensity before and after fusion. Frame time (seconds) is shown at the top of each image. Scale bar= 0.3 μ m. **D.** Quantification of fluorescence intensity changes of satellites shown in C. Fusion of satellites resulted in an increase of signal intensity at 45 s and remained at this level until the complex split at 135 s.

3.4.6 The Directed Motility and Random Movement of Satellites are Mediated by Microtubules

The linear directed motility exhibited by a small subset of satellites and the speed of their motility is reminiscent of molecular motor-mediated transport along microtubules. To test this, we investigated the effects of microtubule depolymerization on satellite dynamics. In nocodazole-treated cells, we did not observe any satellites with long, directional motility, confirming the requirement for microtubules for this type of motility (n=92 satellites in 4 cells) (Movie 6). Instead, satellites had randomized Brownian-type movement, with a low persistent ratio of 0.20, direct displacement value of $0.39 \mu\text{m} \pm 0.04$ and mean average speed of $0.28 \pm 0.02 \mu\text{m}/\text{sec}$ (mean $\pm$ SEM) (Figure 33A-D). To visualize the movement of satellites along microtubules, we performed time-lapse imaging of RPE1::GFP-CCDC66 cells stained with SIR-tubulin that labels microtubules (Figure 33F and 33G). We tracked a subset of CCDC66-positive satellites moving along microtubules interrupted with pauses and showed the color-coded trajectories of satellites with high persistence in superimposed images (Figure 33F) (Movie 7). The orientation of the microtubules strongly aligned with the trajectory of satellites and some of them moved directionally towards or away from the centrosome (Figure 33G). Together, our data confirms the requirement for microtubules for the persistent directional motility of satellites, which occurs in a subset of the population.

There is heterogeneity in the composition of individual satellites, and CCDC66 localizes only to a subset of satellites (D. Conkar et al., 2017; Firat-Karalar, Sante, et al., 2014; G. D. Gupta et al., 2015). To investigate whether the dynamics of CCDC66-positive satellites are representative of all satellites in cells, we performed time-lapse imaging experiments in HeLa Kyoto cells stably expressing GFP-PCM1 from a bacterial artificial chromosome (BAC) transgene, which was recently used to study the function of FGFR1 oncogene partner (FOP) on satellite dynamics (Cabaud et al., 2018; Poser et al., 2008). Representative satellites and their trajectories are shown in Figure 36A (Movie 8). Analogous to CCDC66, the majority of PCM1-positive granules (113 of 150 or $\approx 76\%$ of the total population, cyan trajectories) had diffusive motility with an average speed of $0.48 \mu\text{m}/\text{sec} \pm 0.02$, a direct displacement value of $0.75 \mu\text{m} \pm 0.06$ and a persistence ratio with a single peak at 0.2 (mean $\pm$ SEM) (Figure 36B-D). A smaller fraction (37 of 150 or $\approx 24\%$ of the total population, magenta trajectories) had a bimodal persistence ratio distribution of two peaks with mean values of 0.29 and 0.72 (Figure 36B). Their mean average velocity was $0.74 \mu\text{m}/\text{sec} \pm 0.06$, direct displacement value was $2.9 \mu\text{m} \pm 0.41$ (mean $\pm$ SEM) (Figure 36C), and a percentage of them moved towards or away from the centrosome (Figure 36E). Upon nocodazole treatment, satellites exhibited Brownian-type diffusive motility, with a low persistent ratio of 0.18, direct displacement value of $0.73 \mu\text{m} \pm 0.06$ and mean average speed of $0.68 \mu\text{m}/\text{sec} \pm 0.04$ (n=136) (mean $\pm$ SEM) (Figure 36A-D) (Movie 9). Collectively, these results suggest that the heterogeneity in the composition of satellites is not reflected by changes in their dynamic behavior and in different cell types.

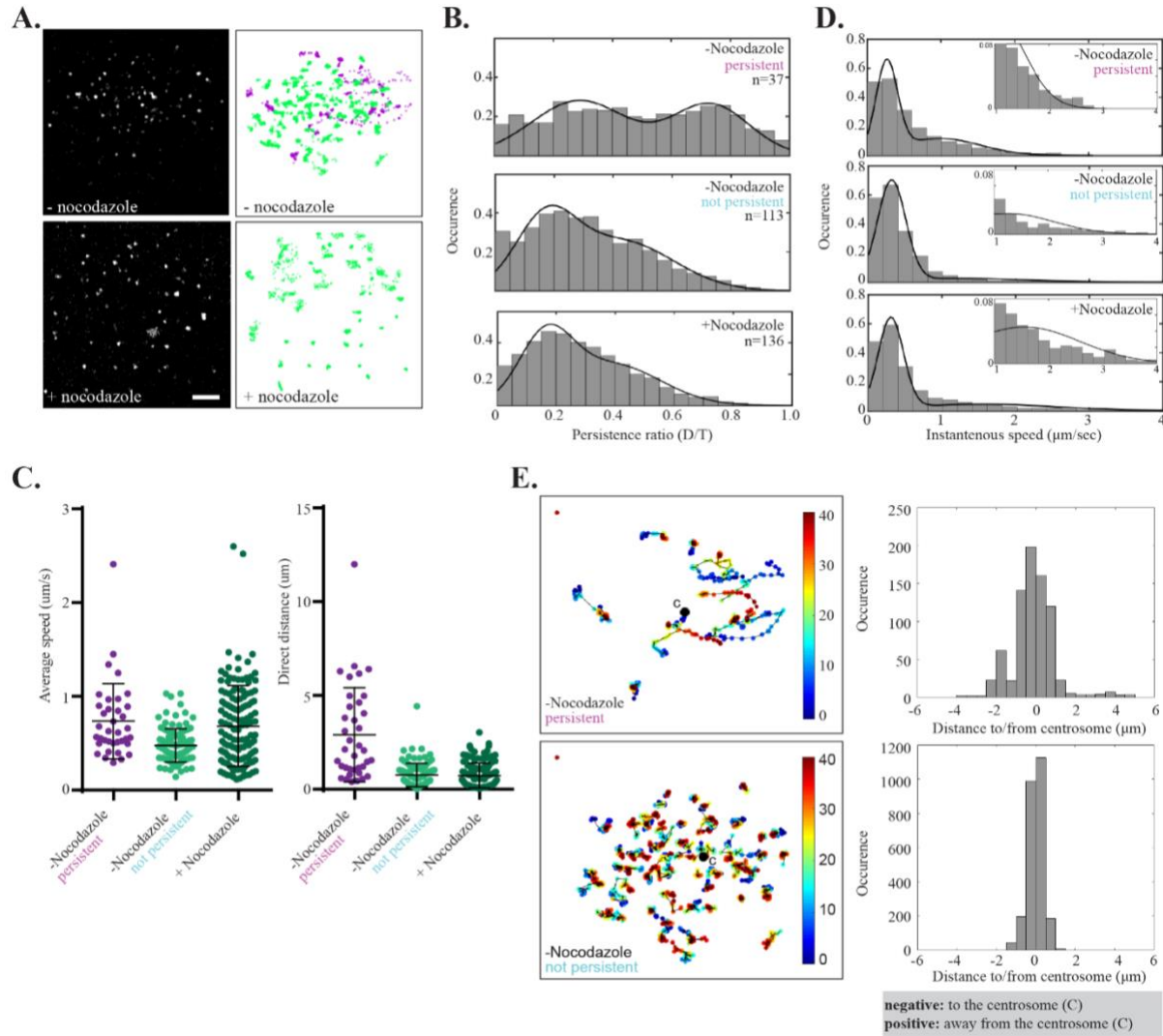


Figure 36. PCM1-positive centriolar satellites exhibit both diffusive and microtubule-mediated directional motility

A. Representative fluorescence images of satellites from time-lapse videos of HeLa::GFP-PCM1 cells (top-left and bottom-left panels) and corresponding trajectories of satellites as a function of time (top-right and bottom-right panels). PCM1-positive satellites were identified and tracked using the single-particle tracking algorithms detailed in Materials and Methods. Satellites were classified into persistent (magenta) and diffusive (green) motility groups using a persistence ratio cutoff value of 0.5. **B.** The distribution of the persistence ratio was used to determine the different motility groups. Persistence histogram (gray bars) were fitted with a single or double Gaussian function (black line). **C.** Average speed and direct distance of satellites. **D.** The distribution of

satellite instant speed. The changes at higher instant speed values were shown in the inset. **E.** Time-colored trajectories of persistent (top-left) and diffusive (bottom-left) satellites and analysis of their directionality to the centrosome (right-hand graphs). Corresponding distribution of distance from/to centroids were plotted to determine the directed motility of satellites. Centroids were marked as C, indicating the localization of the centrosome. Negative values indicate movement towards the centrosome and positive values indicate movement away from the centrosome.



3.5 Discussion

The dynamic alterations in the biogenesis, maintenance and function of the centrosome/cilium complex in response to physiological stimuli requires tight spatiotemporal control of the identity, quantity and modifications of the resident proteins. Defects in this regulation cause diseases like ciliopathies. In this study, we quantitatively assayed the dynamic behavior of CCDC66 at its different cellular locations, and elucidated the interplay between microtubules, motors, and satellites in regulating the targeting of CCDC66 to centrosomes and cilia. Since defective targeting of proteins to centrosomes and cilia contribute to disease phenotypes of ciliopathies, our results provide insight into cellular defects associated with retinal degeneration.

Comparative analysis of CCDC66 at its specific subcellular locations revealed different dynamics at these sites, likely indicative of the different functions of these compartments. Centrosomal CCDC66 was highly mobile with fast recovery time, which contrasts with the relatively immobile structural components of the centrosome, like centrin-2 (Hames et al., 2005; Mahjoub et al., 2010). This dynamic exchange of CCDC66 between the centrosome and cytoplasm might be important for its ciliary and/or mitotic functions, which awaits further investigation. In contrast, ciliary CCDC66 was relatively immobile. Given that ciliary receptors and transport complexes studied so far including HTR6, SSTR3, IFT88 and BBS4 have fast ciliary dynamics (Q. Hu et al., 2010), we propose that CCDC66 may function as a structural component of the axoneme at the cilium, likely through its direct microtubule binding affinity. Interestingly, our STED analysis revealed that CCDC66 localized along the axoneme in a punctate pattern with irregular spacing, in contrast to the rather homogenous localization of acetylated tubulin, which may be linked to its function.

Although microtubules, molecular motors, and centriolar satellites all regulate protein targeting to the centrosomes, the interplay between them is poorly understood. We found that while microtubule organization and molecular motors were required for CCDC66 recruitment and kinetics at the centrosome, satellites inhibited it. We propose that microtubule dependent CCDC66 targeting to the centrosome is mediated by two complementary mechanisms, which together will facilitate timely and rapid protein targeting. First, CCDC66 is actively transported to the centrosome along microtubules in a dynein-dependent manner, either by directly interacting with microtubules or indirectly by PCM1-negative protein complexes. Active transport mechanisms

were previously reported for other centrosome proteins including CDK5RAP2 (Jia et al., 2013; S. Lee & Rhee, 2010) and Par6alpha (Kodani et al., 2010). Second, microtubule ends and dynein complex components like p150^{glued} at the centrosome provide binding sites for CCDC66 through protein-protein interactions. This mechanism is supported by the interactions we identified for CCDC66 with microtubules and dynein complex components.

Previous studies identified requirements for centriolar satellites in centrosomal targeting of various proteins including centrin and pericentrin (Dammermann & Merdes, 2002; Hori et al., 2016; Kubo et al., 1999). In contrast, we identified inhibitory functions for satellites in regulating centrosomal CCDC66 abundance and kinetics. This inhibition might be explained by their function as sequestration sites to limit CCDC66 centrosomal recruitment, which was previously also suggested but not tested, for regulation of the ubiquitin ligase Mib1 (W. J. Wang et al., 2013). Loss of satellites likely results in an increase in the cytoplasmic soluble pool of CCDC66, which will be available for incorporation into the centrosome. An alternative explanation could be satellite-mediated removal of CCDC66 from the centrosome. Although our quantitation of satellite dynamics identified satellites with persistent motility away from the centrosomes, these events were infrequent. We would also like to highlight that satellite components CEP72 and CEP290 both had inhibitory roles in CCDC66 centrosomal targeting, like PCM1. This is interesting as depletion of these proteins result in tighter clustering of satellites around the centrosome, which is a very different phenotype to the loss of satellites observed in PCM1-depleted cells (T. R. Stowe et al., 2012). Given that CCDC66 interacts with CEP72 and CEP290, this result might be explained by putative functions for CEP72 and CEP290 in mediating the interaction between PCM1 and CCDC66 (D. Conkar et al., 2017).

The trafficking model for centriolar satellites was based on directed motility reported for a subset of satellites and changes in the centrosomal levels of various proteins in satellite-less cells (Cabaud et al., 2018; Dammermann & Merdes, 2002; Hori & Toda, 2017; Kubo et al., 1999; L. Wang et al., 2016). The lack of quantitative analysis of satellite dynamics in cells in an unbiased way has made this model incomplete. In this study, we quantified the persistence, mean and instantaneous speeds and directionality of CCDC66- and PCM1-positive satellites using live imaging combined with single particle tracking algorithms. Our analysis represents the first systematic quantitative characterization of the dynamic behavior of satellites and provides insight

into their molecular mechanism of action. Supporting the trafficking function of satellites, we identified a subset of them to exhibit bimodal motility characterized by fast processive long-range motility towards or away from the centrosomes interrupted by pauses. However, these events were very infrequent, and the majority of satellites displayed short-range diffusive motility. Although previous studies described the bimodal motility for satellites qualitatively, the predominant diffusive behavior of satellites has not been reported (Cabaud et al., 2018; Hall et al., 2013; Kubo et al., 1999). This result has important implications for our understanding of satellite mechanisms. For example, satellite dynamics in specific contexts may be linked to specific cell types and functions or to different signals received by cells. Additionally, satellites might exert different modes of regulation for different centrosome and cilium proteins.

The higher ratio of non-directional diffusive satellites relative to directional ones is analogous to the dynamic behavior of other microtubule-associated cargoes such as autophagosomes, endosomes and peroxisomes (Arnette et al., 2016). However, only satellites cluster around the centrosome, and the molecular basis and functional significance of this clustering is not known. Various minus and plus end-directed molecular motors including BICD1, BICD2, DYNLL1, KIF7, KIF14, KIF20A were identified as components of satellites in proteomics studies (G. D. Gupta et al., 2015; Hein et al., 2015). The clustered satellite organization is likely a consequence of the minus end-directed motors dominating over plus end-directed motors during the tug of war between them. The binding affinities of satellites to different motors and the adaptors that mediate these interactions should be identified to elucidate how this tug of war ensures proximity of satellites to the centrosome.

Our findings reveal that satellites use different mechanisms to regulate protein targeting and that the interplay among these mechanisms change for different satellite-associated centrosome and cilium proteins (Figure 37). Alongside active transport along microtubules, we here demonstrate a “sequestration-based mechanism” for regulating CCDC66 centrosomal and ciliary abundance. Despite the presence of a small population of CCDC66-positive satellites with persistent directional motility towards or away from the centrosome, the net effect of this trafficking did not dominate over sequestration. We speculate that satellites store protein around the centrosome in order to ensure their timely and rapid incorporation at the centrosome and cilia when required. Satellite-mediated storage and trafficking of the proteins might be advantageous

for regulating protein targeting in response to physiological stimuli as it overcomes the limitations associated with lack of directionality in diffusion-based transport. An important next step in advancing this model will be the identification of the molecular basis of the release of proteins from the satellites, as well as the specific signals that initiates that release.

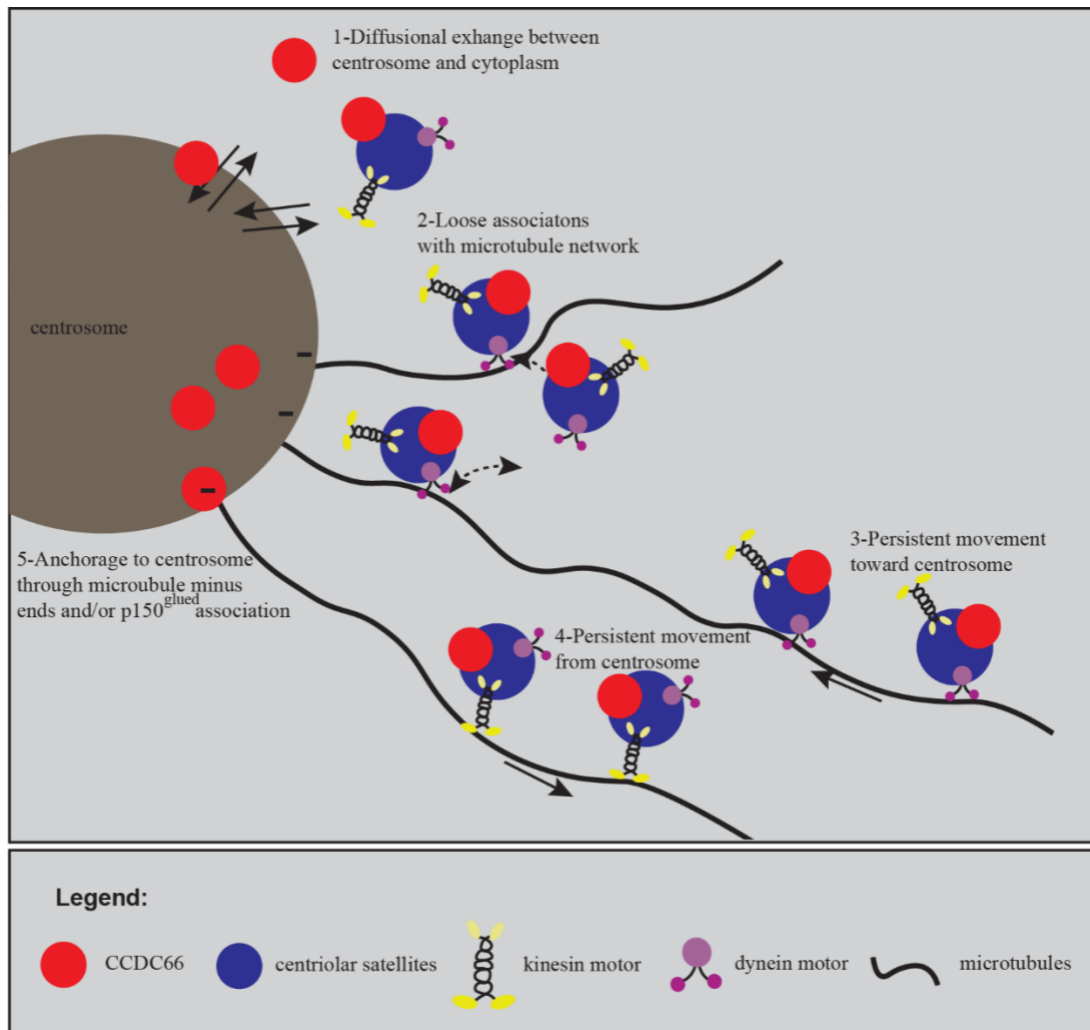


Figure 37. Different modes of CCDC66 targeting to the centrosome

1- Diffusional exchange between centrosome and cytoplasm: Cytoplasmic CCDC66 exchanges with its centrosomal pool by passive diffusion. The exchange could either be with the soluble cytoplasmic pool or with the pool associated with centriolar satellites. In the latter case, satellites act as storage sites.

2- Association and dissociation with microtubules: Satellites associate and dissociate with microtubules and the switch between these states in response to signals might regulate the interplay between diffusive and persistent motilities.

3-4 Persistent, directional movement toward and away from centrosome: Centriolar satellites move on microtubules towards the centrosome in a dynein-dependent manner and away from the centrosome in a kinesin-dependent manner. This pool comprises a small percentage within the total satellite population.

5- Anchorage to centrosome through microtubule minus end and/or p150^{glued} association: Microtubule ends and/or microtubule associated proteins like p150^{glued} might provide binding sites for CCDC66 at the centrosome.

Finally, we anticipate our findings to provide important insight into our understanding of cellular compartmentalization through membrane-less structures. A major difference of satellites from most microtubule-associated cargoes is their membrane-less nature and consequential liquid-like behavior (Rai et al., 2018). Importantly, satellites are regulators of the biogenesis and function of centrosomes, another membrane-less condensate (Woodruff et al., 2017). Consistent with the liquid-like behavior of satellites, we observed fusion and splitting events between satellites and the centrosome, as well as between individual satellites (Figure 35). These events were more frequent around the centrosome where satellites clustered and phase-separation might be a way for satellites to exchange material with the centrosome (Banani et al., 2017). The trafficking and storage-mediated functions of satellites identify them as likely transit sites for centrosome and cilium proteins. This is analogous to the roles of the vesicular trafficking pathway in storing and targeting their cargoes. Satellites may represent a novel way of mediating communication between membrane-less compartments. Defining the full repertoire of satellite interactions, functions and mechanisms will be an important next step in testing this model.

Bibliography

Adams, G. M., Huang, B., & Luck, D. J. (1982). Temperature-Sensitive, Assembly-Defective Flagella Mutants of CHLAMYDOMONAS REINHARDTII. *Genetics*, 100(4), 579–586.

Adams, N. A., Awadein, A., & Toma, H. S. (2007). The retinal ciliopathies. *Ophthalmic Genet*, 28(3), 113–125. <https://doi.org/782473199> [pii] 10.1080/13816810701537424

Andersen, J. S., Wilkinson, C. J., Mayor, T., Mortensen, P., Nigg, E. A., & Mann, M. (2003). Proteomic characterization of the human centrosome by protein correlation profiling. *Nature*, 426(6966), 570–574. <https://doi.org/10.1038/nature02166> nature02166 [pii]

Anderson, R. G. (1972). The three-dimensional structure of the basal body from the rhesus monkey oviduct. *The Journal of Cell Biology*, 54(2), 246–265. <https://doi.org/10.1083/jcb.54.2.246>

Arnette, C., Frye, K., & Kaverina, I. (2016). Microtubule and Actin Interplay Drive Intracellular c-Src Trafficking. *PLoS One*, 11(2), e0148996. <https://doi.org/10.1371/journal.pone.0148996>

Avidor-Reiss, T., & Gopalakrishnan, J. (2013). Cell Cycle Regulation of the Centrosome and Cilium. *Drug Discov Today Dis Mech*, 10(3–4), e119–e124. <https://doi.org/10.1016/j.ddmec.2013.03.002>

Avidor-Reiss, T., Maer, A. M., Koundakjian, E., Polyanovsky, A., Keil, T., Subramaniam, S., & Zuker, C. S. (2004). Decoding cilia function: Defining specialized genes required for compartmentalized cilia biogenesis. *Cell*, 117(4), 527–539. <https://doi.org/S009286740400412X> [pii]

Avidor-Reiss, Tomer, Maer, A. M., Koundakjian, E., Polyanovsky, A., Keil, T., Subramaniam, S., & Zuker, C. S. (2004). Decoding cilia function: Defining specialized genes required for compartmentalized cilia biogenesis. *Cell*, 117(4), 527–539. [https://doi.org/10.1016/s0092-8674\(04\)00412-x](https://doi.org/10.1016/s0092-8674(04)00412-x)

Aydin, O., Aksoy, B., Akalin, O. B., Bayraktar, H., & Alaca, B. E. (2016). Time-resolved local strain tracking microscopy for cell mechanics. *Rev Sci Instrum*, 87(2), 023905. <https://doi.org/10.1063/1.4941715>

Badgandi, H. B., Hwang, S.-H., Shimada, I. S., Lorient, E., & Mukhopadhyay, S. (2017). Tubby family proteins are adapters for ciliary trafficking of integral membrane proteins. *The Journal of Cell Biology*, 216(3), 743–760. <https://doi.org/10.1083/jcb.201607095>

Banani, S. F., Lee, H. O., Hyman, A. A., & Rosen, M. K. (2017). Biomolecular condensates: Organizers of cellular biochemistry. *Nat Rev Mol Cell Biol*, 18(5), 285–298. <https://doi.org/10.1038/nrm.2017.7>

Bangs, F., & Anderson, K. V. (2017). Primary Cilia and Mammalian Hedgehog Signaling. *Cold Spring Harbor Perspectives in Biology*, 9(5). <https://doi.org/10.1101/cshperspect.a028175>

- Barenz, F., Mayilo, D., & Gruss, O. J. (2011). Centriolar satellites: Busy orbits around the centrosome. *Eur J Cell Biol*, 90(12), 983–989. <https://doi.org/10.1016/j.ejcb.2011.07.007> S0171-9335(11)00134-8 [pii]
- Berbari, N. F., O'Connor, A. K., Haycraft, C. J., & Yoder, B. K. (2009). The primary cilium as a complex signaling center. *Curr Biol*, 19(13), R526-35. <https://doi.org/10.1016/j.cub.2009.05.025> S0960-9822(09)01126-9 [pii]
- Berbari, Nicolas F., Lewis, J. S., Bishop, G. A., Askwith, C. C., & Mykityn, K. (2008). Bardet-Biedl syndrome proteins are required for the localization of G protein-coupled receptors to primary cilia. *Proceedings of the National Academy of Sciences of the United States of America*, 105(11), 4242–4246. <https://doi.org/10.1073/pnas.0711027105>
- Bergman, K., Goodenough, U. W., Goodenough, D. A., Jawitz, J., & Martin, H. (1975). Gametic differentiation in *Chlamydomonas reinhardtii*. II. Flagellar membranes and the agglutination reaction. *The Journal of Cell Biology*, 67(3), 606–622. <https://doi.org/10.1083/jcb.67.3.606>
- Bertelli, E., & Regoli, M. (1994). A morphological study of the primary cilia in the rat pancreatic ductal system: Ultrastructural features and variability. *Acta Anatomica*, 151(3), 194–197. <https://doi.org/10.1159/000147663>
- Betleja, E., & Cole, D. G. (2010). Ciliary trafficking: CEP290 guards a gated community. *Curr Biol*, 20(21), R928-31. <https://doi.org/10.1016/j.cub.2010.09.058> S0960-9822(10)01215-7 [pii]
- Bettencourt-Dias, M., Hildebrandt, F., Pellman, D., Woods, G., & Godinho, S. A. (2011). Centrosomes and cilia in human disease. *Trends Genet*, 27(8), 307–315. <https://doi.org/10.1016/j.tig.2011.05.004> S0168-9525(11)00065-5 [pii]
- Bettencourt-Dias, Mónica, Hildebrandt, F., Pellman, D., Woods, G., & Godinho, S. A. (2011). Centrosomes and cilia in human disease. *Trends in Genetics: TIG*, 27(8), 307–315. <https://doi.org/10.1016/j.tig.2011.05.004>
- Blacque, O. E., & Sanders, A. A. (2014). Compartments within a compartment: What *C. elegans* can tell us about ciliary subdomain composition, biogenesis, function, and disease. *Organogenesis*, 10(1), 126–137. <https://doi.org/10.4161/org.28830>
- Blacque, Oliver E., Perens, E. A., Boroevich, K. A., Inglis, P. N., Li, C., Warner, A., Khattra, J., Holt, R. A., Ou, G., Mah, A. K., McKay, S. J., Huang, P., Swoboda, P., Jones, S. J. M., Marra, M. A., Baillie, D. L., Moerman, D. G., Shaham, S., & Leroux, M. R. (2005). Functional genomics of the cilium, a sensory organelle. *Current Biology: CB*, 15(10), 935–941. <https://doi.org/10.1016/j.cub.2005.04.059>
- Bornens, M. (2002). Centrosome composition and microtubule anchoring mechanisms. *Curr Opin Cell Biol*, 14(1), 25–34.
- Bornens, Michel. (2002). Centrosome composition and microtubule anchoring mechanisms. *Current Opinion in Cell Biology*, 14(1), 25–34. [https://doi.org/10.1016/s0955-0674\(01\)00290-3](https://doi.org/10.1016/s0955-0674(01)00290-3)
- Braun, D. A., & Hildebrandt, F. (2017). Ciliopathies. *Cold Spring Harb Perspect Biol*, 9(3).

<https://doi.org/10.1101/cshperspect.a028191>

Breslow, D. K., Hoogendoorn, S., Kopp, A. R., Morgens, D. W., Vu, B. K., Kennedy, M. C., Han, K., Li, A., Hess, G. T., Bassik, M. C., Chen, J. K., & Nachury, M. V. (2018). A CRISPR-based screen for Hedgehog signaling provides insights into ciliary function and ciliopathies. *Nature Genetics*, 50(3), 460–471. <https://doi.org/10.1038/s41588-018-0054-7>

Broadhead, R., Dawe, H. R., Farr, H., Griffiths, S., Hart, S. R., Portman, N., Shaw, M. K., Ginger, M. L., Gaskell, S. J., McKean, P. G., & Gull, K. (2006). Flagellar motility is required for the viability of the bloodstream trypanosome. *Nature*, 440(7081), 224–227. <https://doi.org/10.1038/nature04541>

Cabaud, O., Roubin, R., Comte, A., Bascunana, V., Serge, A., Sedjai, F., Birnbaum, D., Rosnet, O., & Acquaviva, C. (2018). Mutation of FOP/FGFR1OP in mice recapitulates human short rib-polydactyly ciliopathy. *Hum Mol Genet*, 27(19), 3377–3391. <https://doi.org/10.1093/hmg/ddy246>

Cai, D., McEwen, D. P., Martens, J. R., Meyhofer, E., & Verhey, K. J. (2009). Single molecule imaging reveals differences in microtubule track selection between Kinesin motors. *PLoS Biol*, 7(10), e1000216. <https://doi.org/10.1371/journal.pbio.1000216>

Carvalho-Santos, Z., Machado, P., Branco, P., Tavares-Cadete, F., Rodrigues-Martins, A., Pereira-Leal, J. B., & Bettencourt-Dias, M. (2010). Stepwise evolution of the centriole-assembly pathway. *Journal of Cell Science*, 123(Pt 9), 1414–1426. <https://doi.org/10.1242/jcs.064931>

Chang, B., Khanna, H., Hawes, N., Jimeno, D., He, S., Lillo, C., Parapuram, S. K., Cheng, H., Scott, A., Hurd, R. E., Sayer, J. A., Otto, E. A., Attanasio, M., O'Toole, J. F., Jin, G., Shou, C., Hildebrandt, F., Williams, D. S., Heckenlively, J. R., & Swaroop, A. (2006). In-frame deletion in a novel centrosomal/ciliary protein CEP290/NPHP6 perturbs its interaction with RPGR and results in early-onset retinal degeneration in the rd16 mouse. *Hum Mol Genet*, 15(11), 1847–1857. <https://doi.org/ddl107> [pii] 10.1093/hmg/ddl107

Chen, C. T., Hehnly, H., Yu, Q., Farkas, D., Zheng, G., Redick, S. D., Hung, H. F., Samtani, R., Jurczyk, A., Akbarian, S., Wise, C., Jackson, A., Bober, M., Guo, Y., Lo, C., & Doxsey, S. (2014). A unique set of centrosome proteins requires pericentrin for spindle-pole localization and spindle orientation. *Curr Biol*, 24(19), 2327–2334. <https://doi.org/10.1016/j.cub.2014.08.029>

Chen, C.-L., & Perrimon, N. (2017). Proximity-dependent labeling methods for proteomic profiling in living cells. *Wiley Interdisciplinary Reviews. Developmental Biology*, 6(4). <https://doi.org/10.1002/wdev.272>

Chih, B., Liu, P., Chinn, Y., Chalouni, C., Komuves, L. G., Hass, P. E., Sandoval, W., & Peterson, A. S. (2011). A ciliopathy complex at the transition zone protects the cilia as a privileged membrane domain. *Nature Cell Biology*, 14(1), 61–72. <https://doi.org/10.1038/ncb2410>

Conkar, D., Culfa, E., Odabasi, E., Rauniyar, N., Yates, J. R., & Firat-Karalar, E. N. (2017). Centriolar satellite protein CCDC66 interacts with CEP290 and functions in cilium formation and trafficking. *J Cell Sci*. <https://doi.org/10.1242/jcs.196832>

- Conkar, Deniz, Culfa, E., Odabasi, E., Rauniyar, N., Yates, J. R., & Firat-Karalar, E. N. (2017). The centriolar satellite protein CCDC66 interacts with CEP290 and functions in cilium formation and trafficking. *Journal of Cell Science*, 130(8), 1450–1462. <https://doi.org/10.1242/jcs.196832>
- Crocker, J. C., & Grier, D. G. (1996). Methods of digital video microscopy for colloidal studies. *Journal of Colloid and Interface Science*, 179(1), 298–310. <https://doi.org/DOI10.1006/jcis.1996.0217>
- Dammermann, A., & Merdes, A. (2002). Assembly of centrosomal proteins and microtubule organization depends on PCM-1. *J Cell Biol*, 159(2), 255–266. <https://doi.org/10.1083/jcb.200204023> jcb.200204023 [pii]
- De Brabander, M., Geuens, G., Nuydens, R., Willebrords, R., Aerts, F., & De Mey, J. (1986). Microtubule dynamics during the cell cycle: The effects of taxol and nocodazole on the microtubule system of Pt K2 cells at different stages of the mitotic cycle. *Int Rev Cytol*, 101, 215–274.
- Dean, S., Moreira-Leite, F., Varga, V., & Gull, K. (2016). Cilium transition zone proteome reveals compartmentalization and differential dynamics of ciliopathy complexes. *Proceedings of the National Academy of Sciences of the United States of America*, 113(35), E5135–5143. <https://doi.org/10.1073/pnas.1604258113>
- Deane, J. A., Cole, D. G., Seeley, E. S., Diener, D. R., & Rosenbaum, J. L. (2001). Localization of intraflagellar transport protein IFT52 identifies basal body transitional fibers as the docking site for IFT particles. *Current Biology: CB*, 11(20), 1586–1590. [https://doi.org/10.1016/s0960-9822\(01\)00484-5](https://doi.org/10.1016/s0960-9822(01)00484-5)
- Dekomien, G., Vollrath, C., Petrasch-Parwez, E., Boeve, M. H., Akkad, D. A., Gerding, W. M., & Epplen, J. T. (2010). Progressive retinal atrophy in Schapendoes dogs: Mutation of the newly identified CCDC66 gene. *Neurogenetics*, 11(2), 163–174. <https://doi.org/10.1007/s10048-009-0223-z>
- Diener, D. R., Lupetti, P., & Rosenbaum, J. L. (2015). Proteomic analysis of isolated ciliary transition zones reveals the presence of ESCRT proteins. *Current Biology: CB*, 25(3), 379–384. <https://doi.org/10.1016/j.cub.2014.11.066>
- Efimenko, E., Bubb, K., Mak, H. Y., Holzman, T., Leroux, M. R., Ruvkun, G., Thomas, J. H., & Swoboda, P. (2005). Analysis of xbx genes in *C. elegans*. *Development (Cambridge, England)*, 132(8), 1923–1934. <https://doi.org/10.1242/dev.01775>
- Field, M. C., & Carrington, M. (2009). The trypanosome flagellar pocket. *Nature Reviews. Microbiology*, 7(11), 775–786. <https://doi.org/10.1038/nrmicro2221>
- Firat-Karalar, E. N., Rauniyar, N., Yates, J. R., & Stearns, T. (2014). Proximity interactions among centrosome components identify regulators of centriole duplication. *Curr Biol*, 24(6), 664–670. <https://doi.org/10.1016/j.cub.2014.01.067> S0960-9822(14)00132-8 [pii]
- Firat-Karalar, E. N., Sante, J., Elliott, S., & Stearns, T. (2014). Proteomic analysis of mammalian

sperm cells identifies new components of the centrosome. *J Cell Sci.* <https://doi.org/jcs.157008> [pii] 10.1242/jcs.157008

Firat-Karalar, E. N., & Stearns, T. (2015). Probing mammalian centrosome structure using BioID proximity-dependent biotinylation. *Methods Cell Biol*, 129, 153–170. <https://doi.org/10.1016/bs.mcb.2015.03.016>

Fisch, C., & Dupuis-Williams, P. (2011). Ultrastructure of cilia and flagella—Back to the future! *Biology of the Cell*, 103(6), 249–270. <https://doi.org/10.1042/BC20100139>

Flores-Rodriguez, N., Rogers, S. S., Kenwright, D. A., Waigh, T. A., Woodman, P. G., & Allan, V. J. (2011). Roles of dynein and dynactin in early endosome dynamics revealed using automated tracking and global analysis. *PLoS One*, 6(9), e24479. <https://doi.org/10.1371/journal.pone.0024479>

Fogeron, M. L., Muller, H., Schade, S., Dreher, F., Lehmann, V., Kuhnel, A., Scholz, A. K., Kashofer, K., Zerck, A., Fauler, B., Lurz, R., Herwig, R., Zatloukal, K., Lehrach, H., Gobom, J., Nordhoff, E., & Lange, B. M. (2013). LGALS3BP regulates centriole biogenesis and centrosome hypertrophy in cancer cells. *Nat Commun*, 4, 1531. <https://doi.org/10.1038/ncomms2517>

Follit, J. A., Tuft, R. A., Fogarty, K. E., & Pazour, G. J. (2006). The Intraflagellar Transport Protein IFT20 Is Associated with the Golgi Complex and Is Required for Cilia Assembly. *Molecular Biology of the Cell*, 17, 12.

Fry, A. M., Meraldi, P., & Nigg, E. A. (1998). A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. *EMBO J*, 17(2), 470–481. <https://doi.org/10.1093/emboj/17.2.470>

Fry, A. M., Sampson, J., Shak, C., & Shackleton, S. (2017). Recent advances in pericentriolar material organization: Ordered layers and scaffolding gels. *F1000Res*, 6, 1622. <https://doi.org/10.12688/f1000research.11652.1>

Fu, W., Wang, L., Kim, S., Li, J., & Dynlacht, B. D. (2016). Role for the IFT-A Complex in Selective Transport to the Primary Cilium. *Cell Reports*, 17(6), 1505–1517. <https://doi.org/10.1016/j.celrep.2016.10.018>

Gaglio, T., Saredi, A., Bingham, J. B., Hasbani, M. J., Gill, S. R., Schroer, T. A., & Compton, D. A. (1996). Opposing motor activities are required for the organization of the mammalian mitotic spindle pole. *J Cell Biol*, 135(2), 399–414.

Garcia-Gonzalo, F. R., Corbit, K. C., Sirerol-Piquer, M. S., Ramaswami, G., Otto, E. A., Noriega, T. R., Seol, A. D., Robinson, J. F., Bennett, C. L., Josifova, D. J., García-Verdugo, J. M., Katsanis, N., Hildebrandt, F., & Reiter, J. F. (2011). A transition zone complex regulates mammalian ciliogenesis and ciliary membrane composition. *Nature Genetics*, 43(8), 776–784. <https://doi.org/10.1038/ng.891>

Gerding, W. M., Schreiber, S., Schulte-Middelmann, T., de Castro Marques, A., Atorf, J., Akkad, D. A., Dekomien, G., Kremers, J., Dermietzel, R., Gal, A., Rulicke, T., Ibrahim, S., Epplen, J. T.,

& Petrasch-Parwez, E. (2011). Ccdc66 null mutation causes retinal degeneration and dysfunction. *Hum Mol Genet*, 20(18), 3620–3631. <https://doi.org/10.1093/hmg/ddr282> [pii]

Ghossoub, R., Molla-Herman, A., Bastin, P., & Benmerah, A. (2011). The ciliary pocket: A once-forgotten membrane domain at the base of cilia. *Biology of the Cell*, 103(3), 131–144. <https://doi.org/10.1042/BC20100128>

Gillingham, A. K., & Munro, S. (2000). The PACT domain, a conserved centrosomal targeting motif in the coiled-coil proteins AKAP450 and pericentrin. *EMBO Rep*, 1(6), 524–529. <https://doi.org/10.1093/embo-reports/kvd105>

Goetz, S. C., Bangs, F., Barrington, C. L., Katsanis, N., & Anderson, K. V. (2017). The Meckel syndrome- associated protein MKS1 functionally interacts with components of the BBSome and IFT complexes to mediate ciliary trafficking and hedgehog signaling. *PloS One*, 12(3), e0173399. <https://doi.org/10.1371/journal.pone.0173399>

Gupta, G. D., Coyaud, E., Goncalves, J., Mojarad, B. A., Liu, Y., Wu, Q., Gheiratmand, L., Comartin, D., Tkach, J. M., Cheung, S. W., Bashkurov, M., Hasegan, M., Knight, J. D., Lin, Z. Y., Schueler, M., Hildebrandt, F., Moffat, J., Gingras, A. C., Raught, B., & Pelletier, L. (2015). A Dynamic Protein Interaction Landscape of the Human Centrosome-Cilium Interface. *Cell*, 163(6), 1484–1499. <https://doi.org/10.1016/j.cell.2015.10.065>

Gupta, Gagan D., Coyaud, É., Gonçalves, J., Mojarad, B. A., Liu, Y., Wu, Q., Gheiratmand, L., Comartin, D., Tkach, J. M., Cheung, S. W. T., Bashkurov, M., Hasegan, M., Knight, J. D., Lin, Z.-Y., Schueler, M., Hildebrandt, F., Moffat, J., Gingras, A.-C., Raught, B., & Pelletier, L. (2015). A Dynamic Protein Interaction Landscape of the Human Centrosome-Cilium Interface. *Cell*, 163(6), 1484–1499. <https://doi.org/10.1016/j.cell.2015.10.065>

Hall, E. A., Keighren, M., Ford, M. J., Davey, T., Jarman, A. P., Smith, L. B., Jackson, I. J., & Mill, P. (2013). Acute versus chronic loss of mammalian Azi1/Cep131 results in distinct ciliary phenotypes. *PLoS Genet*, 9(12), e1003928. <https://doi.org/10.1371/journal.pgen.1003928> PGENETICS-D-13-01720 [pii]

Hamel, V., Steib, E., Hamelin, R., Armand, F., Borgers, S., Fluckiger, I., Busso, C., Olieric, N., Sorzano, C. O. S., Steinmetz, M. O., Guichard, P., & Gonczy, P. (2017). Identification of Chlamydomonas Central Core Centriolar Proteins Reveals a Role for Human WDR90 in Ciliogenesis. *Curr Biol*, 27(16), 2486–2498 e6. <https://doi.org/10.1016/j.cub.2017.07.011>

Hames, R. S., Crookes, R. E., Straatman, K. R., Merdes, A., Hayes, M. J., Faragher, A. J., & Fry, A. M. (2005). Dynamic recruitment of Nek2 kinase to the centrosome involves microtubules, PCM-1, and localized proteasomal degradation. *Mol Biol Cell*, 16(4), 1711–1724. <https://doi.org/10.1091/mbc.E04-08-0688> [pii]

Hao, L., Acar, S., Evans, J., Ou, G., & Scholey, J. M. (2009). Analysis of intraflagellar transport in *C. elegans* sensory cilia. *Methods in Cell Biology*, 93, 235–266. [https://doi.org/10.1016/S0091-679X\(08\)93013-2](https://doi.org/10.1016/S0091-679X(08)93013-2)

Hehnlly, H., & Doxsey, S. (2014). Rab11 endosomes contribute to mitotic spindle organization

and orientation. *Dev Cell*, 28(5), 497–507. <https://doi.org/10.1016/j.devcel.2014.01.014>

Hein, M. Y., Hubner, N. C., Poser, I., Cox, J., Nagaraj, N., Toyoda, Y., Gak, I. A., Weisswange, I., Mansfeld, J., Buchholz, F., Hyman, A. A., & Mann, M. (2015). A human interactome in three quantitative dimensions organized by stoichiometries and abundances. *Cell*, 163(3), 712–723. <https://doi.org/10.1016/j.cell.2015.09.053>

Heydeck, W., Fievet, L., Davis, E. E., & Katsanis, N. (2018). The complexity of the cilium: Spatiotemporal diversity of an ancient organelle. *Curr Opin Cell Biol*, 55, 139–149. <https://doi.org/10.1016/j.ceb.2018.08.001>

Hildebrandt, F., Benzing, T., & Katsanis, N. (2011). Ciliopathies. *N Engl J Med*, 364(16), 1533–1543. <https://doi.org/10.1056/NEJMra1010172>

Hirano, T., Katoh, Y., & Nakayama, K. (2017). Intraflagellar transport-A complex mediates ciliary entry and retrograde trafficking of ciliary G protein-coupled receptors. *Molecular Biology of the Cell*, 28(3), 429–439. <https://doi.org/10.1091/mbc.E16-11-0813>

Hodges, M. E., Scheumann, N., Wickstead, B., Langdale, J. A., & Gull, K. (2010). Reconstructing the evolutionary history of the centriole from protein components. *J Cell Sci*, 123(Pt 9), 1407–1413. <https://doi.org/10.1242/jcs.064873> jcs.064873 [pii]

Hori, A., Barnouin, K., Snijders, A. P., & Toda, T. (2016). A non-canonical function of Plk4 in centriolar satellite integrity and ciliogenesis through PCM1 phosphorylation. *EMBO Rep*, 17(3), 326–337. <https://doi.org/10.15252/embr.201541432>

Hori, A., & Toda, T. (2017). Regulation of centriolar satellite integrity and its physiology. *Cell Mol Life Sci*, 74(2), 213–229. <https://doi.org/10.1007/s00018-016-2315-x>

Hu, Q., Milenkovic, L., Jin, H., Scott, M. P., Nachury, M. V., Spiliotis, E. T., & Nelson, W. J. (2010). A septin diffusion barrier at the base of the primary cilium maintains ciliary membrane protein distribution. *Science*, 329(5990), 436–439. <https://doi.org/10.1126/science.1191054>

Hu, Qicong, Milenkovic, L., Jin, H., Scott, M. P., Nachury, M. V., Spiliotis, E. T., & Nelson, W. J. (2010). A septin diffusion barrier at the base of the primary cilium maintains ciliary membrane protein distribution. *Science (New York, N.Y.)*, 329(5990), 436–439. <https://doi.org/10.1126/science.1191054>

Huang, B.-R., Tsai, C.-F., Lin, H.-Y., Tseng, W.-P., Huang, S.-S., Wu, C.-R., Lin, C., Yeh, W.-L., & Lu, D.-Y. (2013). Interaction of inflammatory and anti-inflammatory responses in microglia by *Staphylococcus aureus*-derived lipoteichoic acid. *Toxicology and Applied Pharmacology*, 269(1), 43–50. <https://doi.org/10.1016/j.taap.2013.03.004>

Huangfu, D., & Anderson, K. V. (2005). Cilia and Hedgehog responsiveness in the mouse. *Proceedings of the National Academy of Sciences of the United States of America*, 102(32), 11325–11330. <https://doi.org/10.1073/pnas.0505328102>

Huangfu, D., Liu, A., Rakeman, A. S., Murcia, N. S., Niswander, L., & Anderson, K. V. (2003). Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature*, 426(6962),

83–87. <https://doi.org/10.1038/nature02061>

Hunnicut, G. R., Kosfisz, M. G., & Snell, W. J. (1990). Cell body and flagellar agglutinins in *Chlamydomonas reinhardtii*: The cell body plasma membrane is a reservoir for agglutinins whose migration to the flagella is regulated by a functional barrier. *The Journal of Cell Biology*, 111(4), 1605–1616. <https://doi.org/10.1083/jcb.111.4.1605>

Inglis, P. N., Ou, G., Leroux, M. R., & Scholey, J. M. (2007). The sensory cilia of *Caenorhabditis elegans*. *WormBook: The Online Review of C. Elegans Biology*, 1–22. <https://doi.org/10.1895/wormbook.1.126.2>

Ishikawa, H., Thompson, J., Yates, J. R., & Marshall, W. F. (2012). Proteomic analysis of mammalian primary cilia. *Curr Biol*, 22(5), 414–419. <https://doi.org/10.1016/j.cub.2012.01.031> S0960-9822(12)00061-9 [pii]

Ishikawa, Hiroaki, Thompson, J., Yates, J. R., & Marshall, W. F. (2012). Proteomic analysis of mammalian primary cilia. *Current Biology: CB*, 22(5), 414–419. <https://doi.org/10.1016/j.cub.2012.01.031>

Janke, C., & Bulinski, J. C. (2011). Post-translational regulation of the microtubule cytoskeleton: Mechanisms and functions. *Nature Reviews. Molecular Cell Biology*, 12(12), 773–786. <https://doi.org/10.1038/nrm3227>

Jia, Y., Fong, K. W., Choi, Y. K., See, S. S., & Qi, R. Z. (2013). Dynamic recruitment of CDK5RAP2 to centrosomes requires its association with dynein. *PLoS One*, 8(7), e68523. <https://doi.org/10.1371/journal.pone.0068523>

Jiang, S. T., Chiou, Y. Y., Wang, E., Lin, H. K., Lee, S. P., Lu, H. Y., Wang, C. K., Tang, M. J., & Li, H. (2008). Targeted disruption of *Nphp1* causes male infertility due to defects in the later steps of sperm morphogenesis in mice. *Hum Mol Genet*, 17(21), 3368–3379. <https://doi.org/10.1093/hmg/ddn231>

Jiang, Y.-Y., Lechtreck, K., & Gaertig, J. (2015). Total internal reflection fluorescence microscopy of intraflagellar transport in *Tetrahymena thermophila*. *Methods in Cell Biology*, 127, 445–456. <https://doi.org/10.1016/bs.mcb.2015.01.001>

Jin, H., White, S. R., Shida, T., Schulz, S., Aguiar, M., Gygi, S. P., Bazan, J. F., & Nachury, M. V. (2010). The conserved Bardet-Biedl syndrome proteins assemble a coat that traffics membrane proteins to cilia. *Cell*, 141(7), 1208–1219. <https://doi.org/10.1016/j.cell.2010.05.015>

Khodjakov, A., & Rieder, C. L. (1999). The sudden recruitment of gamma-tubulin to the centrosome at the onset of mitosis and its dynamic exchange throughout the cell cycle, do not require microtubules. *J Cell Biol*, 146(3), 585–596.

Kim, J. C., Badano, J. L., Sibold, S., Esmail, M. A., Hill, J., Hoskins, B. E., Leitch, C. C., Venner, K., Ansley, S. J., Ross, A. J., Leroux, M. R., Katsanis, N., & Beales, P. L. (2004). The Bardet-Biedl protein BBS4 targets cargo to the pericentriolar region and is required for microtubule anchoring and cell cycle progression. *Nat Genet*, 36(5), 462–470.

<https://doi.org/10.1038/ng1352> ng1352 [pii]

Kim, J., Krishnaswami, S. R., & Gleeson, J. G. (2008). CEP290 interacts with the centriolar satellite component PCM-1 and is required for Rab8 localization to the primary cilium. *Hum Mol Genet*, 17(23), 3796–3805. <https://doi.org/10.1093/hmg/ddn277> ddn277 [pii]

Kim, Jun Chul, Badano, J. L., Sibold, S., Esmail, M. A., Hill, J., Hoskins, B. E., Leitch, C. C., Venner, K., Ansley, S. J., Ross, A. J., Leroux, M. R., Katsanis, N., & Beales, P. L. (2004). The Bardet-Biedl protein BBS4 targets cargo to the pericentriolar region and is required for microtubule anchoring and cell cycle progression. *Nature Genetics*, 36(5), 462–470. <https://doi.org/10.1038/ng1352>

Kim, K., Lee, K., & Rhee, K. (2012). CEP90 is required for the assembly and centrosomal accumulation of centriolar satellites, which is essential for primary cilia formation. *PLoS One*, 7(10), e48196. <https://doi.org/10.1371/journal.pone.0048196> PONE-D-12-20571 [pii]

Kim, K., & Rhee, K. (2011). The pericentriolar satellite protein CEP90 is crucial for integrity of the mitotic spindle pole. *J Cell Sci*, 124(Pt 3), 338–347. <https://doi.org/10.1242/jcs.078329> jcs.078329 [pii]

Kim, S., & Dynlacht, B. D. (2013). Assembling a primary cilium. *Curr Opin Cell Biol*, 25(4), 506–511. <https://doi.org/10.1016/j.ceb.2013.04.011> S0955-0674(13)00074-4 [pii]

Klink, B. U., Zent, E., Juneja, P., Kuhlee, A., Raunser, S., & Wittinghofer, A. (2017). A recombinant BBSome core complex and how it interacts with ciliary cargo. *ELife*, 6. <https://doi.org/10.7554/eLife.27434>

Kobayashi, T., Tsang, W. Y., Li, J., Lane, W., & Dynlacht, B. D. (2011). Centriolar kinesin Kif24 interacts with CP110 to remodel microtubules and regulate ciliogenesis. *Cell*, 145(6), 914–925. <https://doi.org/10.1016/j.cell.2011.04.028>

Kodani, A., Tonthat, V., Wu, B., & Sutterlin, C. (2010). Par6 alpha interacts with the dynactin subunit p150 Glued and is a critical regulator of centrosomal protein recruitment. *Mol Biol Cell*, 21(19), 3376–3385. <https://doi.org/10.1091/mbc.E10-05-0430> E10-05-0430 [pii]

Kodani, A., Yu, T. W., Johnson, J. R., Jayaraman, D., Johnson, T. L., Al-Gazali, L., Sztriha, L., Partlow, J. N., Kim, H., Krup, A. L., Dammermann, A., Krogan, N. J., Walsh, C. A., & Reiter, J. F. (2015). Centriolar satellites assemble centrosomal microcephaly proteins to recruit CDK2 and promote centriole duplication. *Elife*, 4. <https://doi.org/10.7554/eLife.07519>

Kohli, P., Höhne, M., Jüngst, C., Bertsch, S., Ebert, L. K., Schauss, A. C., Benzing, T., Rinschen, M. M., & Schermer, B. (2017). The ciliary membrane-associated proteome reveals actin-binding proteins as key components of cilia. *EMBO Reports*, 18(9), 1521–1535. <https://doi.org/10.15252/embr.201643846>

Kozminski, K. G., Johnson, K. A., Forscher, P., & Rosenbaum, J. L. (1993). A motility in the eukaryotic flagellum unrelated to flagellar beating. *Proceedings of the National Academy of Sciences of the United States of America*, 90(12), 5519–5523.

<https://doi.org/10.1073/pnas.90.12.5519>

Krupp, M., Marquardt, J. U., Sahin, U., Galle, P. R., Castle, J., & Teufel, A. (2012). RNA-Seq Atlas—A reference database for gene expression profiling in normal tissue by next-generation sequencing. *Bioinformatics*, 28(8), 1184–1185. <https://doi.org/10.1093/bioinformatics/bts084> [pii]

Kubo, A., Sasaki, H., Yuba-Kubo, A., Tsukita, S., & Shiina, N. (1999). Centriolar satellites: Molecular characterization, ATP-dependent movement toward centrioles and possible involvement in ciliogenesis. *J Cell Biol*, 147(5), 969–980.

Langousis, G., Shimogawa, M. M., Saada, E. A., Vashisht, A. A., Spreafico, R., Nager, A. R., Barshop, W. D., Nachury, M. V., Wohlschlegel, J. A., & Hill, K. L. (2016). Loss of the BBSome perturbs endocytic trafficking and disrupts virulence of *Trypanosoma brucei*. *Proceedings of the National Academy of Sciences of the United States of America*, 113(3), 632–637. <https://doi.org/10.1073/pnas.1518079113>

Lechtreck, K. F. (2015). IFT-Cargo Interactions and Protein Transport in Cilia. *Trends in Biochemical Sciences*, 40(12), 765–778. <https://doi.org/10.1016/j.tibs.2015.09.003>

Lechtreck, K. F., Brown, J. M., Sampaio, J. L., Craft, J. M., Shevchenko, A., Evans, J. E., & Witman, G. B. (2013). Cycling of the signaling protein phospholipase D through cilia requires the BBSome only for the export phase. *The Journal of Cell Biology*, 201(2), 249–261. <https://doi.org/10.1083/jcb.201207139>

Lechtreck, K.-F., Johnson, E. C., Sakai, T., Cochran, D., Ballif, B. A., Rush, J., Pazour, G. J., Ikebe, M., & Witman, G. B. (2009). The *Chlamydomonas reinhardtii* BBSome is an IFT cargo required for export of specific signaling proteins from flagella. *The Journal of Cell Biology*, 187(7), 1117–1132. <https://doi.org/10.1083/jcb.200909183>

Lee, J. Y., & Stearns, T. (2013). FOP is a centriolar satellite protein involved in ciliogenesis. *PLoS One*, 8(3), e58589. <https://doi.org/10.1371/journal.pone.0058589> PONE-D-12-26824 [pii]

Lee, S., & Rhee, K. (2010). CEP215 is involved in the dynein-dependent accumulation of pericentriolar matrix proteins for spindle pole formation. *Cell Cycle*, 9(4), 774–783.

Li, J. B., Gerdes, J. M., Haycraft, C. J., Fan, Y., Teslovich, T. M., May-Simera, H., Li, H., Blacque, O. E., Li, L., Leitch, C. C., Lewis, R. A., Green, J. S., Parfrey, P. S., Leroux, M. R., Davidson, W. S., Beales, P. L., Guay-Woodford, L. M., Yoder, B. K., Stormo, G. D., ... Dutcher, S. K. (2004). Comparative genomics identifies a flagellar and basal body proteome that includes the BBS5 human disease gene. *Cell*, 117(4), 541–552. <https://doi.org/S0092867404004507> [pii]

Liu, A., Wang, B., & Niswander, L. A. (2005). Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. *Development (Cambridge, England)*, 132(13), 3103–3111. <https://doi.org/10.1242/dev.01894>

Liu, Q., Tan, G., Levenkova, N., Li, T., Pugh, E. N., Rux, J. J., Speicher, D. W., & Pierce, E. A. (2007). The proteome of the mouse photoreceptor sensory cilium complex. *Molecular & Cellular*

Proteomics: MCP, 6(8), 1299–1317. <https://doi.org/10.1074/mcp.M700054-MCP200>

Long, H., Zhang, F., Xu, N., Liu, G., Diener, D. R., Rosenbaum, J. L., & Huang, K. (2016). Comparative Analysis of Ciliary Membranes and Ectosomes. *Current Biology: CB*, 26(24), 3327–3335. <https://doi.org/10.1016/j.cub.2016.09.055>

Lopes, C. A., Prosser, S. L., Romio, L., Hirst, R. A., O'Callaghan, C., Woolf, A. S., & Fry, A. M. (2011). Centriolar satellites are assembly points for proteins implicated in human ciliopathies, including oral-facial-digital syndrome 1. *J Cell Sci*, 124(Pt 4), 600–612. <https://doi.org/10.1242/jcs.077156> [pii]

Luders, J., & Stearns, T. (2007). Microtubule-organizing centres: A re-evaluation. *Nat Rev Mol Cell Biol*, 8(2), 161–167. <https://doi.org/nrm2100> [pii] 10.1038/nrm2100

Mahjoub, M. R., Xie, Z., & Stearns, T. (2010). Cep120 is asymmetrically localized to the daughter centriole and is essential for centriole assembly. *J Cell Biol*, 191(2), 331–346. <https://doi.org/10.1083/jcb.201003009> [pii]

Martinez-Garay, I., Rustom, A., Gerdes, H. H., & Kutsche, K. (2006). The novel centrosomal associated protein CEP55 is present in the spindle midzone and the midbody. *Genomics*, 87(2), 243–253. <https://doi.org/10.1016/j.ygeno.2005.11.006>

Mayer, U., Küller, A., Daiber, P. C., Neudorf, I., Warnken, U., Schnölzer, M., Frings, S., & Möhrle, F. (2009). The proteome of rat olfactory sensory cilia. *Proteomics*, 9(2), 322–334. <https://doi.org/10.1002/pmic.200800149>

Mayer, U., Ungerer, N., Klimmeck, D., Warnken, U., Schnölzer, M., Frings, S., & Möhrle, F. (2008). Proteomic analysis of a membrane preparation from rat olfactory sensory cilia. *Chemical Senses*, 33(2), 145–162. <https://doi.org/10.1093/chemse/bjm073>

McClintock, T. S., Glasser, C. E., Bose, S. C., & Bergman, D. A. (2008). Tissue expression patterns identify mouse cilia genes. *Physiol Genomics*, 32(2), 198–206. <https://doi.org/00128.2007> [pii] 10.1152/physiolgenomics.00128.2007

Mick, D. U., Rodrigues, R. B., Leib, R. D., Adams, C. M., Chien, A. S., Gygi, S. P., & Nachury, M. V. (2015). Proteomics of Primary Cilia by Proximity Labeling. *Dev Cell*, 35(4), 497–512. <https://doi.org/10.1016/j.devcel.2015.10.015>

Mick, David U., Rodrigues, R. B., Leib, R. D., Adams, C. M., Chien, A. S., Gygi, S. P., & Nachury, M. V. (2015). Proteomics of Primary Cilia by Proximity Labeling. *Developmental Cell*, 35(4), 497–512. <https://doi.org/10.1016/j.devcel.2015.10.015>

Miyamoto, T., Hosoba, K., Ochiai, H., Royba, E., Izumi, H., Sakuma, T., Yamamoto, T., Dynlacht, B. D., & Matsuura, S. (2015). The Microtubule-Depolymerizing Activity of a Mitotic Kinesin Protein KIF2A Drives Primary Cilia Disassembly Coupled with Cell Proliferation. *Cell Reports*, 10(5), 664–673. <https://doi.org/10.1016/j.celrep.2015.01.003>

Molday, R. S., & Goldberg, A. F. X. (2017). Peripherin diverts ciliary ectosome release to photoreceptor disc morphogenesis. *The Journal of Cell Biology*, 216(5), 1227–1229.

<https://doi.org/10.1083/jcb.201703020>

Molla-Herman, A., Ghossoub, R., Blisnick, T., Meunier, A., Serres, C., Silbermann, F., Emmerson, C., Romeo, K., Bourdoncle, P., Schmitt, A., Saunier, S., Spassky, N., Bastin, P., & Benmerah, A. (2010). The ciliary pocket: An endocytic membrane domain at the base of primary and motile cilia. *Journal of Cell Science*, 123(Pt 10), 1785–1795.

<https://doi.org/10.1242/jcs.059519>

Moudjou, M., Bordes, N., Paintrand, M., & Bornens, M. (1996). gamma-Tubulin in mammalian cells: The centrosomal and the cytosolic forms. *J Cell Sci*, 109 (Pt 4), 875–887.

Mykytyn, K., Mullins, R. F., Andrews, M., Chiang, A. P., Swiderski, R. E., Yang, B., Braun, T., Casavant, T., Stone, E. M., & Sheffield, V. C. (2004). Bardet-Biedl syndrome type 4 (BBS4)-null mice implicate Bbs4 in flagella formation but not global cilia assembly. *Proc Natl Acad Sci U S A*, 101(23), 8664–8669. <https://doi.org/10.1073/pnas.0402354101>

Nachury, M. V. (2008). Tandem affinity purification of the BBSome, a critical regulator of Rab8 in ciliogenesis. *Methods Enzymol*, 439, 501–513. [https://doi.org/10.1016/S0076-6879\(07\)00434-X](https://doi.org/10.1016/S0076-6879(07)00434-X)

Nachury, M. V., Loktev, A. V., Zhang, Q., Westlake, C. J., Peranen, J., Merdes, A., Slusarski, D. C., Scheller, R. H., Bazan, J. F., Sheffield, V. C., & Jackson, P. K. (2007). A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. *Cell*, 129(6), 1201–1213. [https://doi.org/S0092-8674\(07\)00534-X](https://doi.org/S0092-8674(07)00534-X) [pii] 10.1016/j.cell.2007.03.053

Nachury, M. V., & Mick, D. U. (2019). Establishing and regulating the composition of cilia for signal transduction. *Nat Rev Mol Cell Biol*. <https://doi.org/10.1038/s41580-019-0116-4>

Nachury, Maxence V., Loktev, A. V., Zhang, Q., Westlake, C. J., Peränen, J., Merdes, A., Slusarski, D. C., Scheller, R. H., Bazan, J. F., Sheffield, V. C., & Jackson, P. K. (2007). A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. *Cell*, 129(6), 1201–1213. <https://doi.org/10.1016/j.cell.2007.03.053>

Nager, A. R., Goldstein, J. S., Herranz-Perez, V., Portran, D., Ye, F., Garcia-Verdugo, J. M., & Nachury, M. V. (2017). An Actin Network Dispatches Ciliary GPCRs into Extracellular Vesicles to Modulate Signaling. *Cell*, 168(1–2), 252–263 e14. <https://doi.org/10.1016/j.cell.2016.11.036>

Nager, Andrew R., Goldstein, J. S., Herranz-Pérez, V., Portran, D., Ye, F., Garcia-Verdugo, J. M., & Nachury, M. V. (2017). An Actin Network Dispatches Ciliary GPCRs into Extracellular Vesicles to Modulate Signaling. *Cell*, 168(1–2), 252–263.e14. <https://doi.org/10.1016/j.cell.2016.11.036>

Nakayama, K., & Katoh, Y. (2018). Ciliary protein trafficking mediated by IFT and BBSome complexes with the aid of kinesin-2 and dynein-2 motors. *Journal of Biochemistry*, 163(3), 155–164. <https://doi.org/10.1093/jb/mvx087>

Narita, K., Kozuka-Hata, H., Nonami, Y., Ao-Kondo, H., Suzuki, T., Nakamura, H., Yamakawa, K., Oyama, M., Inoue, T., & Takeda, S. (2012). Proteomic analysis of multiple primary cilia

reveals a novel mode of ciliary development in mammals. *Biology Open*, 1(8), 815–825.
<https://doi.org/10.1242/bio.20121081>

Nigg, E. A., & Holland, A. J. (2018). Once and only once: Mechanisms of centriole duplication and their deregulation in disease. *Nat Rev Mol Cell Biol*, 19(5), 297–312.
<https://doi.org/10.1038/nrm.2017.127>

Nigg, E. A., & Raff, J. W. (2009). Centrioles, centrosomes, and cilia in health and disease. *Cell*, 139(4), 663–678. <https://doi.org/10.1016/j.cell.2009.10.036> S0092-8674(09)01362-2 [pii]

Nigg, Erich A., Čajánek, L., & Arquint, C. (2014). The centrosome duplication cycle in health and disease. *FEBS Letters*, 588(15), 2366–2372. <https://doi.org/10.1016/j.febslet.2014.06.030>

Nigg, Erich A., & Stearns, T. (2011). The centrosome cycle: Centriole biogenesis, duplication and inherent asymmetries. *Nature Cell Biology*, 13(10), 1154–1160.
<https://doi.org/10.1038/ncb2345>

Pampliega, O., Orhon, I., Patel, B., Sridhar, S., Diaz-Carretero, A., Beau, I., Codogno, P., Satir, B. H., Satir, P., & Cuervo, A. M. (2013). Functional interaction between autophagy and ciliogenesis. *Nature*, 502(7470), 194–200. <https://doi.org/10.1038/nature12639> nature12639 [pii]

Pazour, G. J., Agrin, N., Leszyk, J., & Witman, G. B. (2005). Proteomic analysis of a eukaryotic cilium. *The Journal of Cell Biology*, 170(1), 103–113. <https://doi.org/10.1083/jcb.200504008>

Pedersen, L. B., & Akhmanova, A. (2014). Kif7 keeps cilia tips in shape. *Nature Cell Biology*, 16(7), 623–625. <https://doi.org/10.1038/ncb2997>

Pedersen, L. B., Mogensen, J. B., & Christensen, S. T. (2016). Endocytic Control of Cellular Signaling at the Primary Cilium. *Trends in Biochemical Sciences*, 41(9), 784–797.
<https://doi.org/10.1016/j.tibs.2016.06.002>

Pedersen, L. B., Schröder, J. M., Satir, P., & Christensen, S. T. (2012). The ciliary cytoskeleton. *Comprehensive Physiology*, 2(1), 779–803. <https://doi.org/10.1002/cphy.c110043>

Plotnikova, O. V., Nikonova, A. S., Loskutov, Y. V., Kozyulina, P. Y., Pugacheva, E. N., & Golemis, E. A. (2012). Calmodulin activation of Aurora-A kinase (AURKA) is required during ciliary disassembly and in mitosis. *Molecular Biology of the Cell*, 23(14), 2658–2670.
<https://doi.org/10.1091/mbc.E11-12-1056>

Poser, I., Sarov, M., Hutchins, J. R., Heriche, J. K., Toyoda, Y., Pozniakovsky, A., Weigl, D., Nitzsche, A., Hegemann, B., Bird, A. W., Pelletier, L., Kittler, R., Hua, S., Naumann, R., Augsburg, M., Sykora, M. M., Hofemeister, H., Zhang, Y., Nasmyth, K., ... Hyman, A. A. (2008). BAC TransgeneOmics: A high-throughput method for exploration of protein function in mammals. *Nat Methods*, 5(5), 409–415. <https://doi.org/10.1038/nmeth.1199>

Pratt, M. B., Titlow, J. S., Davis, I., Barker, A. R., Dawe, H. R., Raff, J. W., & Roque, H. (2016). *Drosophila* sensory cilia lacking MKS proteins exhibit striking defects in development but only subtle defects in adults. *Journal of Cell Science*, 129(20), 3732–3743.
<https://doi.org/10.1242/jcs.194621>

- Price, H. P., Paape, D., Hodgkinson, M. R., Farrant, K., Doehl, J., Stark, M., & Smith, D. F. (2013). The *Leishmania major* BBSome subunit BBS1 is essential for parasite virulence in the mammalian host. *Molecular Microbiology*, 90(3), 597–611. <https://doi.org/10.1111/mmi.12383>
- Pugacheva, E. N., Jablonski, S. A., Hartman, T. R., Henske, E. P., & Golemis, E. A. (2007). HEF1-dependent Aurora A activation induces disassembly of the primary cilium. *Cell*, 129(7), 1351–1363. <https://doi.org/10.1016/j.cell.2007.04.035>
- Pusapati, G. V., Kong, J. H., Patel, B. B., Krishnan, A., Sagner, A., Kinnebrew, M., Briscoe, J., Aravind, L., & Rohatgi, R. (2018). CRISPR Screens Uncover Genes that Regulate Target Cell Sensitivity to the Morphogen Sonic Hedgehog. *Developmental Cell*, 44(1), 113–129.e8. <https://doi.org/10.1016/j.devcel.2017.12.003>
- Quintyne, N. J., Gill, S. R., Eckley, D. M., Crego, C. L., Compton, D. A., & Schroer, T. A. (1999). Dynactin is required for microtubule anchoring at centrosomes. *J Cell Biol*, 147(2), 321–334.
- Rai, A. K., Chen, J. X., Selbach, M., & Pelkmans, L. (2018). Kinase-controlled phase transition of membraneless organelles in mitosis. *Nature*, 559(7713), 211–216. <https://doi.org/10.1038/s41586-018-0279-8>
- Ran, J., Yang, Y., Li, D., Liu, M., & Zhou, J. (2015). Deacetylation of α -tubulin and cortactin is required for HDAC6 to trigger ciliary disassembly. *Scientific Reports*, 5, 12917. <https://doi.org/10.1038/srep12917>
- Reck-Peterson, S. L., Redwine, W. B., Vale, R. D., & Carter, A. P. (2018). The cytoplasmic dynein transport machinery and its many cargoes. *Nat Rev Mol Cell Biol*, 19(6), 382–398. <https://doi.org/10.1038/s41580-018-0004-3>
- Redwine, W. B., DeSantis, M. E., Hollyer, I., Htet, Z. M., Tran, P. T., Swanson, S. K., Florens, L., Washburn, M. P., & Reck-Peterson, S. L. (2017). The human cytoplasmic dynein interactome reveals novel activators of motility. *Elife*, 6. <https://doi.org/10.7554/eLife.28257>
- Reiter, J. F., & Leroux, M. R. (2017). Genes and molecular pathways underpinning ciliopathies. *Nat Rev Mol Cell Biol*, 18(9), 533–547. <https://doi.org/10.1038/nrm.2017.60>
- Reiter, Jeremy F., & Leroux, M. R. (2017). Genes and molecular pathways underpinning ciliopathies. *Nature Reviews. Molecular Cell Biology*, 18(9), 533–547. <https://doi.org/10.1038/nrm.2017.60>
- Rhein, L. D., & Cagan, R. H. (1980). Biochemical studies of olfaction: Isolation, characterization, and odorant binding activity of cilia from rainbow trout olfactory rosettes. *Proceedings of the National Academy of Sciences of the United States of America*, 77(8), 4412–4416. <https://doi.org/10.1073/pnas.77.8.4412>
- Rosenbaum, J. L., & Witman, G. B. (2002). Intraflagellar transport. *Nature Reviews. Molecular Cell Biology*, 3(11), 813–825. <https://doi.org/10.1038/nrm952>
- Roux, K. J., Kim, D. I., & Burke, B. (2013). BioID: a screen for protein-protein interactions.

Curr Protoc Protein Sci, 74, Unit 19 23. <https://doi.org/10.1002/0471140864.ps1923s74>

Salinas, R. Y., Pearing, J. N., Ding, J.-D., Spencer, W. J., Hao, Y., & Arshavsky, V. Y. (2017). Photoreceptor discs form through peripherin-dependent suppression of ciliary ectosome release. *The Journal of Cell Biology*, 216(5), 1489–1499. <https://doi.org/10.1083/jcb.201608081>

Sang, L., Miller, J. J., Corbit, K. C., Giles, R. H., Brauer, M. J., Otto, E. A., Baye, L. M., Wen, X., Scales, S. J., Kwong, M., Huntzicker, E. G., Sfakianos, M. K., Sandoval, W., Bazan, J. F., Kulkarni, P., Garcia-Gonzalo, F. R., Seol, A. D., O'Toole, J. F., Held, S., ... Jackson, P. K. (2011). Mapping the NPHP-JBTS-MKS protein network reveals ciliopathy disease genes and pathways. *Cell*, 145(4), 513–528. <https://doi.org/10.1016/j.cell.2011.04.019>

Santi-Rocca, J., Chenouard, N., Fort, C., Lagache, T., Olivo-Marin, J.-C., & Bastin, P. (2015). Imaging intraflagellar transport in trypanosomes. *Methods in Cell Biology*, 127, 487–508. <https://doi.org/10.1016/bs.mcb.2015.01.005>

Satir, P. (1965). STUDIES ON CILIA: II. Examination of the Distal Region of the Ciliary Shaft and the Role of the Filaments in Motility. *The Journal of Cell Biology*, 26(3), 805–834. <https://doi.org/10.1083/jcb.26.3.805>

Satir, P. (1968). Studies on cilia. 3. Further studies on the cilium tip and a “sliding filament” model of ciliary motility. *The Journal of Cell Biology*, 39(1), 77–94. <https://doi.org/10.1083/jcb.39.1.77>

Satish Tammana, T. V., Tammana, D., Diener, D. R., & Rosenbaum, J. (2013). Centrosomal protein CEP104 (Chlamydomonas FAP256) moves to the ciliary tip during ciliary assembly. *Journal of Cell Science*, 126(Pt 21), 5018–5029. <https://doi.org/10.1242/jcs.133439>

Scherft, J. P., & Daems, W. T. (1967). Single cilia in chondrocytes. *Journal of Ultrastructure Research*, 19(5), 546–555. [https://doi.org/10.1016/s0022-5320\(67\)80080-7](https://doi.org/10.1016/s0022-5320(67)80080-7)

Scholey, J. M. (2008). Intraflagellar transport motors in cilia: Moving along the cell's antenna. *J Cell Biol*, 180(1), 23–29. <https://doi.org/10.1083/jcb.200709133>

Schroer, T. A., Schnapp, B. J., Reese, T. S., & Sheetz, M. P. (1988). The role of kinesin and other soluble factors in organelle movement along microtubules. *J Cell Biol*, 107(5), 1785–1792.

Schroer, T. A., Steuer, E. R., & Sheetz, M. P. (1989). Cytoplasmic dynein is a minus end-directed motor for membranous organelles. *Cell*, 56(6), 937–946.

Seo, S., Zhang, Q., Bugge, K., Breslow, D. K., Searby, C. C., Nachury, M. V., & Sheffield, V. C. (2011). A novel protein LZTFL1 regulates ciliary trafficking of the BBSome and Smoothed. *PLoS Genetics*, 7(11), e1002358. <https://doi.org/10.1371/journal.pgen.1002358>

Sepulveda, G., Antkowiak, M., Brust-Mascher, I., Mahe, K., Ou, T., Castro, N. M., Christensen, L. N., Cheung, L., Jiang, X., Yoon, D., Huang, B., & Jao, L. E. (2018). Co-translational protein targeting facilitates centrosomal recruitment of PCNT during centrosome maturation in vertebrates. *Elife*, 7. <https://doi.org/10.7554/eLife.34959>

- Sharp, J. A., Plant, J. J., Ohsumi, T. K., Borowsky, M., & Blower, M. D. (2011). Functional analysis of the microtubule-interacting transcriptome. *Mol Biol Cell*, 22(22), 4312–4323. <https://doi.org/10.1091/mbc.E11-07-0629> mbc.E11-07-0629 [pii]
- Shimizu, T., & Johnson, K. A. (1983). Presteady state kinetic analysis of vanadate-induced inhibition of the dynein ATPase. *J Biol Chem*, 258(22), 13833–13840.
- Sigg, M. A., Menchen, T., Lee, C., Johnson, J., Jungnickel, M. K., Choksi, S. P., Garcia, G., Busengdal, H., Dougherty, G. W., Pennekamp, P., Werner, C., Rentzsch, F., Florman, H. M., Krogan, N., Wallingford, J. B., Omran, H., & Reiter, J. F. (2017). Evolutionary Proteomics Uncovers Ancient Associations of Cilia with Signaling Pathways. *Dev Cell*, 43(6), 744–762 e11. <https://doi.org/10.1016/j.devcel.2017.11.014>
- Sigg, Monika Abedin, Menchen, T., Lee, C., Johnson, J., Jungnickel, M. K., Choksi, S. P., Garcia, G., Busengdal, H., Dougherty, G. W., Pennekamp, P., Werner, C., Rentzsch, F., Florman, H. M., Krogan, N., Wallingford, J. B., Omran, H., & Reiter, J. F. (2017). Evolutionary Proteomics Uncovers Ancient Associations of Cilia with Signaling Pathways. *Developmental Cell*, 43(6), 744–762.e11. <https://doi.org/10.1016/j.devcel.2017.11.014>
- Silva, E., Betleja, E., John, E., Spear, P., Moresco, J. J., Zhang, S., Yates, J. R., Mitchell, B. J., & Mahjoub, M. R. (2016). Ccdc11 is a novel centriolar satellite protein essential for ciliogenesis and establishment of left-right asymmetry. *Mol Biol Cell*, 27(1), 48–63. <https://doi.org/10.1091/mbc.E15-07-0474> mbc.E15-07-0474 [pii]
- Smith, J. C., Northey, J. G. B., Garg, J., Pearlman, R. E., & Siu, K. W. M. (2005). Robust method for proteome analysis by MS/MS using an entire translated genome: Demonstration on the ciliome of *Tetrahymena thermophila*. *Journal of Proteome Research*, 4(3), 909–919. <https://doi.org/10.1021/pr050013h>
- Sorokin, S. (1962). Centrioles and the formation of rudimentary cilia by fibroblasts and smooth muscle cells. *The Journal of Cell Biology*, 15, 363–377. <https://doi.org/10.1083/jcb.15.2.363>
- Spencer, M., Detwiler, P. B., & Bunt-Milam, A. H. (1988). Distribution of membrane proteins in mechanically dissociated retinal rods. *Investigative Ophthalmology & Visual Science*, 29(7), 1012–1020.
- Staples, C. J., Myers, K. N., Beveridge, R. D., Patil, A. A., Howard, A. E., Barone, G., Lee, A. J., Swanton, C., Howell, M., Maslen, S., Skehel, J. M., Boulton, S. J., & Collis, S. J. (2014). Ccdc13 is a novel human centriolar satellite protein required for ciliogenesis and genome stability. *J Cell Sci*, 127(Pt 13), 2910–2919. <https://doi.org/10.1242/jcs.147785>
- Staples, C. J., Myers, K. N., Beveridge, R. D., Patil, A. A., Lee, A. J., Swanton, C., Howell, M., Boulton, S. J., & Collis, S. J. (2012). The centriolar satellite protein Cep131 is important for genome stability. *J Cell Sci*, 125(Pt 20), 4770–4779. <https://doi.org/10.1242/jcs.104059> jcs.104059 [pii]
- Stepanek, L., & Pigino, G. (2016). Microtubule doublets are double-track railways for intraflagellar transport trains. *Science (New York, N.Y.)*, 352(6286), 721–724.

<https://doi.org/10.1126/science.aaf4594>

Stowe, T. R., Wilkinson, C. J., Iqbal, A., & Stearns, T. (2012). The centriolar satellite proteins Cep72 and Cep290 interact and are required for recruitment of BBS proteins to the cilium. *Mol Biol Cell*, 23(17), 3322–3335. <https://doi.org/10.1091/mbc.E12-02-0134> mbc.E12-02-0134 [pii]

Stowe, Timothy R., Wilkinson, C. J., Iqbal, A., & Stearns, T. (2012). The centriolar satellite proteins Cep72 and Cep290 interact and are required for recruitment of BBS proteins to the cilium. *Molecular Biology of the Cell*, 23(17), 3322–3335. <https://doi.org/10.1091/mbc.E12-02-0134>

Su, X., Driscoll, K., Yao, G., Raed, A., Wu, M., Beales, P. L., & Zhou, J. (2014). Bardet-Biedl syndrome proteins 1 and 3 regulate the ciliary trafficking of polycystic kidney disease 1 protein. *Human Molecular Genetics*, 23(20), 5441–5451. <https://doi.org/10.1093/hmg/ddu267>

Sung, C.-H., & Leroux, M. R. (2013). The roles of evolutionarily conserved functional modules in cilia-related trafficking. *Nature Cell Biology*, 15(12), 1387–1397. <https://doi.org/10.1038/ncb2888>

Swoboda, P., Adler, H. T., & Thomas, J. H. (2000). The RFX-type transcription factor DAF-19 regulates sensory neuron cilium formation in *C. elegans*. *Molecular Cell*, 5(3), 411–421. [https://doi.org/10.1016/s1097-2765\(00\)80436-0](https://doi.org/10.1016/s1097-2765(00)80436-0)

Szymanska, K., & Johnson, C. A. (2012). The transition zone: An essential functional compartment of cilia. *Cilia*, 1(1), 10. <https://doi.org/10.1186/2046-2530-1-10>

Tang, Z., Lin, M. G., Stowe, T. R., Chen, S., Zhu, M., Stearns, T., Franco, B., & Zhong, Q. (2013). Autophagy promotes primary ciliogenesis by removing OFD1 from centriolar satellites. *Nature*, 502(7470), 254–257. <https://doi.org/10.1038/nature12606> nature12606 [pii]

Tayeh, M. K., Yen, H.-J., Beck, J. S., Searby, C. C., Westfall, T. A., Griesbach, H., Sheffield, V. C., & Slusarski, D. C. (2008). Genetic interaction between Bardet-Biedl syndrome genes and implications for limb patterning. *Human Molecular Genetics*, 17(13), 1956–1967. <https://doi.org/10.1093/hmg/ddn093>

Tokuyasu, K., & Yamada, E. (1959). The fine structure of the retina studied with the electron microscope. IV. Morphogenesis of outer segments of retinal rods. *The Journal of Biophysical and Biochemical Cytology*, 6, 225–230. <https://doi.org/10.1083/jcb.6.2.225>

Tollenaere, M. A., Mailand, N., & Bekker-Jensen, S. (2015). Centriolar satellites: Key mediators of centrosome functions. *Cell Mol Life Sci*, 72(1), 11–23. <https://doi.org/10.1007/s00018-014-1711-3>

Tollenaere, M. A. X., Villumsen, B. H., Blasius, M., Nielsen, J. C., Wagner, S. A., Bartek, J., Beli, P., Mailand, N., & Bekker-Jensen, S. (2015). P38- and MK2-dependent signalling promotes stress-induced centriolar satellite remodelling via 14-3-3-dependent sequestration of CEP131/AZI1. *Nat Commun*, 6, 10075. <https://doi.org/10.1038/ncomms10075>

Uzbekov and Alieva. (2013). *Centrosome – history of investigations and new discoveries. From*

cytoplasmic granule to the center of intracellular regulation. Moscow University Press.

Uzbekov, R. E., Maurel, D. B., Aveline, P. C., Pallu, S., Benhamou, C. L., & Rochefort, G. Y. (2012). Centrosome Fine Ultrastructure of the Osteocyte Mechanosensitive Primary Cilium. *Microscopy and Microanalysis*, 18(6), 1430–1441. <https://doi.org/10.1017/S1431927612013281>

Valentine, M. S., Rajendran, A., Yano, J., Weeraratne, S. D., Beisson, J., Cohen, J., Koll, F., & Van Houten, J. (2012). Paramecium BBS genes are key to presence of channels in Cilia. *Cilia*, 1(1), 16. <https://doi.org/10.1186/2046-2530-1-16>

van der Horst, A., Simmons, J., & Khanna, K. K. (2009). Cep55 stabilization is required for normal execution of cytokinesis. *Cell Cycle*, 8(22), 3742–3749. <https://doi.org/10.4161/cc.8.22.10047>

Villumsen, B. H., Danielsen, J. R., Povlsen, L., Sylvestersen, K. B., Merdes, A., Beli, P., Yang, Y. G., Choudhary, C., Nielsen, M. L., Mailing, N., & Bekker-Jensen, S. (2013). A new cellular stress response that triggers centriolar satellite reorganization and ciliogenesis. *EMBO J*, 32(23), 3029–3040. <https://doi.org/10.1038/emboj.2013.223> [pii]

Vitre, B. D., & Cleveland, D. W. (2012). Centrosomes, chromosome instability (CIN) and aneuploidy. *Curr Opin Cell Biol*, 24(6), 809–815. <https://doi.org/10.1016/j.ceb.2012.10.006> S0955-0674(12)00169-X [pii]

Wang, J., & Barr, M. M. (2016). Ciliary Extracellular Vesicles: Txt Msg Organelles. *Cellular and Molecular Neurobiology*, 36(3), 449–457. <https://doi.org/10.1007/s10571-016-0345-4>

Wang, L., Lee, K., Malonis, R., Sanchez, I., & Dynlacht, B. D. (2016). Tethering of an E3 ligase by PCM1 regulates the abundance of centrosomal KIAA0586/Talpid3 and promotes ciliogenesis. *Elife*, 5. <https://doi.org/10.7554/eLife.12950>

Wang, Q., Pan, J., & Snell, W. J. (2006). Intraflagellar transport particles participate directly in cilium-generated signaling in Chlamydomonas. *Cell*, 125(3), 549–562. <https://doi.org/10.1016/j.cell.2006.02.044>

Wang, W. J., Tay, H. G., Soni, R., Perumal, G. S., Goll, M. G., Macaluso, F. P., Asara, J. M., Amack, J. D., & Tsou, M. F. (2013). CEP162 is an axoneme-recognition protein promoting ciliary transition zone assembly at the cilia base. *Nat Cell Biol*, 15(6), 591–601. <https://doi.org/10.1038/ncb2739> [pii]

Waters, A. M., & Beales, P. L. (2011). Ciliopathies: An expanding disease spectrum. *Pediatr Nephrol*, 26(7), 1039–1056. <https://doi.org/10.1007/s00467-010-1731-7>

Werner, S., Pimenta-Marques, A., & Bettencourt-Dias, M. (2017). Maintaining centrosomes and cilia. *J Cell Sci*, 130(22), 3789–3800. <https://doi.org/10.1242/jcs.203505>

Wheway, G., Parry, D. A., & Johnson, C. A. (2014). The role of primary cilia in the development and disease of the retina. *Organogenesis*, 10(1), 69–85. <https://doi.org/10.4161/org.26710> [pii]

Wheway, Gabrielle, Nazlamova, L., & Hancock, J. T. (2018). Signaling through the Primary Cilium. *Frontiers in Cell and Developmental Biology*, 6, 8.

<https://doi.org/10.3389/fcell.2018.00008>

Williams, C. L., Li, C., Kida, K., Inglis, P. N., Mohan, S., Semenec, L., Bialas, N. J., Stupay, R. M., Chen, N., Blacque, O. E., Yoder, B. K., & Leroux, M. R. (2011). MKS and NPHP modules cooperate to establish basal body/transition zone membrane associations and ciliary gate function during ciliogenesis. *The Journal of Cell Biology*, 192(6), 1023–1041.

<https://doi.org/10.1083/jcb.201012116>

Williams, C. L., McIntyre, J. C., Norris, S. R., Jenkins, P. M., Zhang, L., Pei, Q., Verhey, K., & Martens, J. R. (2014). Direct evidence for BBSome-associated intraflagellar transport reveals distinct properties of native mammalian cilia. *Nature Communications*, 5, 5813.

<https://doi.org/10.1038/ncomms6813>

Winey, M., & O'Toole, E. (2014). Centriole structure. *Philos Trans R Soc Lond B Biol Sci*, 369(1650). <https://doi.org/10.1098/rstb.2013.0457>

Won, J., Marin de Evsikova, C., Smith, R. S., Hicks, W. L., Edwards, M. M., Longo-Guess, C., Li, T., Naggert, J. K., & Nishina, P. M. (2011). NPHP4 is necessary for normal photoreceptor ribbon synapse maintenance and outer segment formation, and for sperm development. *Hum Mol Genet*, 20(3), 482–496. <https://doi.org/10.1093/hmg/ddq494>

Wood, C. R., Huang, K., Diener, D. R., & Rosenbaum, J. L. (2013). The cilium secretes bioactive ectosomes. *Current Biology: CB*, 23(10), 906–911. <https://doi.org/10.1016/j.cub.2013.04.019>

Wood, C. R., & Rosenbaum, J. L. (2015). Ciliary ectosomes: Transmissions from the cell's antenna. *Trends in Cell Biology*, 25(5), 276–285. <https://doi.org/10.1016/j.tcb.2014.12.008>

Woodruff, J. B., Ferreira Gomes, B., Widlund, P. O., Mahamid, J., Honigsmann, A., & Hyman, A. A. (2017). The Centrosome Is a Selective Condensate that Nucleates Microtubules by Concentrating Tubulin. *Cell*, 169(6), 1066–1077 e10. <https://doi.org/10.1016/j.cell.2017.05.028>

Wright, A. F., Chakarova, C. F., Abd El-Aziz, M. M., & Bhattacharya, S. S. (2010). Photoreceptor degeneration: Genetic and mechanistic dissection of a complex trait. *Nat Rev Genet*, 11(4), 273–284. <https://doi.org/10.1038/nrg2717>

Wu, C.-T., Chen, H.-Y., & Tang, T. K. (2018). Myosin-Va is required for preciliary vesicle transportation to the mother centriole during ciliogenesis. *Nature Cell Biology*, 20(2), 175–185. <https://doi.org/10.1038/s41556-017-0018-7>

Yang, T. T., Su, J., Wang, W.-J., Craige, B., Witman, G. B., Tsou, M.-F. B., & Liao, J.-C. (2015). Superresolution Pattern Recognition Reveals the Architectural Map of the Ciliary Transition Zone. *Scientific Reports*, 5, 14096. <https://doi.org/10.1038/srep14096>

Ye, F., Nager, A. R., & Nachury, M. V. (2018). BBSome trains remove activated GPCRs from cilia by enabling passage through the transition zone. *The Journal of Cell Biology*, 217(5), 1847–1868. <https://doi.org/10.1083/jcb.201709041>

Yee, L. E., Garcia-Gonzalo, F. R., Bowie, R. V., Li, C., Kennedy, J. K., Ashrafi, K., Blacque, O. E., Leroux, M. R., & Reiter, J. F. (2015). Conserved Genetic Interactions between Ciliopathy Complexes Cooperatively Support Ciliogenesis and Ciliary Signaling. *PLoS Genetics*, *11*(11), e1005627. <https://doi.org/10.1371/journal.pgen.1005627>

Young, A., Dichtenberg, J. B., Purohit, A., Tuft, R., & Doxsey, S. J. (2000). Cytoplasmic dynein-mediated assembly of pericentrin and gamma tubulin onto centrosomes. *Mol Biol Cell*, *11*(6), 2047–2056. <https://doi.org/10.1091/mbc.11.6.2047>

Yuan, S., & Sun, Z. (2013). Expanding horizons: Ciliary proteins reach beyond cilia. *Annual Review of Genetics*, *47*, 353–376. <https://doi.org/10.1146/annurev-genet-111212-133243>

Zhang, Q., Nishimura, D., Seo, S., Vogel, T., Morgan, D. A., Searby, C., Bugge, K., Stone, E. M., Rahmouni, K., & Sheffield, V. C. (2011). Bardet-Biedl syndrome 3 (Bbs3) knockout mouse model reveals common BBS-associated phenotypes and Bbs3 unique phenotypes. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(51), 20678–20683. <https://doi.org/10.1073/pnas.1113220108>

Zhang, Q., Seo, S., Bugge, K., Stone, E. M., & Sheffield, V. C. (2012). BBS proteins interact genetically with the IFT pathway to influence SHH-related phenotypes. *Human Molecular Genetics*, *21*(9), 1945–1953. <https://doi.org/10.1093/hmg/dds004>

Zimmerman, W., & Doxsey, S. J. (2000). Construction of centrosomes and spindle poles by molecular motor-driven assembly of protein particles. *Traffic*, *1*(12), 927–934.

Zybailov, B., Mosley, A. L., Sardi, M. E., Coleman, M. K., Florens, L., & Washburn, M. P. (2006). Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. *J Proteome Res*, *5*(9), 2339–2347. <https://doi.org/10.1021/pr060161n>

Vita

Deniz Çonkar was born in Rize, Turkey on July 27, 1990. She graduated from The Koç School in 2009 and received her B.Sc. degree in Molecular Biology and Genetics from Bilkent University, Ankara in 2014. From September 2014 to July 2020, she worked as a teaching and research assistant in Koç University, Istanbul and performed her Ph.D. studies in the Cytoskeleton Laboratory under the supervision of Dr. Elif Nur Firat-Karalar. Her Ph.D. research was on “Functional and Molecular Dissection of the Retinal Degeneration Protein CCDC66”.

Publications

Conkar D and Firat-Karalar EN. (2020) Microtubule-associated proteins and emerging links to primary cilium structure, assembly and maintenance. *The FEBS Journal*

Sadehi S, Melikov R, **Conkar D**, Firat-Karalar EN, Nizamoglu S. (2020) Ultra-Efficient and High-Quality White Light-Emitting Devices using Fluorescent Proteins in Aqueous Medium. *Advanced Materials Technologies*

Conkar D, Bayraktar H, Firat-Karalar EN. (2019) Centrosomal and ciliary targeting of CCDC66 requires cooperative action of centriolar satellites, microtubules and molecular motors. *Scientific Reports*

Conkar D and Firat-Karalar EN. (2018) Chapter 6 - Structure and Function of the Primary Cilium. Flagella and Cilia: Types, Structure and Functions. *Nova Science Publishers*. pp 129-167

Conkar D, Culfa E, Odabasi E, Rauniyar N, Yates JR 3rd, Firat-Karalar EN. (2017) The centriolar satellite protein CCDC66 interacts with CEP290 and functions in cilium formation and trafficking. s in cilium formation and trafficking. *Journal of Cell Science*.

Dogru IB, Min K, Umar M, Bahmani H, Bagar E, **Conkar D**, Firat-Karalar EN, Kim S, Nizamoglu S. (2017) Single transverse mode protein laser. *Applied Physics Letters*

Dogru IB, Kosak Soz C, Press DA, Melikov R, Bagar E, **Conkar D**, Firat-Karalar EN, Yilgor E, Yilgor, Nizamoglu S. (2017) 3D Coffee Stains. *Materials Chemistry Frontiers*

Press DA, Melikov R, **Conkar D**, Firat-Karalar EN, Nizamoglu S. (2016) Fluorescent integrated white LEDs for displays. *Nanotechnology*

Appendix A

Primers for CCDC66 deletion mutants		
1	CCDC66_isoform1_F	GGGGACAACCTTTGTACAAAAAAGTTGAT ATGAACTTGGGAGATGGTTTAAAGCTTG
2	CCDC66_(363)_R	GGGGACAACCTTTGTACAAGAAAGTTGT TTAAGTTTTTTTGAATAATCCCATAATG
3	CCDC66_(364)_F	GGGGACAACCTTTGTACAAAAAAGTTGAT GAAATGGTTTCATCTGTCCCAGC
4	CCDC66_(591)_R	GGGGACAACCTTTGTACAAGAAAGTTGT TTATTCTTTTTCTTTCTTCTTTAAAGC
5	CCDC66_(966)_R	GGGGACAACCTTTGTACAAGAAAGTTGT TTAACAAACACTGCTGACATCAGCCAGC
6	CCDC66_(967)_F	GGGGACAACCTTTGTACAAAAAAGTTGAT ACACCTACAACCGGAAGCCAGGTTG
7	CCDC66_(1138)_F	GGGGACAACCTTTGTACAAAAAAGTTGAT GAGCATCAGAAAGCCATCACTGCC
8	CCDC66_(1434)_R	GGGGACAACCTTTGTACAAGAAAGTTGT TTATCTAGAAGTGTCATGTCCCTTTTG
9	CCDC66_(1449)_R	GGGGACAACCTTTGTACAAGAAAGTTGT TTAAAGATTTTAAATCAGTCTAGAAGTG
10	CCDC66_(1450)_F	GGGGACAACCTTTGTACAAAAAAGTTG ATGGTGTTGATACAATACAAATGG
11	CCDC66_(2056)_F	GGGGACAACCTTTGTACAAAAAAGTTGAT AGAATAGAGAAACAAACAAAACAC
12	CCDC66_(2271)_R	GGGGACAACCTTTGTACAAGAAAGTTGT TTATGAAATGCCTCTGTTTTGAGAGAGG
13	CCDC66_(2272)_F	GGGGACAACCTTTGTACAAAAAAGTTGAT CCAGAAATTTTTCATTCATCTCATC
14	CCDC66_(2532)_R	GGGGACAACCTTTGTACAAGAAAGTTGT TTAGGATGATTCAGAAATCCCAGATGAT

15	CCDC66_(2592)_R	GGGGACAACCTTTGTACAAGAAAGTTGT TTAAAATGAAGAACCAAACTCTCTTC
16	siRNA resistant CCDC66_F	TTAGAAAAAGAAAACAATCGGTGTAATGAC CAGTGCAACCAATTTACAAGAATAGAGAAA CAAACAAAACACATGAAGA
17	siRNA resistant CCDC66_R	TCTTCATGTGTTTTGTTTGTCTCTATTCT TGTAATTGGTTGCACTGGTCATTACACCG ATTGTTTTCTTTTCTAA
CCDC66 siRNA: CAGTGTAATCAGTTCACAATT		

Appendix B

Antibodies used for immunofluorescence		
Rabbit anti-CCDC66	A303-339A; Bethyl Laboratories, Inc.	1:500
Mouse anti-CCDC66	SAB1408484; Sigma-Aldrich	1:750
Goat anti-PCM1	sc-50164; Santa Cruz Biotechnology	1:1000
Mouse anti- α -tubulin	GTU-88; T5326; Sigma-Aldrich	1:4000
Mouse anti-GFP	3e6; A-11120; Invitrogen	1:750
Mouse anti-Myc	9e10; M4439; Sigma-Aldrich	1:500
Rabbit anti-CEP290	ab84870; Abcam	1:1000
Mouse anti-polyglutamylated tubulin	GT335; AG-20B-0020-C100; Adipogen	1:500
Rabbit anti-Arl13b	17711-1-AP, Proteintech	1:100
Mouse anti- acetylated tubulin	clone 6-11B, 32270, Thermo Fischer	1:10000
Mouse anti- α tubulin	DM1A, Sigma	1:1000
Rabbit anti- CEP152	A302-480A; Bethyl Laboratories, Inc.	1:500
Mouse anti-p150 ^{glued}	610473BD; Biosciences	1:1000
All secondary antibodies are AlexaFluor 488-, 568- or 633-coupled.		1:2000
Antibodies used for western blotting		
Rabbit anti-CCDC66	sc-102420; Santa Cruz Biotechnology	1:2500
Mouse anti-Myc	sc-789; Santa Cruz Biotechnolo	1:2000
rabbit anti-CEP290	ab84870; Abcam	1:1000
Rabbit anti-CEP72	19928-1-AP; Proteintech	1:500
Rabbit anti-GFP	Hatch et al., 2010	0.15 μ g/ml
Rabbit anti-PCM1	19856-1-AP; Proteintech	1:1000
Rabbit anti-p38	sc-535; Santa Cruz Biotechnology	1:2000
Mouse anti-p150 ^{glued}	610473BD; Biosciences	1:1000
Rabbit anti-BBS4	12766-1-AP; Proteintech	1:500
Mouse anti-beta-actin	60008-1-Ig; Proteintech	1:10000
Mouse anti- α tubulin	DM1A, Sigma	1:1000