Investigating the roles of a few centrosomal proteins in ciliogenesis/maintenance

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled **"Investigating the roles of a few centrosomal proteins in ciliogenesis/maintenance"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Merrin Vincent** at National Center for Biological Sciences, Bangalore under the supervision of **Dr. Swadhin C Jana**, NCBS-TIFR, India (& IGC-FCT, Portugal) during the academic year 2022-2023.

Dr. Swadhin Chandra Jana

Date: 10 April, 2023

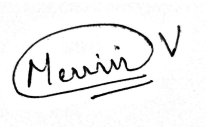
Committee:

Dr. Swadhin C Jana

Dr. Richa Rikhy

Declaration

I hereby declare that the matter embodied in the report entitled **"*Investigating the roles of a few centrosomal proteins in ciliogenesis/maintenance*"** are the results of the work carried out by me at the National Center for Biological Sciences, Bangalore under the supervision of **Dr. Swadhin C Jana**, NCBS-TIFR, India & IGC-FCT, Portugal during the academic year 2022-2023 and the same has not been submitted elsewhere for any other degree.



Merrin Vincent

Date: 10-04-2023

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Abbreviations

Abbreviations	Definition	Abbreviations	Definition
MTs	Microtubules	PC-1	polycystin-1
IP	Immunoprecipitation	NPHP	Nephronophthisis
RPE1	Retinal Pigment Epithelium	MCPH	Microcephaly
MDCK	Madin-Darby Canine Kidney	CPAP	centrosomal P4.1-associated protein
Cnn	Centrosomin	UAS	Upstream Activation Sequence
Pde1c	Phosphodiesterase 1C	TTBK2	Tau tubulin kinase 2
Sas4	Spindle assembly abnormal 4	DMEM	Dulbecco's Modified Eagle Medium
Alms1	Alstrom syndrome 1	FBS	Fetal bovine serum
SAS6	Spindle assembly abnormal protein 6	BSA	Bovine serum albumin
Dm	Drosophila melanogaster	RT	Room Temperature
PCM	Pericentriolar Material	PFA	Paraformaldehyde
CEP135	Centrosomal protein 135 kDa	ARL-13	adenosine diphosphate ribosylation factor-like protein 13
CEP164	Centrosomal protein 164 kDa	APS	Ammonium Persulfate
PCNT	Pericentrin	TEMED	Tetramethylethylenediamine
RNAi	RNA interference	GFP	Green fluorescent protein
PLP	Pericentrin-like protein	BL#	Bloomington number
Y-tubulin	Gamma-tubulin	Chat	Choline acetyltransferase
SPD-2	Spindle-defective protein 2	PMSF	phenylmethylsulfonyl

			fluoride
CDK5RAP2	cyclin-dependent kinase 5 regulatory subunit-associated protein 2	HCl	Hydrochloric acid
BB	Basal body	NaCl	Sodium chloride
TZ	Transition Zone	NP-40	nonionic polyoxyethylene
TF	Transition Fibre	SDS	Sodium dodecyl sulfate
IFT	Intraflagellar transport	DTT	Dithiothreitol
CDK	cyclin-dependent kinase	SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
PLK	Polo-like kinases	TBST	Tris-buffered saline with triton x
CCPP-1	Cytosolic carboxypeptidase protein 1	ECL	Enhanced chemiluminescence
HDAC6	Histone deacetylase 6	FA/AA	formaldehyde/ acrylamide
PCD	primary ciliary dyskinesia	mCD8GFP	mouse lymphocyte marker CD8
ADPKD	Autosomal dominant polycystic kidney disease	MS	Mass Spectroscopy

Abstract

Centrioles are cylindrical structures, which play an instrumental role in forming centrosomes and cilium. Centrosomes are critical for the spindle pole formation during mitosis in the cell cycle. During ciliogenesis, usually one of the two centrioles of a centrosome uses specially designed appendages at its distal end to cohere to ciliary vesicles and the cell membrane, forming cilia. Both cilia and centrosomes operate as signalling hubs and are naturally associated with cell cycle progression, cell proliferation, and differentiation. Understanding the operative linkages between centrosomes and cilia are essential because perturbations in these structures have been involved in many diseases, counting cancer and ciliopathies, e.g., Retinal degeneration, Alstrom syndrome and others. However, our knowledge on the molecular mechanisms of centrosome-to-cilium conversion and cilia maintenance has just begun to be explored. The primary challenge exists in designing applicable experimental approaches to distinguish centrosome/cilia biogenesis and maintenance. In this project, we aim to study this conversion of centrosome-to-cilium in mammalian cells through different techniques such as immunofluorescence and expansion microscopy, and genetic perturbations. We also aim to discover new novel interactors by Immunoprecipitation(IP) of important centrosomal/ciliary proteins from Drosophila non-ciliated cells vs ciliated cells, followed by Mass-Spectrometry (MS). Then we developed a strategy for examining the role of three ciliopathy-associated centrosome/ciliary proteins in cilia maintenance by conditional knockdown studies in fruit flies using excellent genetically tractable approaches. By using multifaceted techniques on long-lived ciliated cells in the fruit fly and mammalian cultured cells, my work helped to highlight the following points: a. we could follow Centrosome-to-Cilium conversion in mammalian cells (using confocal and expansion microscopy), b. IP, followed by MS studies of three proteins centrosome/cilium proteins identified a few potential common interactors between all the three proteins and its functions have to be further studied in future and c. we identified at least two of the centriolar proteins knocked down are critical for ciliary function maintenance in adult flies.

Acknowledgments

I express my sincere gratitude to **Dr. Swadhin C Jana** for granting me the opportunity to work in his lab. I am extremely thankful of his support, valuable time and guidance that he provided me all throughout this project. Additionally, I would like to extend my thanks to **Dr. Richa Rikhy**, my expert member, for her suggestions. I am grateful to my lab members **Ashmita Kumari, Minita Desai, Pranjali Priya** and **Ramsi** for their exceptional teachings, scientific and technical inputs, and for answering my numerous queries during the project. Working alongside **Anupama, Sagnik, Riddhi, Diksha, Lavina, Anwesha, Ragini** and **Girish** has been an incredible experience, and I cannot imagine working with a better group of individuals. I also acknowledge the fly facility,lab support, lab kitchen, CIFF (Central Imaging & Flow Cytometry Facility) and all other facilities at **NCBS Bangalore**. I would also like to thank Bloomington Drosophila Stock center and Vienna Drosophila resource center for providing the right flies for the project. Last but not least, I am grateful to my family and friends for their unwavering support and motivation throughout.

Contributions

Contributor name	Contributor role
SCJ	Conceptualization Ideas
MV, SCJ, MD, RN, AK	Methodology
-	Software
MV, SCJ, RN	Validation
-	Formal analysis
MV	Investigation
SCJ, Bloomington,VDRC	Resources
MV, SCJ	Data Curation
MV, SCJ, PP	Writing - original draft preparation
MV, SCJ	Writing - review and editing
MV, SCJ, PP, MD	Visualization
SCJ	Supervision
SCJ	Project administration
SCJ	Funding acquisition

**Abbreviated names are listed in the table next page.*

SCJ	Dr. Swadhin C Jana
MV	Merrin Vincent
MD	Minita Desai
RN	Ramsi Nilopher
PP	Pranjali Priya
AK	Ashmita Kumari

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

Chapter 1 Introduction

Centrosome structure

Centrosomes contain a mature or mother centriole and a daughter centriole which are placed orthogonally, along with pericentriolar material (PCM) surrounding them. The centrioles have a barrel-shaped structure, with symmetric distribution of nine triplets in MT located at the core of PCM, as shown in Figure 1. However, there are exceptions to this arrangement in *Drosophila melanogaster* and *Caenorhabditis elegans*. In *C. elegans*, the centriole is made of singlet MTs, while in *Drosophila* embryos, the centrioles contain doublets of microtubule (Brito et al., 2012; Debec et al., 2010; Enjolras et al., 2012).

The centrioles have MTs that are symmetrically distributed radially and originate from a structure called cartwheel. The cartwheel is composed of SAS6 and Bld10/CEP135 proteins and at the proximal region of the daughter centriole is called procentriole, as initially observed in *Paramecium* and *Chlamydomonas*. This arrangement ceases at the mature centriole (Brito et al., 2012). Nine pair of radial spokes in the centriole create linkers among the central hub and the microtubule triplets in the cartwheel (Robbins et al., 1968).

Additionally, mature centriole is found to be with structures like sub-distal and distal appendages, protruding from microtubule triplets. The former serve as supporting MTs and their number, width, and arrangement can vary significantly. On the other hand, the distal appendages exhibit a constant and radially symmetric arrangement of nine-fold, and they play a crucial role in attaching the mother centriole to the cell membrane. In our study (Figure 1), we utilized a protein called CEP164, which is part of the distal appendages (Jana, 2021).

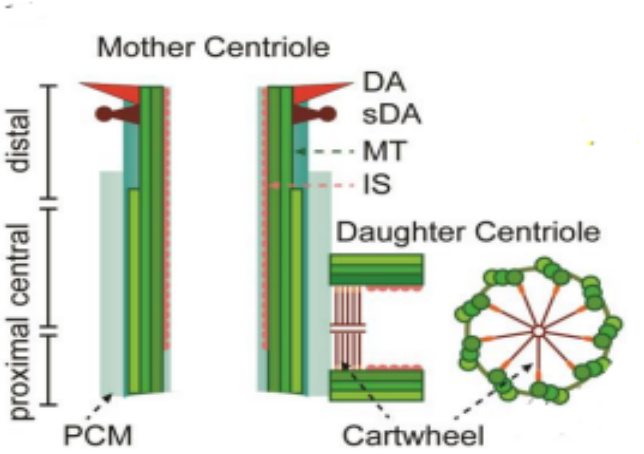


FIGURE 1: The scheme of ultrastructure of the centrosome with longitudinal and cross section views. The figure shows the longitudinal view(left) and cross sectional view(right) of the structure of centriole. Different parts of centrioles including DA (Distal appendages), sDA (sub distal appendages), MTs (microtubules), PCM (peri centriolar matrix), IS (inner segment) are marked in the figure (Jana, 2021)

The first electron microscopy images revealed the presence of centrosomes inside cells with pericentriolar material as a heavily stained, shapeless mass surrounding the clear centrioles (Robbins et al., 1968). The pericentriolar structure acts like a stage for protein-complexes that control spindle assembly, organelle trafficking, and protein degradation (Czarnecki & Shah, 2012). Pericentrin (PCNT) was among the first components of the PCM to be identified in human cells. Subsequent studies involving mass spectrometry, localization screens and large-scale RNAi in *Drosophila*, *C.elegans*, and human cells revealed distinct proteins associated with the centrosome. Among these were core PCM components such as PCNT/D-PLP, Y-tubulin, CDK5RAP2/Cnn, Cep152/Asterless, Cep192/SPD-2, and SPD-5, which were discovered in various organisms (Andersen et al., 2003; Sönnichsen et al., 2005).

Apart from being a part of centrosomes, centrioles also play a dual role in animal cells by aiding in the formation of cilia. In cells with cilia, the mature centriole of the centrosome forms a complex called the basal body (BB), which serves as a template for the assembly of the cilium or flagellum.

Cilia structure

Cilia are organelles in eukaryotic cells that are compartmentalized and composed of microtubules (MTs). They extend from the cell surface, and the fibrillar bundle of the cilium is derived from the mother centriole. Cilia play a role in various processes, for

example sensing, cell motility, fluid flow, and sensory modulation (Nigg & Raff, 2009).

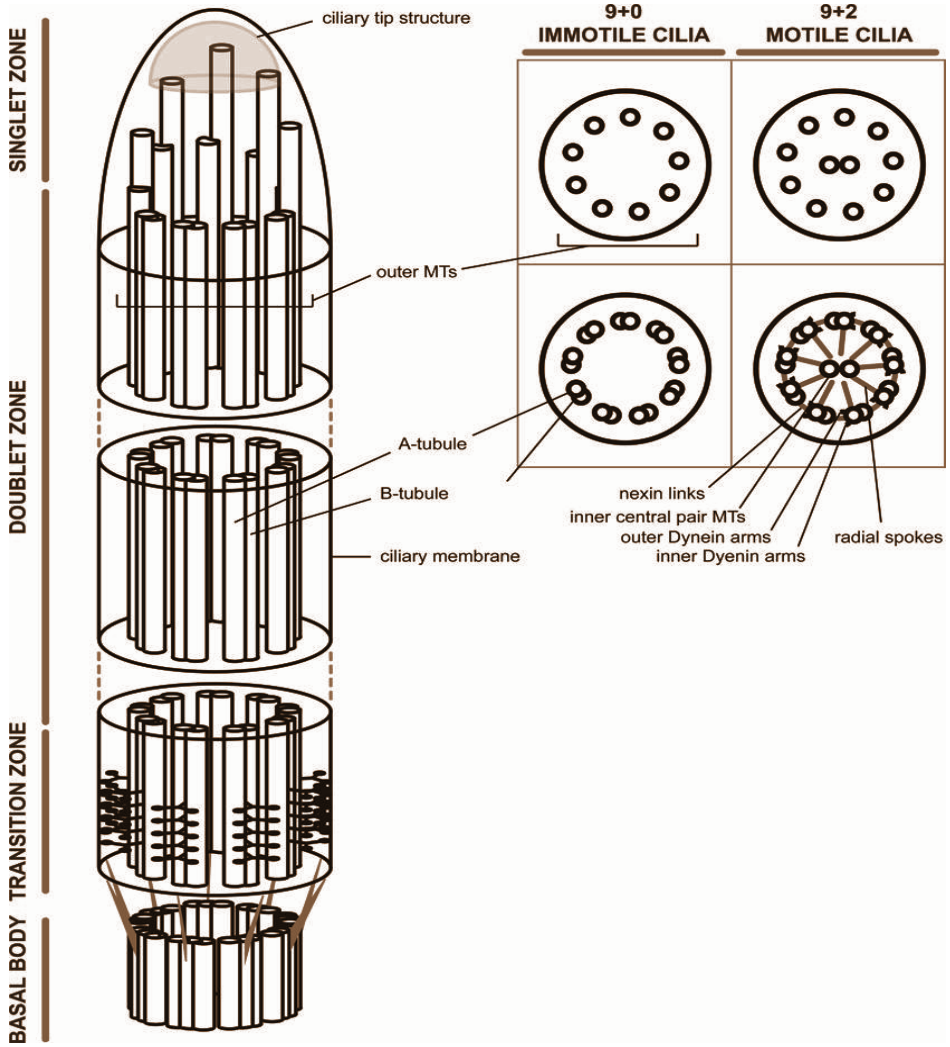


FIGURE 2: Scheme of the ultrastructural organization of a cilium. The figure depicts the structure of cilium and its differences in motile and non motile condition As shown in figure, the microtubular arrangement is different in both conditions i.e 9+2 arrangement in motile cilia and 9+0 arrangement in non motile cilia. The figure depicts different parts of cilia longitudinally and in cross section (adapted from Czarnecki & Shah, 2012).

The cilia can be immotile or motile. (Satir & Christensen, 2007). Cilia can generally be categorized into two structural classes: 9+2, which have nine doublets of MTs radially arranged around a central pair of MTs, and 9+0, which do not have the central pair of MTs(Hoyer-Fender, 2010). The 9+2 structure of cilia is typically associated with motility, while the 9+0 structure is generally linked to immotility.

Within the 9+2 structure, radial spokes and dynein arms typically exist, as shown in Figure 2. Cilia motility is driven by the dynein arms that proceed towards the minus end of microtubules, leading to the bending of the cilia. However, there are

exceptions to this general pattern of mobility associated with the 9+2 structure. For instance, cilia in embryonic node cells in mice, that are involved in establishing left-right body symmetry, are deficient of the central pair of MTs and have a 9+0 structure, yet are still motile. Another case is the cilia in the olfactory neurons in *D. melanogaster,* which have a 9+2 structure but are immotile (Hoyer-Fender, 2010).

The ciliary structure can be divided into ciliary shaft and the ciliary base. The ciliary shaft includes the transition zone (TZ), and the axoneme, whereas the ciliary base consists of a basal body as shown in Figure 2 (adapted from Czarnecki & Shah, 2012). The BB, which is derived from the mother centriole, is located at the foot of the cilium and serves as the scaffold for the formation of the axoneme. The TZ is present at the base of the cilium and contains transition fibers (TF), Y-linkers, and doublet MTs. These components act as a ciliary gate, regulating the entry of molecules into the cilium (Debec et al., 2010). Thus, transition zone connects the doublet MTs of the axoneme and the triplet MTs of the BB.

Centriole Biogenesis

Centriole duplication is coordinated with cell cycle development to ensure that each new cell receives an appropriate number of chromosomes and pair of centrioles in a centrosome (Fırat-Karalar & Stearns, 2014; Zitouni et al., 2014).]. The two pairs of centrioles are a mature mother centriole and a young daughter centriole. During the G1 phase, these two centrioles are joined by a flexible connector known as the centriole linker, which connects their proximal ends (Piel et al., 2000; Bornens et al., 1987). As the cell cycle progresses from G1 to S phase, daughter centriole, oriented perpendicularly to the MT wall, forms adjacent to the proximal side of the existing centriole. These procentrioles continue to elongate, still connected to their parent centrioles via the linker, throughout the S and G2 phases, reaching a length of about 400 nanometers (as shown in Figure 2). The two linked mature centriole is separated near the completion of the G2 phase, the centriole linker of the two old centrioles is cleaved, separating both centrosomes. Each centrosome now contains two centrioles, perpendicular to each other, and these control the formation of the bipolar mitotic apparatus. The individual new centriole detaches from its progenitor centriole and no longer has the cartwheel, which most probably gets degraded during mitosis. The resulting two centrioles in each daughter cell are connected via their proximal

ends using centriole linkers, thus finishing the centriole duplication process (Banterle & Gönczy, 2017; Jana, 2021; Loncarek & Bettencourt-Dias, 2018).There are two proteins ,Polo/Plk1 and Cnn which we are using in this study.For proper centrosome maturation, Polo/Plk1 starts the phosphorylation-dependent construction of a Cnn scaffold around centrioles. In contrast to phosphomimicking mutations, which enable Cnn to multimerize in vitro and spontaneously create cytoplasmic scaffolds in vivo that organise microtubules independently of centrosomes, mutations that block Cnn phosphorylation severely inhibit scaffold building and centrosome maturation(polo(Conduit et al., 2014)

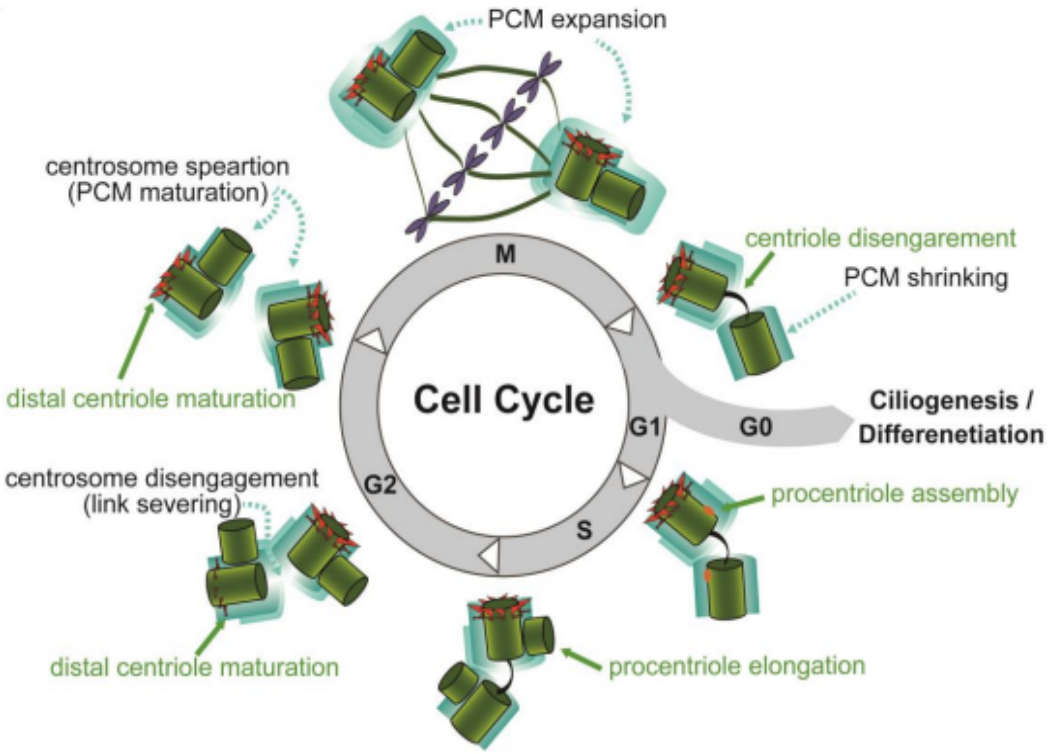


FIGURE 3: Scheme shows the centrosome duplication cycle in context of the cell cycle. The figure depicts the centriole duplication cycle and shows how it is coupled with different stages of the cell cycle. The centriolar duplication starts in S phase and its completed in (adapted from Jana, 2021)

Ciliogenesis

The process of cilia formation, known as ciliogenesis, involves several steps. Initially, cells must exit the mitotic cycle to allow the centrioles to be available for axoneme nucleation. Cilia biogenesis begins with the docking of membrane bound sacs called vesicles (from the Golgi) to the distal appendages of the mother centriole, followed

by the centrosome moving toward the plasma membrane. During this movement, many vesicles from the Golgi fused with the other vesicle already docked on the mature centriole. The transition zone (TZ) is formed by assembling from the mother centriolar distal end surrounded by a vesicular membrane. Subsequently, the mature centriole attached to the plasma membrane using distal appendage, followed by fusion of vesicles to the plasma membrane, and the microtubule based cilium begins to grow (Avasthi & Marshall, 2012; Debec et al., 2010; Pedersen et al., 2016)

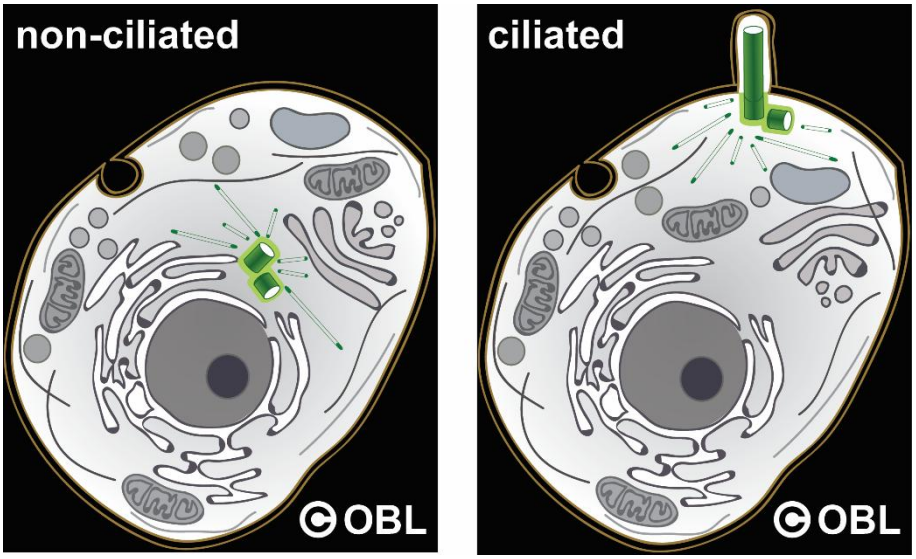


FIGURE 4: Sketches of diverse organelles, including centrosome and cilium in non-ciliated and ciliated cells.In non ciliated cells(left), centrosome is positioned near nucleus and as the ciliogenesis proceeds the centrosome moves towards cell membrane and docks to it.The ciliated cell thus formed is shown in right side image(figure taken from OBL lab website).

Cilia formation proceeds through extension from the cilium tip. Typically, at the basal body, ciliary proteins are loaded as cargo onto the axonemal microtubules via intraflagellar transport (IFT). Anterograde transport of IFT trains from the foot to the cilium tip is facilitated by kinesin-2 motors in association with IFT-B complex proteins, while retrograde transport from the apex to the foot of the cilium is driven by IFT dynein-2 motors along with the IFT-A complex proteins. Most of the cargoes are transported to the growing tip of the cilia prior to their release for fusion into the ciliary structure. Some cargoes may also be delivered during anterograde transport (Carvalho-Santos et al., 2012).

Centrosome Maintenance

For a long time, centrioles were regarded as very stable structures that could endure different types of stress, including the depolymerization of microtubules induced by drugs and cold temperatures, as well as mechanical forces during mitosis (Belmont et al., 1990; Kochanski & Borisy, 1990). Research on *C. elegans* has revealed that centrioles can persist through multiple rounds of cellular division, even following mating with a sperm carrying a solitary paternally-inherited centriole (Delattre & Gönczy, 2004). But centrosomes are lost from most of the metazoan oocytes and can become inactivated in certain cell types during differentiation, such as muscle, neuronal, and epithelial cells. In these cells, centrosomes lose their microtubule-organizing capacity due to the loss of PCM proteins, which can result in MT nucleation and anchoring occurring along the apical-basal axis (Nguyen et al., 2011; Sanchez & Feldman, 2017; Manandhar et al., 2005; Stiess et al., 2010; Tassin et al., 1985). Neuronal cells can extend axons even without active centrosomes, and myocyte differentiation results in the absence of centrosomes in muscle fibers, with PCM proteins instead accumulating at the nuclear periphery for MT nucleation. In many epithelial cell types, MT nucleation can be inactivated or anchoring abolished, leading to MTs generated along the apical-basal axis. These findings propose that the centrosome is subject to a homeostatic maintenance program which can be restricted, giving rise to various microtubule arrays (Przybylski, 1971; Srsen et al., 2009).

The stability of the centrosome is maintained through a homeostatic maintenance program that is controlled by cell cycle regulators such as CDKs and PLKs (Muroyama et al., 2016; Yang & Feldman, 2015). This program is dependent on crucial components of the centrosome, such as the centriole walls, the PCM,and the centriole cartwheel. The cartwheel and PCM are excessive in giving stability to centrioles, compensating for themselves in centrosome protection. However, there may be differences in the degree of redundancy among different species and tissues. The renewal of centrosome components is critical for homeostatic maintenance, as demonstrated by studies on S-phase-arrested Drosophila cells. The interplay between the centriole walls, the cartwheel, and the PCM plays a role in supporting centriole stability (Pimenta-Marques et al., 2016). The post-translational

modifications of tubulin and the function of centrobin are also important in this process (Bobinnec et al., 1998). Kinases, such as CDK1 and PLK1, regulate both centrosome function and maintenance, making it hard to distinguish between the two mechanisms (Muroyama et al., 2016). The existence of centrioles is crucial for centrosome upkeep and nucleation in differentiated and interphase cells. The lack of centrioles leads to the inactivation of the centrosome and disappearance in Drosophila. More research is needed to comprehend how this process is limited at the transcriptional and post-transcriptional levels, and the dynamic nature of the centriole proteins and PCM. In oogenesis, the enforced confinement of Polo to centrioles leads to the maintenance of PCM and the existence of centrioles in the oocyte, which emphasizes the need for the active integration of newly produced Polo and PCM constituents into the centrosome (Werner et al., 2017).

Cilia Maintenance

The effective operation of cilia is dependent on both its structural and signaling integrity. To ensure the synthesis and transportation of its components, it is crucial to make modifications that increase its longevity. Tubulin post-translational modifications are associated with stabilizing the axonemal microtubules within cilia. Additionally, mutations in tubulin deglutamylase CCPP-1 have been linked to the degeneration of axonemal microtubules (O'Hagan et al., 2011; Bosch Grau et al., 2017). Intraflagellar Transport (IFT) is critical in maintaining cilia integrity and function(Milenkovic et al., 2015; Hu et al., 2010; Ye et al., 2013; Trivedi et al., 2012). Continuous intraflagellar transport (IFT) is crucial for maintaining the structural and functional integrity of cilia, even upon complete formation. Inhibition of IFT in green algae resulted in the shortening of previously functional cilia, demonstrating the necessity for constant supply and renewal of ciliary proteins (W. F. Marshall et al., 2005; W. F. Marshall & Rosenbaum, 2001). Although IFT is essential for the function of cilia in most systems, there are exceptions such as Trypanosoma, where IFT is only necessary for flagellar function and not for structural integrity. Additionally, IFT is not required for biogenesis or maintenance of Drosophila sperm and mature mouse sperm (Fort et al., 2016; Han et al., 2003; Sarpal et al., 2003). Controlling the maintenance of cilia may have implications for controlling the cell cycle, as the disassembly of cilia is necessary for the progression of some quiescent ciliated cells

into S phase. Important factors in initiating the disassembly of cilia include Aurora A, PLK1, and HDAC6. Interfering with the cilia maintenance program may also contribute to the disassembly of cilia during the cell cycle (Inoko et al., 2012; Plotnikova et al., 2012; Wang et al., 2013).

Impact of cilia and centrosome in human pathologies

Numerous human cancers, including those affecting the breast, prostate, lung, colon, and brain, have been shown to frequently exhibit centrosome abnormalities. Additionally, several brain development disorders have been linked to proteins that regulate centrosomes and microtubules (Debec et al., 2010; Delattre & Gönczy, 2004).

Ciliary assembly errors have been associated with Kartagener syndrome, which is a disease resulting from defects in motile cilia. The condition is characterised by bronchitis, sinusitis, and sperm immobility due to defects in mucus-clearing cilia. These rare diseases are collectively known as primary ciliary dyskinesias (PCD), and defects in ciliary ultrastructural have been observed in around 90% of PCD cases. It has been demonstrated that ciliary movement is crucial for generating directional flow in the embryos in very early stages, which is necessary for starting the typical left-right developmental mechanism, as evidenced by body symmetry changes seen in about 50% of PCD patients (Bettencourt-Dias et al., 2011; Zariwala et al., 2007).

Over the years, the number of "ciliopathies" has increased due to the identification of mutations in various genes that affect the structure of the cilia and its function, or impact gene products at the centrosome and/or primary cilia. This group includes rare disorders such as nephronophthisis, polycystic kidney disease, Joubert, Bardet-Biedl, retinitis pigmentosa, and Meckel syndromes. These conditions can affect many organs, including the retina, bones, kidney, liver and brain, and can be associated with clinical complications such as blindness, renal failure, liver fibrosis, diabetes and obesity (Zariwala et al., 2007).

The formation of centrosomes requires the recruitment of pericentriolar material (PCM) to centrioles. Any disruption in this process may lead to centrosome

abnormalities, resulting in diseases such as autosomal recessive primary microcephaly (MCPH), characterized by a reduction in brain growth (Zheng et al., 2014). The CPAP/Sas-4/MCPH6, some of the conserved centrosomal proteins, play a crucial part in centriole development, acting as a frame for PCM complexes in cytoplasm to attach and connect to centrioles, ultimately leading to the development of functional centrosomes (Gopalakrishnan et al., 2011).

Alström Syndrome is a monogenic, recessive ciliopathy due to the mutations in ALMS1 gene, and it affects multiple systems including childhood obesity, hearing loss, early cone-rod retinal dystrophy and blindness, type 2 diabetes mellitus, cardiomyopathy, fibrosis, and multiple organ failure. Although the exact role of ALMS1 in these processes is not fully understood yet, it has been found to be involved in cell cycle regulation, endosomal and ciliary transport, and other cellular processes (Marshall et al., 2015).

Tau tubulin kinase 2, also known as Serine/threonine kinase a crucial ciliogenesis regulator, controls the process by attaching to the basal body's distal end and encouraging the removal of CCP110, which covers the mother centriole. This causes the recruitment of IFT proteins, which construct the ciliary axoneme, to take place(Bouskila et al., 2011).There has been several clinical reports of patients with late onset ciliopathies with mutations specific to ALSM1,SAS4 and TTBK2 ((Lo et al., 2019).Hence, in the project we aim study their role in ciliogenesis and its maintenance.

Since ciliopathies are known to be linked with defects in centrosome and ciliary structures and functions, studying how cilia are assembled and maintained are of prime importance in understanding physiology and diseases. Our knowledge on the molecular mechanisms on centrosome-to-cilium conversion and cilia maintenance are quite limited. The main challenge exists in designing appropriate experimental approaches to distinguish centrosome/cilia biogenesis and maintenance. Therefore, in this project aims to establish an appropriate experimental design to study the role of selected centrosomal protein in centrosome to cilia conversion. Figure 5 implicates the cellular process defined in my project.I aim to study the process of

ciliogenesis in normal cells and the process of cilia maintenance in the case of perturbation.

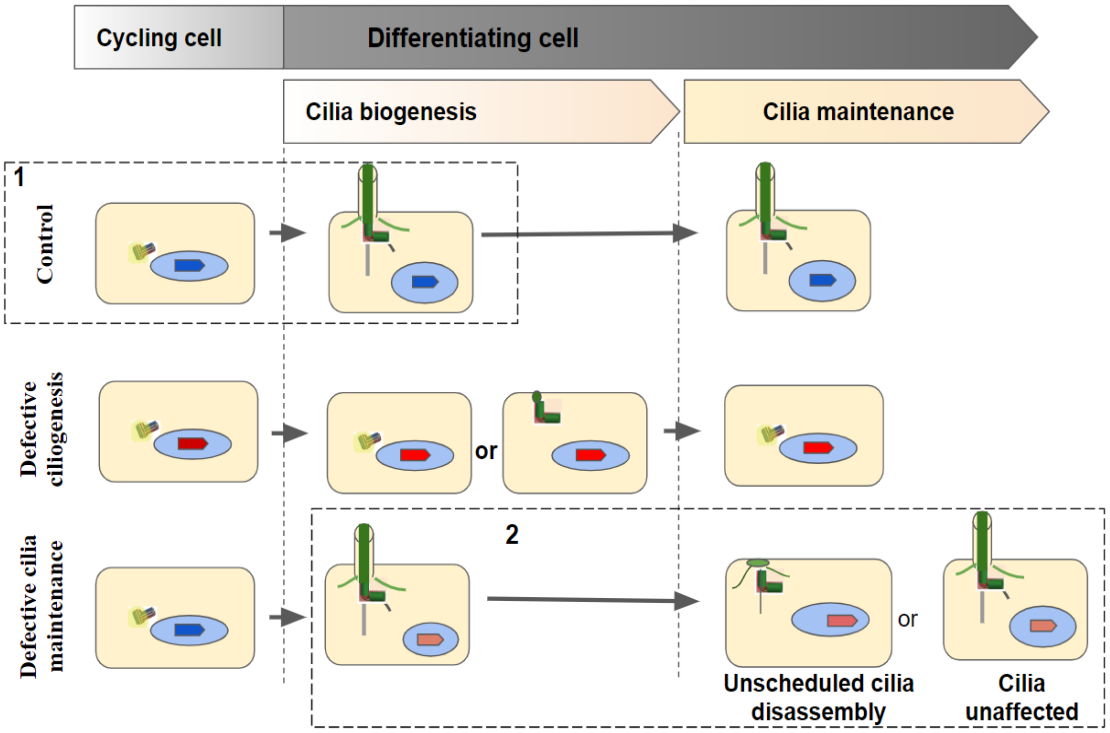


FIGURE 5: The scheme of studies in the project. First part of the study deals with the study of centrosome to cilia conversion in normal RPE1 cells (box marked with number 1 in Figure). Second part deals with the study of how the formed cilia are maintained inside the cells (box marked with number 2). While for Part 1 we used cultured mammalian cells and tissues collected from the fruit fly, *Drosophila* was used as the model organism to perturb and study the changes in Part 2.

Thesis Outline

Aim 1. To establish a combination of immunofluorescence, expansion microscopy and biochemical interaction techniques to describe centrosomal and ciliary components localisation during centrosome-to-cilium conversion (and if possible in maintenance).

1. Task 1: Standardisation of ciliogenesis in hRPE-1 cells and Visualisation of centriolar and ciliary proteins using immunoflourescence assay.

   We first attempted to screen a set of molecules for its localisation at different time points mainly from the point of induction of cilia formation (by serum starvation) to the time points at which the majority of cells would have completed the docking of centriole to plasma membrane. We used, immortal human Retinal Pigment Epithelial-1 (hRPE-1) cell line. These cells can be induced to form cilia upon serum starvation. The main focus was to visualise centriole/pericentriolar materials (PCM) movement and their docking to plasma membrane for the formation of cilia.

2. Task 2: Visualisation of ciliogenesis using expansion microscopy

   Following this our plan was to further study the centrosome structure/components during the same time-points using expansion microscopy to visualise these events with increased (at least 4 times in one dimension) resolution.

3. Task 3: IP-MS approaches for interaction studies

   We also tried Immunoprecipitation followed by mass spectroscopic analysis of known prominent centrosomal proteins such as Polo, Cnn and Pde1c protein from Drosophila tissues to find new interactors which can have a role in centrosome to cilium conversion or its maintenance. Polo and Cnn are known to have a role in the formation of spindle fibre in mitotic cells and Pde1c

belongs to the class of proteins called phosphodiesterases which is important in sensory functioning.

Aim 2. To study the role of three centrosomal proteins in ciliogenesis and maintenance in *Drosophila*

1. Task 1: Negative geotaxis assay for knockdown analysis

   We aimed to study the role of some centrosome/ciliary proteins in aforementioned both processes, independently. For this, we used the UAS-GAL4 system in *Drosophila*. Gal4s expressed under different promoters specific to Centrosome-to-Cilium conversion (e.g., GAl4^{Tubulin}) and cilia maintenance (e.g., for ciliated neurons GAl4^{ChAT}). To analyse the effect of this knockdown we used an assay called called negative geotaxis assay. The assay was standardised according to our knockdown studies with the control mCherryRi knockdown flies.

2. Task 2: Ubiquitous Knockdown

   Different RNAi lines for three centrosome/cilium proteins of our interest and mCherry (for internal control)) were used to express antisense RNAs by respective GAL4 expression. For ubiquitous knockdown, Gal4 was expressed under Tubulin promoter and flies with knockdowns (and controls) were used to study the animal behaviour and ciliated sensory neurons morphology at 1-2 days after eclosion from pupal stage.

3. Task 3: Tissue specific knockdown

   For tissue specific knockdown, Gal4 was expressed under Choline acetyl transferase promoter and flies with knockdowns (and controls) were used to study the animal behaviour and ciliated sensory neurons morphology at 1-2 days,9-10days and 19-20days after eclosion from pupal stage. is a

transferase enzyme responsible for the synthesis of the neurotransmitter acetylcholine(Oda, 1999)

NOTE: All three proteins are centrosomal/ciliary proteins whose mutations are implicated in early as well as late onset ciliopathies. We used flies with knockdown of mCherry as our control for this study as mCherry protein is not naturally expressed in *Drosophila*.

Chapter 2 Materials and Methods

Cell culturing, synchronisation and serum starvation

RPE-1 cells were cultured in DMEM F-12+ Glutamax medium (GIBCO) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Thermo Fisher) at 37°C and 5% CO2.

For synchronisation, 5000 cells were plated on 12mm coverslips for 12-16 hrs.These coverslips were then incubated with 3mM thymidine(Sigma) rich fresh media for 19 hrs.The coverslips were again incubated with the 3mM thymidine rich fresh media for 16hrs after a 4-8 hrs release in normal fresh media.After releasing them again for 12 hrs in fresh media, these coverslips were fed with serum free media(normal media without 10% FBS) until fixation.These coverslips are fixed at 0hr, 6hrs, 12hrs, 24hrs and 72hrs either with 4% formaldehyde for 15 mins at RT or methanol for 7 mins at -20^{o}C.

Immunofluorescence assay

Fixed coverslips of RPE1 cells were put in blocking solution(1%BSA(Puregene), 0.1% TritonX(Sigma) in PBS(Sigma)) for 1hr after 2 washes with PBS.Then the coverslips were incubated with respective primary antibody combinations(Table:1) solutions made in blocking for 16 hrs in 4^{o}C.After 3 washes with blocking solution ,5 mins each,the coverslips were incubated with secondary antibodies solution made in blocking solution along with phalloidin for second combination for 2hrs in RT.After 2 washes with 0.1% triton X in PBS ,the coverslips were incubated with Dapi(Sigma) for 15 mins in RT followed by two PBS washes.The coverslips were then mounted in 70%glycerol solution and sealed with nail polish.These were then imaged using Olympus FV3000 microscope within 2-3 days.

Combination	Fixation	Primary antibody	Dilution	Secondary antibody/Chemicals	Dilution
1	4%PFA	Mouse Acetyl alpha tubulin (Sigma)	1:1500	Donkey anti mouse 488 (Invitrogen)	1:1000
		Rabbit Arl13b (Protein Tech)	1:1500	Donkey anti mouse647 (Invitrogen)	1:1000
		Goat Cep164 (Protein Tech)	1:1000	Donkey anti Goat 568 (Invitrogen)	1:1000
2	100%methanol	Rabbit ɣ tubulin (Sigma)	1:250	Donkey anti Rabbit 647 (Invitrogen)	1:1000
		Mouse Acetyl alpha tubulin (Sigma)	1:1500	Donkey anti mouse 488 (Invitrogen)	1:1000
		-	-	Phalloidin(Invitrogen)	1:1000

Table 1: List of antibodies used in immunostaining at different time points in centrosome to cilia conversion in RPE1 cells.

Expansion Microscopy

$5x10^4$ RPE1/MDCK cells were plated in a 12mm coverslip for 12-16hours in a 24 well plate.These were then incubated with 1ml of 1.4% formaldehyde(Sigma)/2%acrylamide(Sigma) solution in PBS for 5 hours at 37^{o}C.The required amount of monomer solution(19% Sodium acrylate,10% acrylamide,0.1% BIS(Sigma) in 10xPBS(pH 7.4) were prepared around 30mins before gelation step.The humid chamber with parafilm,kept in -20^{o}C for 15mins, was then placed on ice.5 ul of 10% APS(Thermofisher) and 10% TEMED(Thermofisher) were added to 90 ul aliquots of monomer solution just before the incubation with coverslips.The coverslips were incubated with 35ul of this final monomer mix on parafilm for 5mins on ice and then 1 hr at 37^{o}C.After that the coverslips with the gel were transferred to a 6 well plate filled with denaturation buffer(200mM SDS(Sigma), 200mM NaCl(Sigma) and 50mM Tris(Sigma) in water,pH 9).These were then agitated till the coverslips detach from the gel.The gels are then transferred to 1.5ml eppendorf filled with fresh denaturation buffer and heated at 95^{o}C for 1hour 30mins.These were then transferred to chambers filled with distilled water,which is changed in every 15 mins for three times and kept for 12-16 hrs of expansion in fresh distilled water.Then a piece of gel was

cut,transferred to 24 well plate and incubated with 1xPBS for 30 mins with changing PBS solution after 15mins.These were then incubated with 250ul primary antibody solution(Mouse anti acetyl alpha tubulin(Sigma),1:100 dilution in 2%BSA (Purgene) in 1XPBS) for 12-16 hrs at 4^{O}C. Gels were then washed with 250 ul of 0.1% Tween20(Sigma) in 1xPBS for three times,10mins each in RT. The gels were then incubated with 250ul of secondary antibody(Donkey anti mouse 568(Invitrogen),1:100 dilution in 2%BSA in 1XPBS) for 2.5hrs at RT. Gels were then washed with 250 ul of 0.1%tween20 in 1xPBS for three times,10mins each in RT. These were then again incubated with distilled water,which is changed in every 15mins for three times and kept for 12-16hrs of second round expansion in fresh distilled water.These were then placed on coverslips attached small petriplates and imaged using Olympus FV3000 confocal microscope.

Immunoprecipitation assay

The assay was done to pull down 3 proteins-Polo,Cnn and Pde1c proteins from different drosophila tissues.IP was performed from both embryo and heads for Polo and Cnn and from heads for Pde1c according to their expression pattern. All these proteins were tagged with GFP. The flies used for pull down are listed in the table below.

IP	Protein	Fly used	Tissues used for IP	Tissue amount used for IP	Amount of Anti-GFP tagged beads used
1	Polo::GFP	w;PoloGFP;PoloGFP (BL#84275)	Embryo (0-6hrs)	1000 embryos	30ul
2	Polo::GFP	w;PoloGFP;PoloGFP (BL#84275)	Heads (0-1 days old flies)	800 Heads	10ul
3	Cnn::GFP	w;CnnGFP;+ (BL#60266)	Embryo (0-6hrs)	1200 embryos	15ul
4	Cnn::GFP	w;CnnGFP;+(BL#60266)	Heads (0-1 day old flies)	500 Heads	10ul
5	Pde1c::GFP	w;Pde1cGFP;+ (BL#63195)	Heads (0-2 days old flies)	1200 Heads	15ul
6	Control:Free GFP	w;UASGFP;$Gal4^{Tub}$/Tm6B	Embryo (0-8 hrs)	300 Embryos	10ul

7	Control:Free GFP	w;Gal4^{Chat19b}UASGFP	Heads (0-1 day old flies)	200 Heads	10ul

Table 2: List of proteins and the tissue in fruit flies used for IP.

The required tissue collected over 3-4 days (stored at -80^{o}C) were snap freeze in Liquid N_2 and homogenised using a homogeniser.To this homogenised mixture 500ul of Lysis buffer(wash buffer 1 with 1x protease inhibitor(Roche),1x phosphatase inhibitor(Roche) and 1mM PMSF(Roche)) was added, sequentially homogenising and spinning down(4C,13k rpm,5mins) after adding 125ul lysis buffer each time for 4 times. A part of the lysate was saved as input which was the added with same amount of 2x Laemmli,boiled at 95^{o}C and stored in -80^{o}C till Gel run.The remaining lysate was incubated with required volume of anti- GFP tagged magnetic beads (Chromotech) for 16hrs,in 4^{o}C at 35 rpm.A part of the solution was collected as Elute 1 after separating the beads using a magnetic stand .Then the beads were washed with wash buffer 1 for two times and part of each solution separated from beads are collected as Elute 2 and 3.The beads are then again washed with wash buffer 2 for two times.These were then added with 50ul of 2x laemmli and boiled at 95^{o}C for 5 mins.All elutes are added with equal amount of 2x Laemmli buffer and boiled at 95^{o}C for 5 mins and stored at -80^{o}C till western blot is done.

Wash buffer 1		**Wash buffer 2**	
Reagents	**Req conc.**	**Reagents**	**Req conc.**
Tris HCl pH8	50mM	Tris HCL pH 8	50mM
NaCl	250mM	NaCl	250mM
Np-40(Merck)	2%		
SDS(Sigma)	0.5%		
Na Deoxycholate(Sigma)	0.5%		
Digitonin(Sigma)	0.1%		
DTT(Clonetech)	1mM		
Both wash buffer 1 and 2 are adjusted to pH 7.2-7.4			

Table 3: Constituents for wash buffer 1 and 2 in IP.

Western blot Analysis

The samples were boiled at 95°C for 5 minutes and then loaded onto a 12% SDS-PAGE gel. The proteins were then transferred onto a PVDF(Merck) membrane using wet transfer at 0.1 A for 16 hours at 4°C. The membrane was then blocked with 5% Bovine Serum Albumin (BSA) and 0.1% Tween 20 in PBS for 1 hour at room temperature. The blot was then incubated with the Mouse anti GFP primary antibody (SantaCruz) for 12-16 hours at 4°C. Three 0.1% TBST (0.05 M Tris pH 7.4, 0.15 M NaCl, 0.1% triton X) washes of 10 minutes each were given. The blot was then incubated in HRP conjugated secondary antibodies against the mouse, followed by three 0.1% TBST washes. The membrane was then developed using Clarity ECL Western substrate (Biorad) and imaged using LasQuant 4000.

Knockdown studies

Two kinds of knockdown studies for three different centrosomal proteins were performed in the model organism *Drosophila melanogaster.* The following table shows the Uas and Gal4 lines used for the studies

Type of knockdown	**Knockdown protein**	**Uas line(RNAi line,Female)**	**Gal4 line (Male)**
Ubiquitous	Control	w;+;UasmCherryRi (BL#35785)	yw;+;Uasmcd8GFPGal4^{Tub}/Tm 3sb (BL#30030)
	ALMS1	w;UasALMS1RNAi;+ (VDRC#106051/KK)	yw;+;Uasmcd8GFPGal4^{Tub}/Tm 3sb (BL#30030)
	SAS4	w;UasSAS4RNAi;+ (VDRC#106025/KK)	yw;+;Uasmcd8GFPGal4^{Tub}/Tm 3sb (BL#30030)
	TTBK2Ri	w;UasTTBK2RNAi;+ (VDRC#45120/GD)	yw;+;Uasmcd8GFPGal4^{Tub}/Tm 3sb (BL#30030)
Tissue specific	Control	w;+;UasmCherryRi (BL#35785)	w;Gal4^{Chat19b}UasGFP
	ALMS1	w;UasALMS1RNAi;+ (VDRC#106051/KK)	w;Gal4^{Chat19b}UasGFP
	SAS4	w;UasSAS4RNAi;+ (VDRC#106025/KK)	w;Gal4^{Chat19b}UasGFP
	TTBK2Ri	w;UasTTBK2RNAi;+ (VDRC#45120/GD)	w;Gal4^{Chat19b}UasGFP

Table 4: List of flies used in knockdown studies.

The crosses between virgin females from RNAi lines and the males from Gal4 lines were put in the ratio 3:1 respectively.For ubiquitous knockdown,all GFP positive pupas were collected on the first day of pupa formation and kept for 5 days for eclosion.All 0-1 day old flies or 5 days unenclosed pupa were then used for further knock studies such as live imaging or behaviour assays. For tissue specific knockdown, all 0-1 days F1 progenies were collected and transferred to 29^{o}C incubator till it reaches specific age for its behavioural or live imaging studies.

Behavioural assays

The F1 progenies from the above crosses were grown till a specific age and were used for climbing assay to analyse the behavioural changes ,if any, due to the knockdown.These flies were anesthetized on ice for 5mins and divided into sets of 7-10 flies.They were then placed in the behavioural set up for 30mins for acclimatisation and recovery.After 30mins,they were transferred to 100ml glass measuring cylinders and covered with parafilm with minute holes for air supply.These cylinders were then placed in front of camera. After starting recording,the measuring cylinders were given 1-2 bangs such all flies fall below 0ml mark.These were then recorded for 1min and number of flies above 50ml mark were counted every 10seconds.

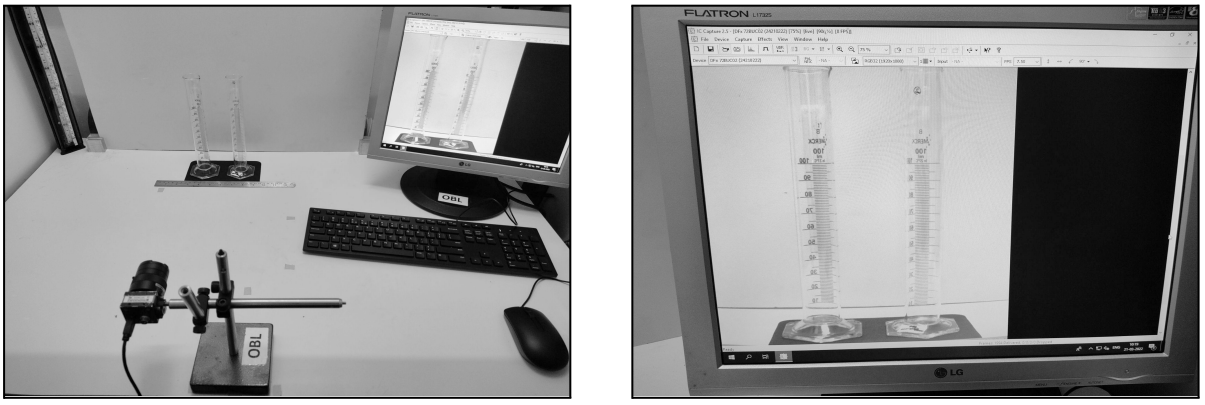


FIGURE 6: Negative geotaxis assay set up in lab.

Imaging of Live tissues

For live sample imaging of the progenies/flies with knockdown at specific age group

were collected and the antenna were dissected from the head.The entire antennal segment was then separated into second and third antennal segments.These were then mounted on slides in halocarbon oil .After properly dipping in oil,vaseline was applied on four corners and coverslips were placed.These samples were then imaged within 30 mins in Olympus FV3000 confocal system using 488 laser.

Statistical Analysis

All statistical analysis in the project was done using unpaired t test in Graph pad.
*** - P< 0.001, ** - P <0.01, * - P<0.05, ns - P >0.05

Chapter 3 Results

Localisation of different centrosome and ciliary proteins during Centrosome-to-Cilium conversion in mammalian cells

As a part of standardisation we did immunostaining of all antibodies using cells fixed with various methods (e.g., 4%PFA,100% Methanol). Ciliogenesis was monitored by using ciliary axoneme(Acetylated alpha tubulin) and membrane markers(Arl13b). Acetylated alpha tubulin localisation suggests ciliogenesis in synchronised cells starts after 6 hrs of serum starvation. At 18 hrs after starvation, a significant percentage of (~40%) cells showed detectable cilia. Though we thought we will be able to confirm cilia formation temporal profile using another commonly used ciliary membrane marker(Arl13b), we noticed early recruitment of Arl13b on the centrosome. This restricted us to conclude on the initial time point of ciliogenesis using this marker. Almost all cells were showing Gamma tubulin staining in all fixations. We could see an increase in recruitment of CEP164 from 18 hours of serum starvation. CEP164 is a distal appendages marker which helps in docking of centiole to the plasma membrane(Graser et al., 2007). Cell synchronisation followed by serum starvation technique is promising to see the synchronised ciliogenesis in hRPE-1 cells. Still based on previous several studies we expected much larger percentage of cells would be found at 18 hour after serum starvation(Pedersen et al., 2008; Smith et al., 2020), suggesting further invigilation would be required to improve synchronisation step.

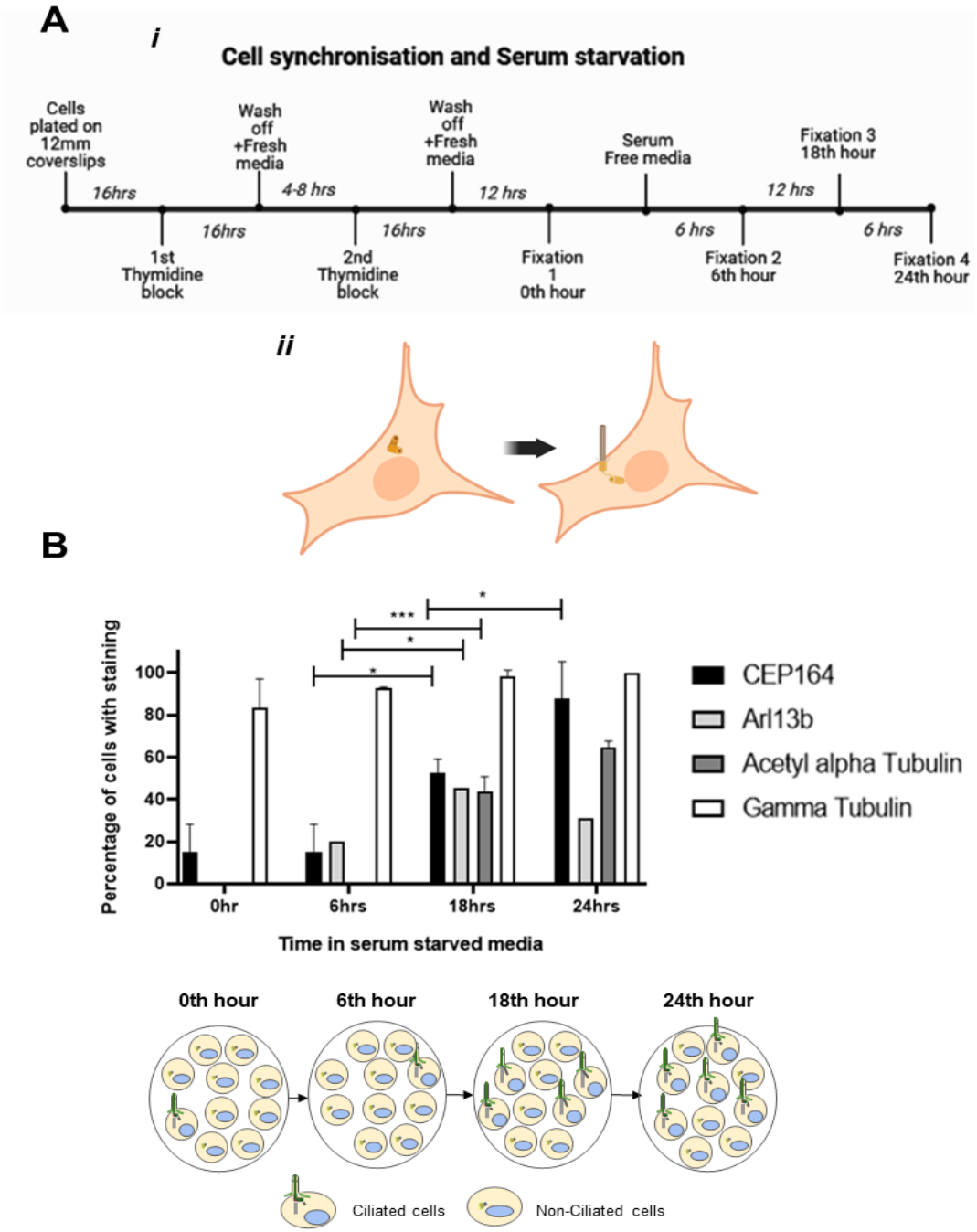
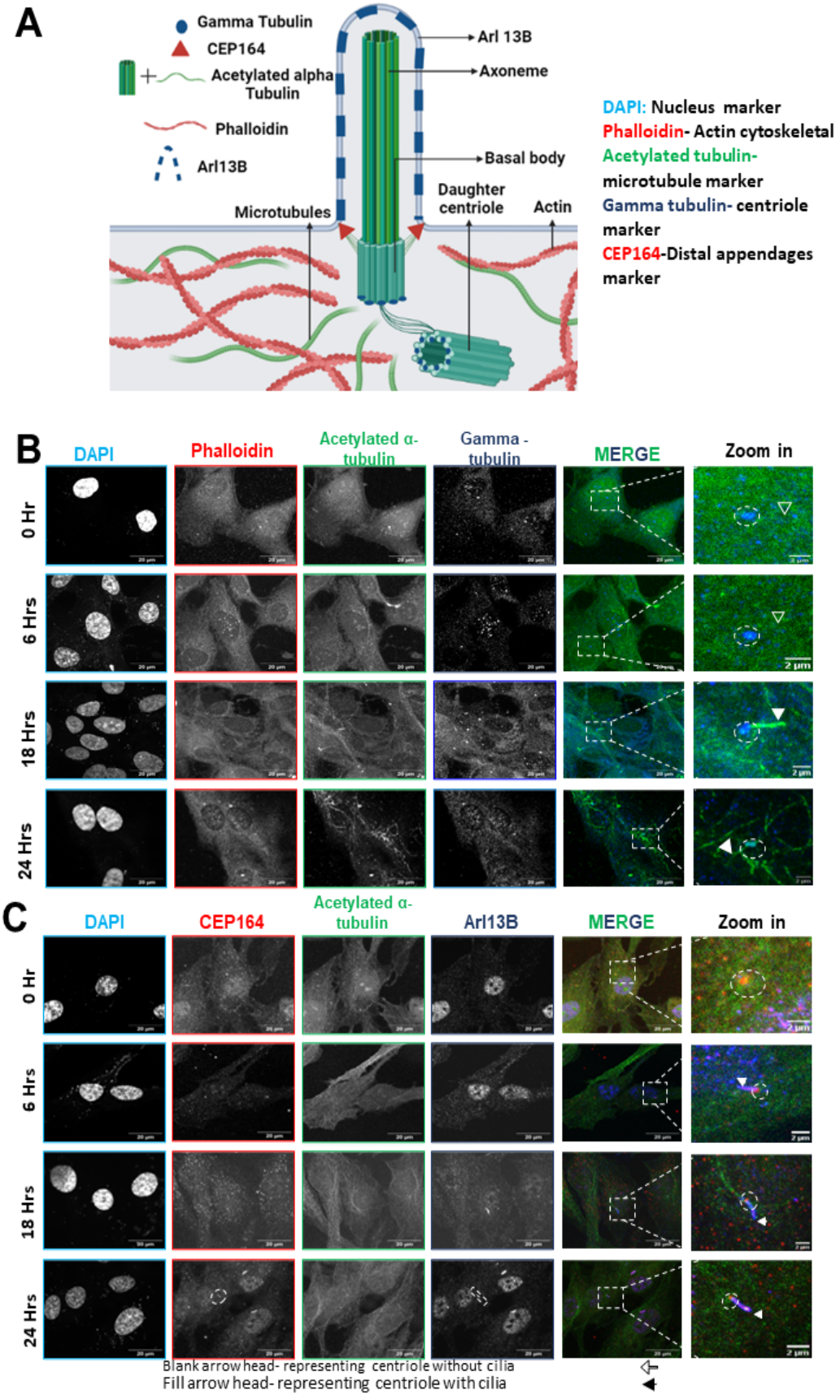


FIGURE 7: Cell synchronisation, serum starvation and ciliogenesis in hRPE-1 cells. **A**.(i)Scheme representing the procedure used for the synchronised development of cilia formation for the study.(ii)Scheme representing cilia formation in cells. **B**.Scheme and graphs showing ciliogenesis at different time points after serum starvation in RPE1 cells.We could see an increse in staining of ciliary proteins from 6th to 18th hour of serum starvation(only significant differences marked in graph,rest all are non significant).


A
Gamma Tubulin
CEP164
Acetylated alpha Tubulin
Phalloidin
Arl13B
Arl 13B
Axoneme
Basal body
Daughter centriole
Actin
Microtubules
DAPI: Nucleus marker
Phalloidin- Actin cytoskeletal
Acetylated tubulin- microtubule marker
Gamma tubulin- centriole marker
CEP164-Distal appendages marker
B
DAPI
Phalloidin
Acetylated α-tubulin
Gamma -tubulin
MERGE
Zoom in
0 Hr
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C
DAPI
CEP164
Acetylated α-tubulin
Arl13B
MERGE
Zoom in
0 Hr
6 Hrs
18 Hrs
24 Hrs
Blank arrow head- representing centriole without cilia
Fill arrow head- representing centriole with cilia

FIGURE 8: Localisation of different centriolar and ciliary proteins in centrosome to cilia conversion. A. Scheme representing the localisation of different protein molecules in cilia. **B.**.Immunofluorescence assay performed for the timely localisation of gamma tubulin(blue), acetylated alpha tubulin(green) and actin(marked by phalloidin,red) in methanol fixed cells.Striking differences in microtubule (green) and gamma tubulin staining were observed as cells changes from non ciliated to ciliated. (Scale bar:20um for all except zoomed in images(2um)). **C.**Immunofluorescence assay performed for the timely localisation of Arl13b(blue), acetylated alpha tubulin(green) and CEP164(red) in 4% Paraformaldehyde fixed cells. Arl13b staining for cilia were observed from 6th hour of serum starvation (Scale bar: 20um for all except zoomed in images(2um))**.**

Establishing expansion microscopy approach to visualise centrioles, during Centrosome-to-Cilium conversion with higher resolution

Expansion microscopy is a technique which can be adopted visualise samples, which are lower than the resolution limit of a normal confocal microscope, using the same conventional microscopic tools. This is acquired by making a network polymer system within the specimen and by swelling and expanding this polymer network with water(Wassie et al., 2019). In this project, we tried using this technique to visualise the conversion of centrosome to cilia conversion with better resolution (for example, 4-5 times higher resolution).We were successful in establishing the expansion technique in cells as we could see a consistent expansion of nucleus. We could also see the expansion in centrioles and cilia but have to standardise the protocol further for the detailed visualisation of symmetrical triplet and doublet microtubular arrangements in these cells. Representative images of (i)nucleus, (ii)centrioles and (iii)cilia show organelles expanded(Figure 9D). As apart of standardisation we did immunostaining of gels with different antibodies such as gamma tubulin,alpha tubulin,acetylated alpha tubulin etc and we could find that anti-acetylated alpha tubulin was staining better using the technique. A further rigorous screening using antibodies using various centrosomal and ciliary proteins would be essential for execution of this project.

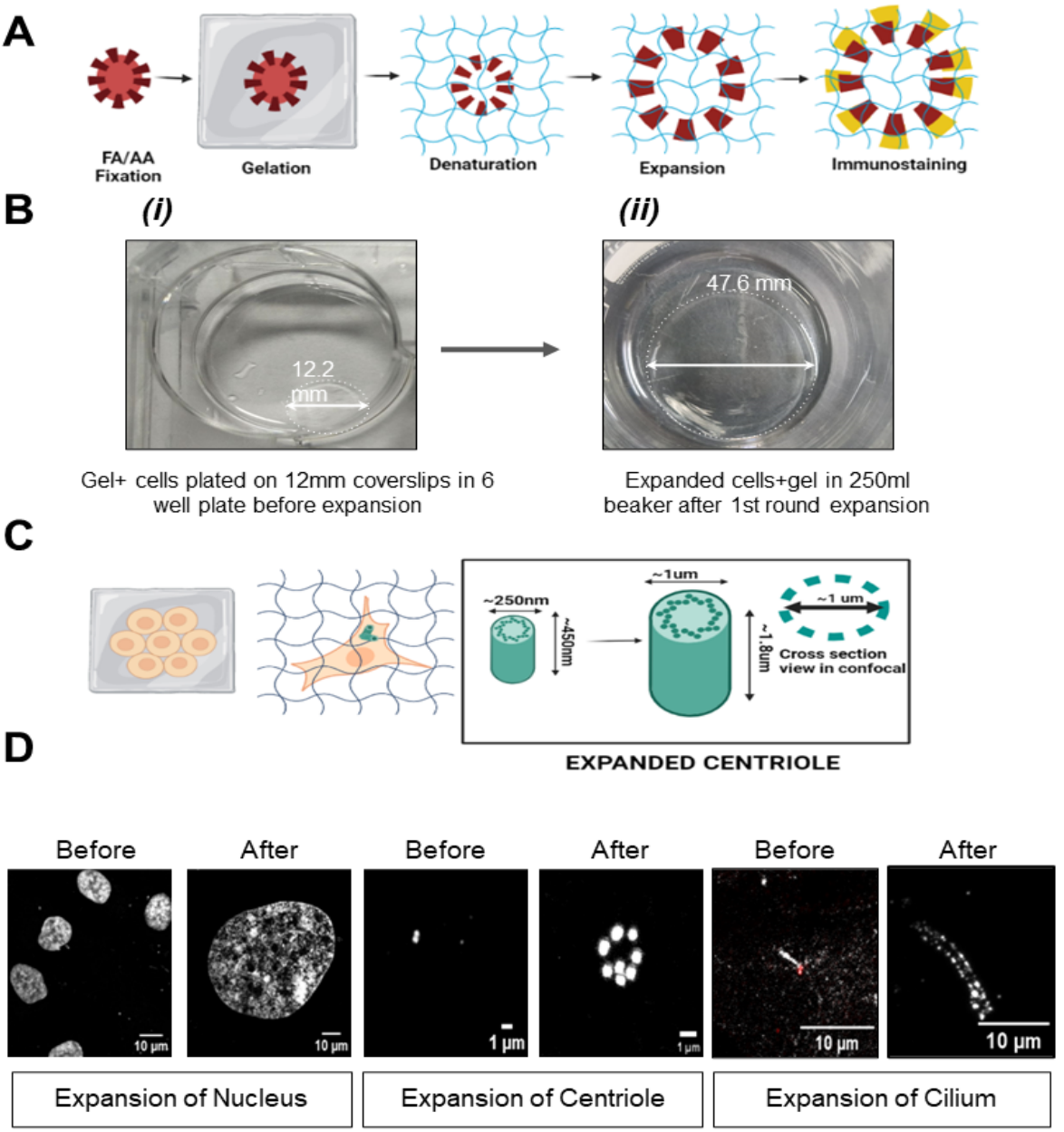
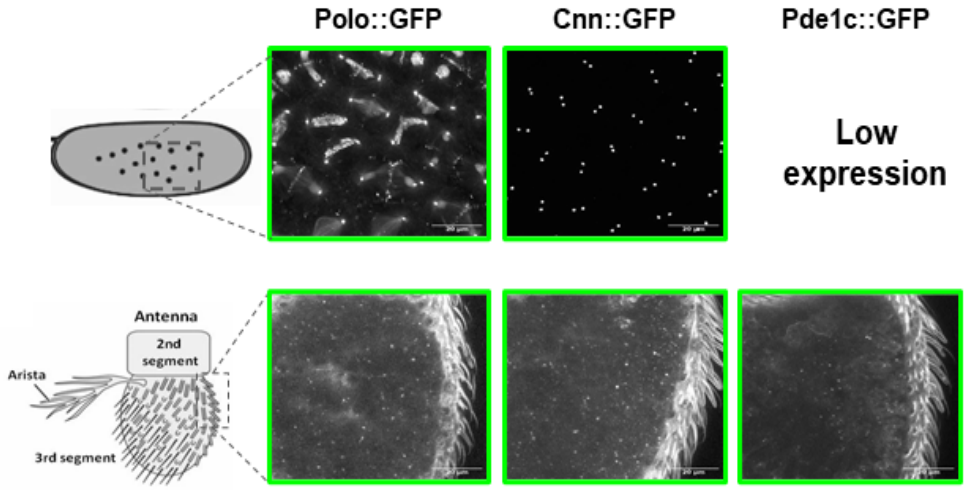


Figure 9: **Expansion Protocol trial for the visualisation of Centrosome to cilia conversion in MDCK cells: A**.Schematic representing the procedure of expansion in cells. **B.** Expansion microscopy protocol tried mitotic cell S2 cell lines for the visualisation of centrioles with higher resolution. We could see a 3.9 fold expansion in the gels with the cells. **C.** Scheme representing the expansion of centrioles in cells. Figure shows the expanded and non expanded centriolar dimensions. **D**. Expanded gels were imaged under confocal and we could see expansion in nucleus(scale bar 10um), centrioles (stained with acetylated alpha tubulin, scale bar 1um) and cilia(stained with acetylated alpha tubulin,scle bar 10um).

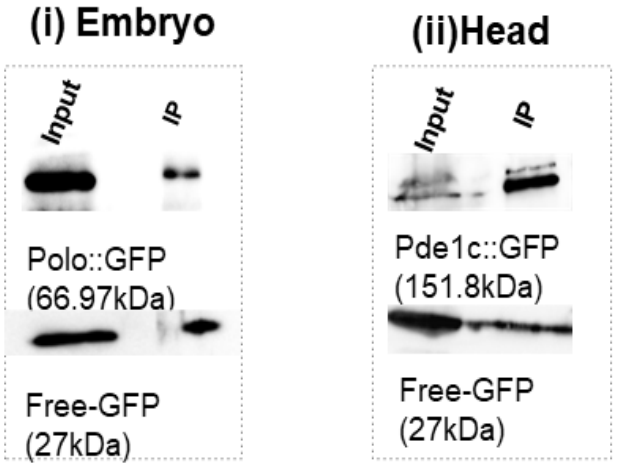
IP-MS with pericentriolar proteins for finding novel interactors in centrosome and cilium

GFP-tagged versions of a set of PCM proteins, including Polo/PLK1, Pdelc and Cnn, which have been already shown to have a significant role in centrosome maturation and ciliogenesis, were expressed in the fruit flies. My first goal was to detect the proteins through western blot analysis. Proteins from the embryo lysates of appropriate flies were separated using SDS PAGE followed by transfer to PVDF membrane and detected for the presence of GFP-tagged proteins using an antibody that recognised GFP. We confirmed the presence of Polo::GFP, Cnn::GFP and Pde1c::GFP expression in the fly tissues. Next we attempted pulling down these proteins from different fly tissues using anti-GFP antibody tagged magnetic beads. Immunoprecipitation assay of Polo::GFP and Cnn::GFP were performed in fly embryos and heads as they have their highest expression in embryonic stage. IP of Pde1c::GFP was done in fly heads. Representative Blots for IP from embryo lysates(Polo::GFP) and head lysates(Pde1c::GFP) along with control IPs($Gal4^{Tubulin}$UASGFP(embryo) and $Gal4^{Chat}$UasGFP(heads) are shown below(Figure:2 B(i & ii). Those IP samples along with IP samples for head lysates of Polo::GFP, Cnn::GFP and Pde1c::GFP were sent for mass spectroscopic analysis to find the intractors of these proteins in these tissues. Pde1c, NinaC and 14-3-3 zeta along with actin, actin interacting proteins and myosins are the common interactors in the list. Polo interactors list has a lot of Rab proteins. Cnn and Pde1c shares a lot of interactors in common(121).

A



B



Mass Spectroscopy assignments failed

Mass Spectroscopy Results analysed

C

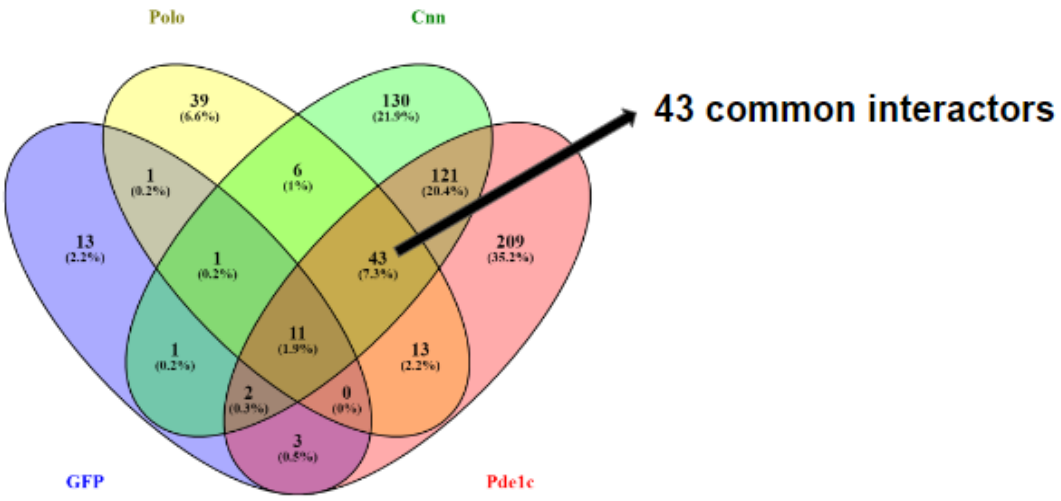


FIGURE 10: Immunoprecipitation done on Drosophila tissue samples. A. Confocal images representing the expression of our proteins of interest(Polo, Cnn, Pde1c) tagged with GFP in ciliated(Antenna) and non ciliated tissues(Embryo). **B.** Representative blots for (i)Polo::GFP and $GAL4^{Tubulin}$UAS GFP embryos and (ii)Pde1c::GFP and $GAL4^{Chat}$ UAS GFP heads immunoprecipitation assays performed. **C.** Interaction plot for the proteins studied along with the control GFP. 43 common interactors were found and the potential candidates among those are listed here.

Estimating the effects of knockdown by negative-gravitaxis assay

To approach the second aim of our project, we attempted to do the knockdown studies of different centrosomal proteins using RNA interference (RNAi/Ri, often used in figures) technique in which their reverse compliment to mRNA is expressed under the UAS promoter.The knockdown levels are then controlled by regulation of Gal4 under different promoters. For example, $Gal4^{chat19b}$ is expressed in all cholinergic neurons and $Gal4^{tubulin}$ is expressed in all ciliated and non-ciliated cells(Fig:8 A). We tried 2 types of knockdown.Figure 11A shows the expression pattern of RNAi under tubulin protein promoter. As tubulin expression is very high throughout the lifetime of a fly and in all cells, the knockdown of the protein using its RNAi is supposed to be in all cells from the very early embryonic stage. The functional analysis of these knockdowns is measured by bang assay. It is used to measure the climbing capacity of flies under different knockdown conditions at a given age (Figure 11 B) after giving it a bang stimulation.The flies expressing mCherryRNAi were taken as a control for all knockdown studies. Figure 11 C shows the climbing ability of the control mCherry RNAi with $Gal4^{tubulin}$ expression. We took 20 seconds as our reference time point for analysis as almost 100% of control knockdown flies managed to climb up the 50ml mark by this time.

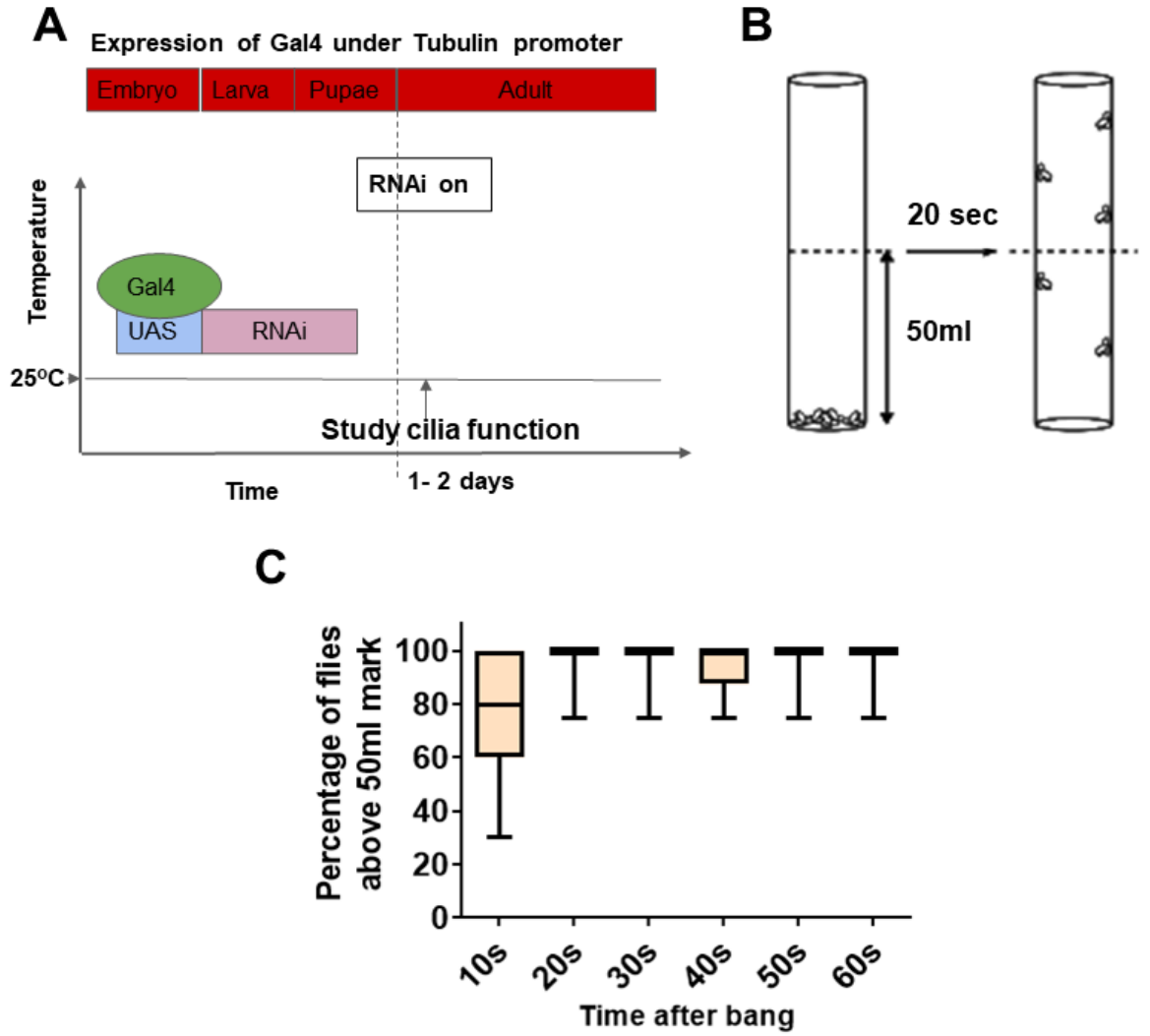
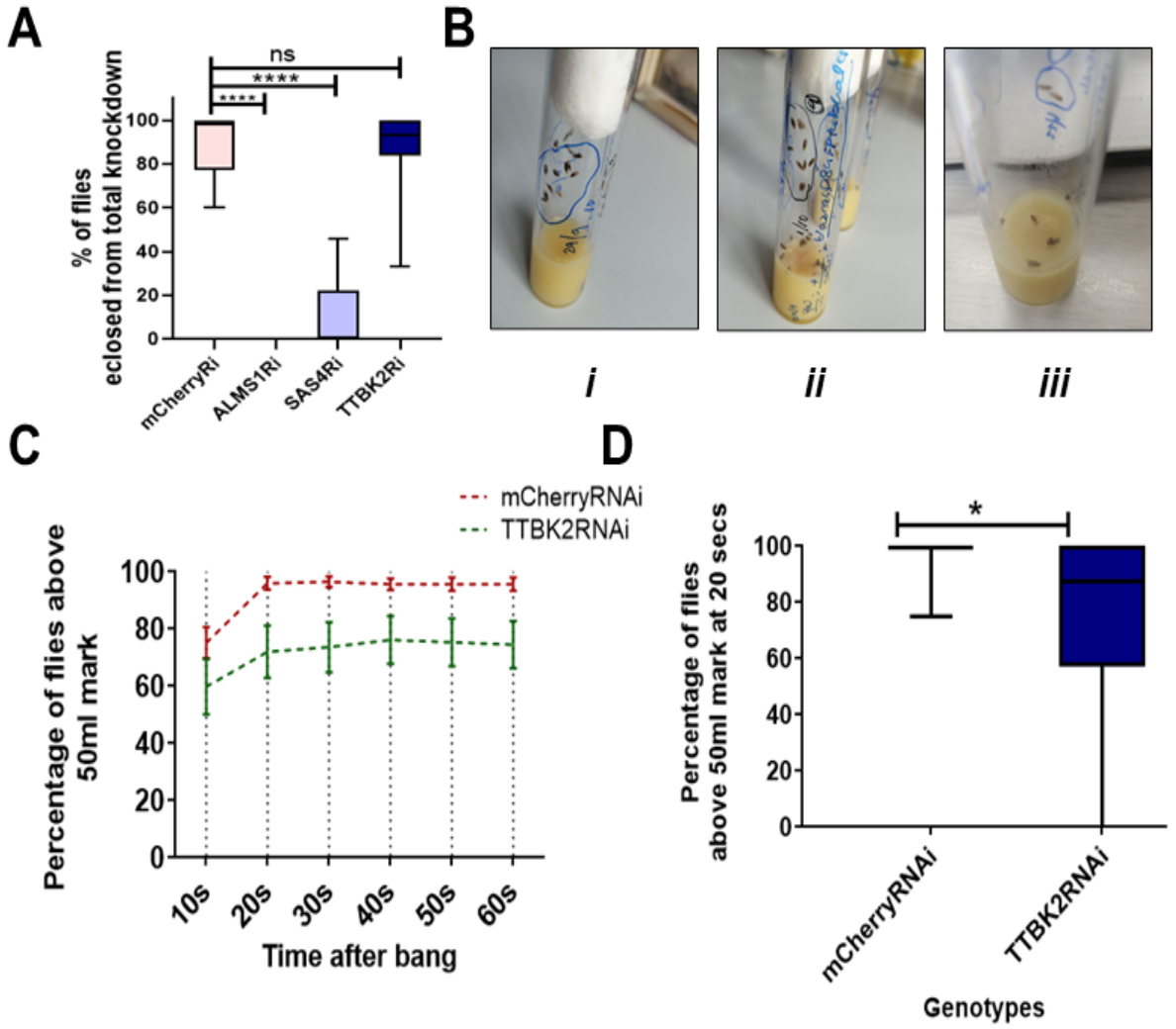


FIGURE 11: Scheme of the approach of the temporal conditional knockdown (target proteins and mCherry RNAi) experiments and behavioural assay. A:1-2 days old F1 fluorescent progenies from the cross between homozygous UAS-RNAi lines in the second chromosome and UAS-mcd8GFP Gal4^{tubulin}/Tm6B in the third chromosomes were used in the shown study. **B.** A schematic representation of the climbing (negative-gravitaxis) assay after bang. The effect on sensory cilia function is approximated by quantifying climbing behavior of the adult flies on specific days (arrows)[*].**C:** The climbing behaviour of 1-2 days old Gal4^{tubulin}UAS-mCherryRi flies.Almost 100% of flies manage to climb above the half mark by 20 seconds.

Ubiquitous knockdowns of *Dm*ALMS1 and *Dm*SAS4 proteins strongly affect the development of pupae and survival in adult flies, but *Dm*TTBK2 is dispensable for the development of pupae

ALMS1(Alstrom syndrome 1), SAS4 (spindle assembly abnormal protein 4) and dASATOR(Tau tubulin kinase 2(TTBK2) homolog in *Drosophila*) were used in the above knockdown studies. The pupal eclosion rate for $Gal4^{tubulin}$ knockdown for these proteins is given below(Figure 12 A). The data shows the development of pupa to fly stage from the total number of knockdown pupa collected. None of the pupa of *Dm*ALMS1 knockdown could develop to fly stage(Figure 12(i)). The very few proportion of *Dm*SAS4 knockdown flies eclosed were found dead on fly food soon after eclosion(Figure 12(ii) & (iii)). Climbing assay was performed with flies that eclosed from *Dm*TTBK2 knockdown and control mCherry RNAi. The *Dm*TTBK2 knockdown flies are showing some defects in climbing compared to the control knockdown flies. Figure 12C shows the Climbing assay data for control and *Dm*TTBK2 knockdown flies and Figure 12D shows the comparative climbing capacity of mCherry knockdown and *Dm*TTBK2 knockdown at 20 secs after bang.There is a defect in climbing in these flies compared to control flies but still more than 80% of flies managed to climb up 50ml mark within 20 seconds. Figure 12 E shows the Confocal live images for second and third antennal segments of 1-2 days old different knockdown flies with the marker mCD8GFP over expressed, under UAS promoter, in cell membranes of these flies..The overall tissue development in the 3rd antennal segment for *Dm*ALMS1 knockdown 5-6 days pupa (equivalent to ~1 day old uneclosed fly) seems to be affected (Figure 12E) *Dm*ALMS1RNAi importantly, cilia development was severely impaired while other two knockdowns were not showing any striking phenotype in confocal imaging.


A
ns
****
****
% of flies eclosed from total knockdown
100
80
60
40
20
0
mCherryRi
ALMS1Ri
SAS4Ri
TTBK2Ri
B
i
ii
iii
C
mCherryRNAi
TTBK2RNAi
Percentage of flies above 50ml mark
100
80
60
40
20
0
10s
20s
30s
40s
50s
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Time after bang
D
*
Percentage of flies above 50ml mark at 20 secs
100
80
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Tissue development is affected by ALMS1 knockdown
SAS4RNAi
TTBK2RNAi

FIGURE 12: Gal4^{tubulin} UAS-RNAi knockdown effects. A.Percentage of pupal eclosion in different Gal^{tubulin} protein knockdown. **B** (i) 5-6days old uneclosed *Dm*ALMS1 knocked down pupa. (ii) Uneclosed pupa in *Dm*SAS4 knockdown progenies.(iii) Eclosed *Dm*SAS4Ri flies seen dead on fly food. **C.**.Climbing assay performed for eclosed 1-2days old flies from mCherryRNAi cross and *Dm*TTBK2RNAi cross. **D.** Comparison of climbing between control and *Dm*TTBK2RNAi at 20 seconds after the bang. **E.** Confocal microscopy image of 2nd and 3rd antennal segment in 1-2 days old Gal4^{tubulin} UAS-mcd8GFP/UAS-mCherryRi flies, 5-6 days UAS-ALMS1Ri/+;Gal4^{tubulin} UAS-mcd8GFP/+ uneclosed pupa, 5-6 days UAS-SAS4Ri/+;Gal4^{tubulin} UAS-mcd8GFP/+ uneclosed pupa and 1-2 days old UAS-TTBK2Ri/+;Gal4^{tubulin} UAS-mcd8GFP/+ flies.Tissue development is seen to be highly affected in *Dm*ALMS1 knockdown flies.

DmALMS1 and DmSAS4 knockdown in cholinergic neurons in the adult flies affects the climbing behavior of flies

Gal4^{Chat19b} based knockdown of the above proteins affects the climbing behaviour of flies with age(Figure 5).Figure 9 A shows the second type of knockdown in which the expression of Gal4 is under choline acetyltransferase promoter.The expression of this protein is high from the time when flies are about to eclose from pupal stage.We choose this promoter to study the role of these proteins in cilia maintenance as its expression window is mainly after cilia formation. We could see a clear climbing defect in flies from the age of 19 days in control flies. The climbing behaviour of mCherry RNAi flies were completely normal in 1-2 days of age and also were not showing much defect in 8-11 days as around 90% of flies managed to climb 50ml mark in 20 secs(Figure 5 A, B and D). But the climbing ability is strikingly defected from the age of 19 days in control flies.Knockdown of ALMS1 and SAS4 proteins are also affecting the climbing behaviour of the flies. They were not showing much defect in 1-2 days as the knockdown of these proteins might have started just before pupal eclosion as the GAL4 is under Chat19b promoter whose expression rises only from eclosion. The defects with knockdown of ALMS1 and SAS4 is clearly visible from 8th day of age in these flies as nearly 60% flies could climb up 50ml mark at this age point . TTBK2 knockdown was not showing much defects compared to other knockdown at any age although it's showing some significant defects in climbing as compared to control mCherryRNAi at 1-2 and 8-11 days of age.

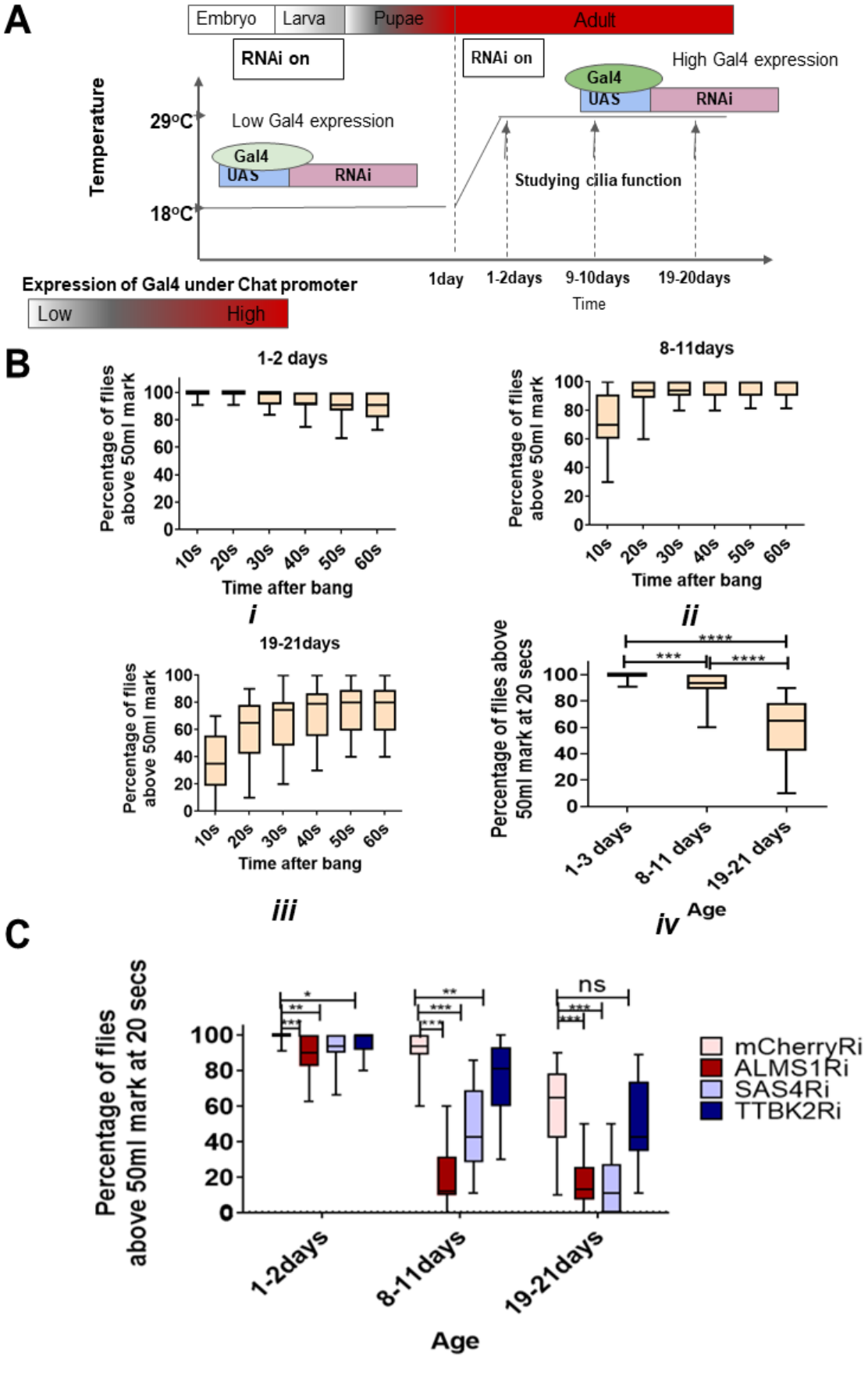

A
Embryo
Larva
Pupae
Adult
RNAi on
RNAi on
High Gal4 expression
Gal4
UAS
RNAi
Temperature
29°C
Low Gal4 expression
Gal4
UAS
RNAi
18°C
Studying cilia function
1day
1-2days
9-10days
19-20days
Time
Expression of Gal4 under Chat promoter
Low
High
B
1-2 days
Percentage of flies above 50ml mark
Time after bang
i
8-11days
Percentage of flies above 50ml mark
Time after bang
ii
19-21days
Percentage of flies above 50ml mark
Time after bang
iii
Percentage of flies above 50ml mark at 20 secs
1-3 days
8-11 days
19-21 days
Age
iv
C
Percentage of flies above 50ml mark at 20 secs
ns
mCherryRi
ALMS1Ri
SAS4Ri
TTBK2Ri
1-2days
8-11days
19-21days
Age

FIGURE 13: Gal4^{Chat19b} UAS-RNAi knockdown effects on behaviour. A.1-2 days,9-10 and 19-20 days F1 progenies from the cross between homozygous UAS-RNAi lines in the second chromosome and homozygous Gal4^{Chat19b}UAS-GFP in the second chromosomes were used in the shown study.**B**.(i)Bang assay analysis for mCherryRi under Chat promoter for 1-2days.(ii)Bang assay analysis for mCherryRi under Chat promoter for 8-11days.Around 100% flies tend to reach above 50ml mark by 20seconds after the bang.(iii).Bang assay analysis for mCherryRi under Chat promoter for 19-21days.The climbing ability of flies seems to be affected with age.(iv)Comparison of climbing behaviour between flies at different age points **C**.Percentage of flies above 50ml at 20 secs after the bang compared over different RNAi knockdowns of different age groups.ALMS1 knockdown highly affects the climbing capability of flies from 8 days of age.SAS4 knockdown also started affecting the climbing from 8 days of age.TTBK2 knockdown has not affected much drastically in the climbing behaviour of flies.

Ciliary gross morphology mostly remained unaffected (with marginal defects) in flies with conditional DmALMS1 and DmSAS4 knockdown

The second and third antennal segments from knockdown flies were dissected and live imaged using a confocal microscope (Fig 7A). We could not see any striking variation between the confoacal images of second and third antennal segments of different knockdown flies at different age points. In second antennal segments, almost all auditory neurons were seen to have two ciliary extensions. All images were taken under the same imaging conditions. In the third antennal segment,there are structures called sensilla where ciliary extensions are present. Each basiconic large sensilla is known to have 2-3 ciliary extensions in it(Figure 15 A). We tried quantifying the amount of GFP expression in each basiconic large sensilla of live sample images of different protein knockdown flies over different ages using ImageJ. We could see some variation in the intensity quantification basiconic sensilla in different knockdown conditions but its not consistent with the behavioural data of auditory neurons. The variations were observed in 1-2days of age can be due the temperature shift from 25ºC to 29ºC on the day of fly eclosion which can induce an increase in protein expression in *Drosophila*.

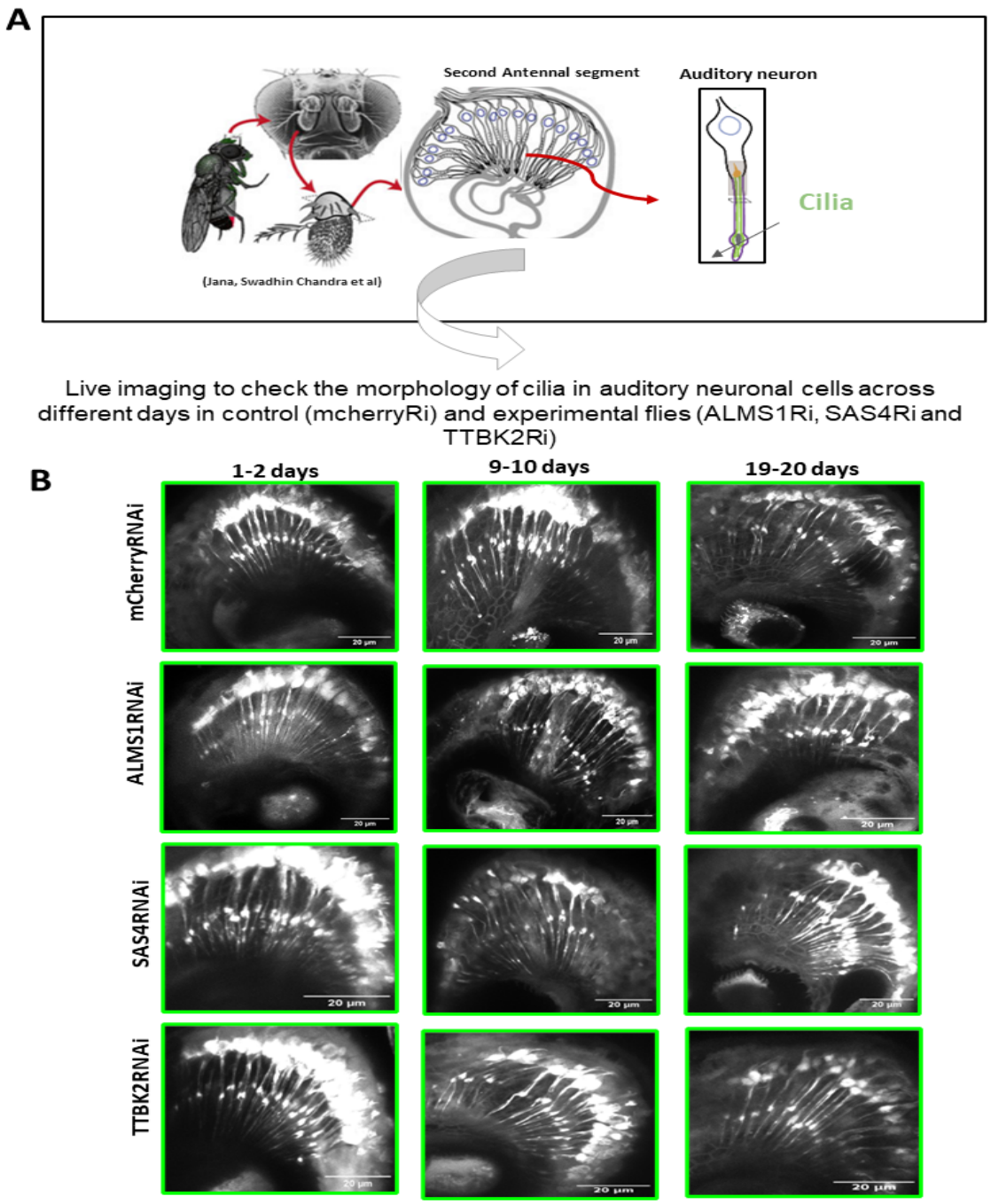
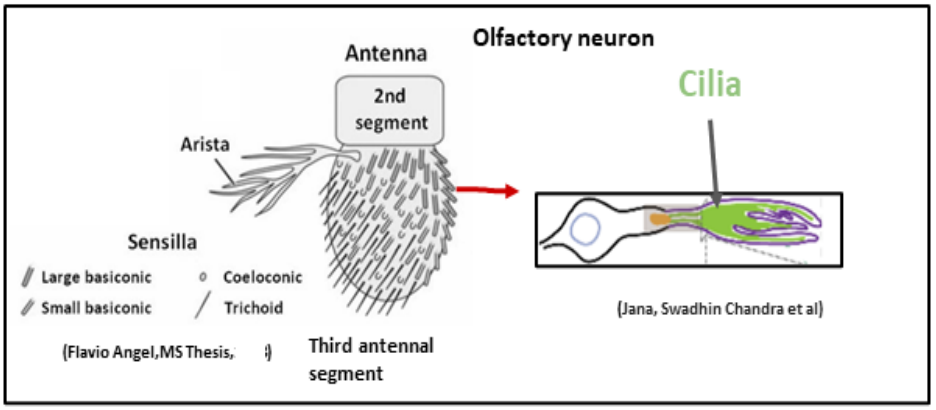
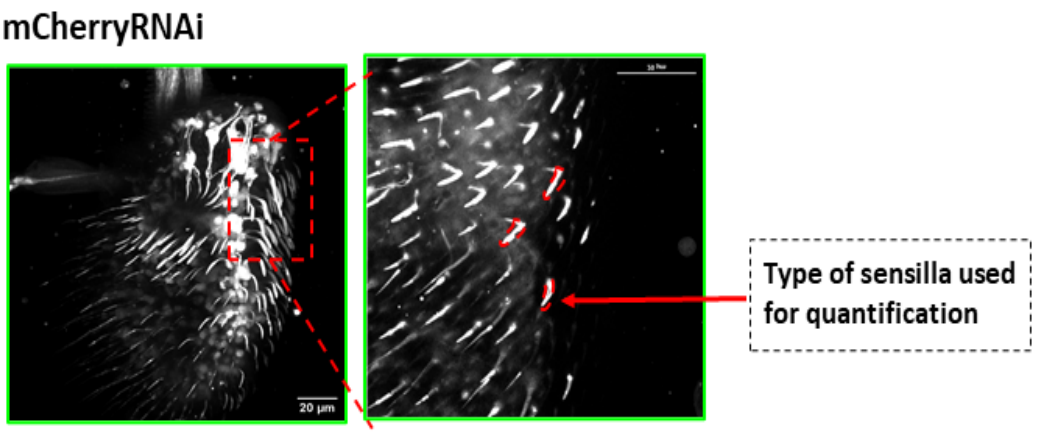


FIGURE 14. Live sample image analysis of second antennal segment for different knockdown experiments: A.Schematic diagram of the dissection, imaging sample and the position of cilia in the second antennal segments in fruit flies.Figures were taken from(Jana et al., 2016).B. Shows the images for auditory neurons in second antennal segments for different knockdowns(column) at different age points(rows).We could not see much variation in the images captured as we saw in their behaviour data. Almost all neurons were having cilia as normal in control flies

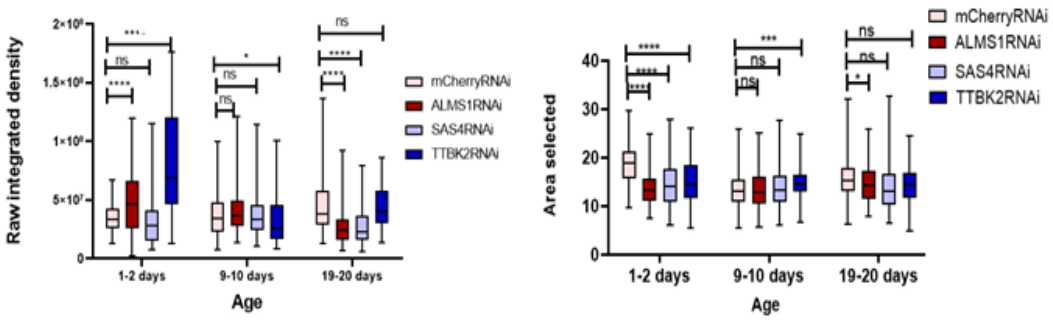
A


Olfactory neuron
Antenna
2nd segment
Arista
Cilia
Sensilla
Large basiconic
Small basiconic
Coeloconic
Trichoid
(Flavio Angel,MS Thesis,)
Third antennal segment
(Jana, Swadhin Chandra et al)

B


mCherryRNAi
20 µm
Type of sensilla used for quantification

C


Raw integrated density
Area selected
mCherryRNAi
ALMS1RNAi
SAS4RNAi
TTBK2RNAi
1-2 days
9-10 days
19-20 days
Age

D

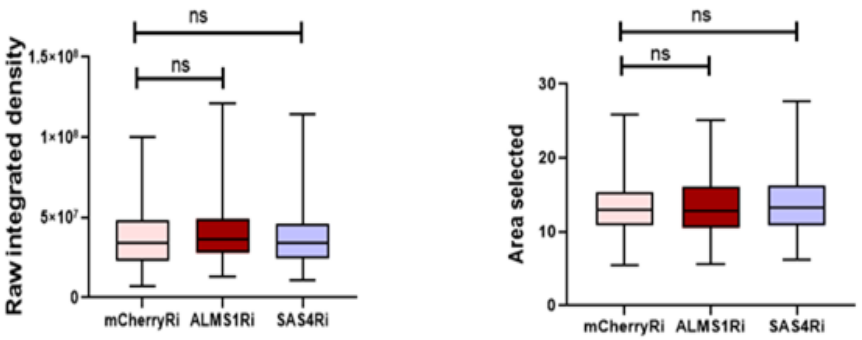

Raw integrated density
Area selected
ns
mCherryRi
ALMS1Ri
SAS4Ri

FIGURE 15. Live sample image analysis of the third antennal segment for different knockdown experiments. A. Schematic diagram of the dissection,imaging sample and the position of cilia in the second antennal segments in fruit flies. Figures were taken from (Angei, 2013; Jana et al., 2016).**B**. Shows the image of third antenatal segment(1x left) and zoomed in image of large basiconic sensilla(2.5x) of 9-10 days old mCherryRNAi flies.The region marked with red dotted line represents the basiconic sensilla which are then quantified using ImageJ software.**C.** Shows the quantification data of intensity in basiconic sensilla for different knockdowns across different age points.The quantification is not showing any consistent trend with knockdown of these proteins. D.It shows the quantification data of intensity in basiconic sensilla for ALMS1 and SAS4 knockdowns across 9- 10 days age points, as we were observing a notable defect in sensory behavior at that age. But the ciliary volume seems to be unaffected

Chapter 4 Discussion

I, by using multifaceted techniques (with help from others in the lab) on mammalian cultured cells and long-lived ciliated cells in the fruit fly, managed to highlight the following points: **a.** we could follow Centrosome-to-Cilium conversion in mammalian cells (using confocal and expansion microscopy), **b.** IP, followed by MS, several centrosome/ciliary proteins from cycling and ciliated Drosophila cells, and **c.** we identified that at least two of the centriolar proteins, such as ALMS1 and SAS4 are critical for ciliary function maintenance in adult flies.

In Part #1 of the project, we could see localisations of different ciliary proteins and its variation in staining in different stages of ciliogenesis. Cell synchronisation followed by serum starvation technique is promising as we saw the synchronised ciliogenesis in hRPE-1 cells between 6 and 18 hrs after serum starvation. We could see a synchronised ciliogenesis in a significant fraction(~40%) of cells. Still, a further standardization on synchronisation followed by extensive analysis of various centrosome/cilia proteins during centrosome-to-cilium conversion would be essential. We established the protocol to study centriole and cilia in cells using ExM. But, a further extensive screening of different centrosome/cilium during the centrosome-to-cilium conversion will be required. In parallel, a study of centrosome-to-cilium conversion within diverse ciliated *Drosophila* cells is done in the lab (by Pranjali Priya, PhD student). She is doing it in *Drosophila* and using electron microscopic techniques to overcome the limit of resolution.

We aim to find novel interactors in the process by pulling down proteins such as Polo, Cnn and Pde1c from both ciliated and non-ciliated tissues. All pull downs were done and were confirmed through western blots. All were sent for mass spectroscopic analysis. Actin, actin interacting molecules, myosin, Pde1c, NinaC and 14-3-3 zeta are found to be common interactors in MS results for all three proteins in the ciliated tissues. The mass spectroscopic assignments for all embryonic samples failed due to detergent contamination. We were able to rectify this issue for all head

tissue samples. Mass spectroscopic analysis for embryonic tissues will be repeated in future. The recent papers show that actin filaments regulate microtubule growth from centrosome(Inoue et al., 2019). The specific roles of these proteins have to be investigated further in future.

In Part #2 of the project, we could see some interesting phenotypes in the knockdown flies.The ubiquitous knockdown of ALMS1 and SAS4 in fruit flies hindered the development of flies from pupal stage to fly stage. The knockout flies for these proteins were known to show same phenotype hence it ensures our knockdown efficiency using the fly line. Moreover,we could read the clear importance of these proteins in the development and eclosion from pupal stage in fruit flies.The TTBK2 knockout flies are not known to survive. Still,the knockdown flies were having slight defects in the behaviour assay (FIGURE 11-13). We have to further confirm the efficiency of each knockdown by RT-qPCR in future.

The method of knockdown is promising in differentiation roles of different proteins in ciliogenesis vs cilia maintenance. We discovered DmALMS1 and DmSAS4 roles in maintaining the ciliary functions apart from its already known role cilia biogenesis. Live tissue imaging of auditory and olfactory neurons showed: (i) most auditory neurons continue to have cilia and (ii) no significant variation in ciliary volume at 9-10 days of age under the knockdown conditions established. Notably, these flies show sever sensory function defects. We hypothesis that sensory function defects can be due to the defects in ultrastructure or homeostasis of sensory receptor/signalling machinery. Further extensive analyses are required to investigate these possibilities.

In the study using ChAT driven GAL4 expression, we could see the flies were almost fine at 1-2 days old in behavioural assays.Since they were showing not much defects in the initial time period we assume that their ciliogenesis part is not affected by the knockdown. Moreover, $Gal4^{Chat19b}$ expression majorly takes place after the ciliogenesis in these neurons are completed.In future we will confirm it once again by further controlling the expression of same Gal4 using temperature sensitive GAL80 expression under an ubiquitous promoter, such as tubulin.The defects that we saw in behaviour in 8-11 days can predict the role of these two proteins in maintaining the cilia once formed in its functionally active state (FIGURE 11-15). This also partly explains the late onset of certain ciliopathies linked with the mutations in these

genes. The climbing ability of flies were also affected with age ,which was clearly visible in control and all the knockdown flies.It can be hypothesised that this defect in climbing is seen because the flies were losing ability to sense the bang stimulation given to them or they were losing ability to climb up as their age increases.The early works show the role of ALMS1 and SAS4 in ciliogenesis and few works also predict the role of ALMS1 in cilia maintenance also.There were not much data available on the role of SAS4 for cilia maintenance yet. Other species like *C.elegans* are known to completely loose SAS4 from their basal body once cilia formation happened(Serwas et al., 2017).The persistence of SAS4 in *Drosophila* cilia can be used to hypothesise its role in cilia maintenance in these species,which we partly agree through our initial results.We would further expand the knockdown molecules to our all candidate genes to elucidate all interactors in the process of cilia maintenance. Therefore, surprisingly, we observed that the SAS4/ALMS1, unlike that in *C. elegans*, plays an important role in the maintenance of the sensory cilia in the fly.

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