Universidade de Aveiro Departamento de Química
2013

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo de Biotecnologia Industrial e Ambiental, realizada sob a orientação científica da Doutora Mónica Bettencourt-Dias, Investigadora Principal do laboratório de Regulação do Ciclo Celular do Instituto Gulbenkian de Ciência e co-orientação da Doutora Maria do Céu Gomes dos Santos, Professora Auxiliar Convidada do Departamento de Biologia da Universidade de Aveiro.

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A todos os que me apoiaram durante este ano.

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palavras-chave Cilios, centriolos, ciliogenese, neurogenese, espermatogenese, CEP164.

resumo

Os cílios/flagelos são organelos constituídos por microtúbulos que emanam da superfície da maioria das células eucariotas. Estes estão envolvidos numa variedade de processos incluindo motilidade celular, movimento de fluidos e mecanismos de perceção de estímulos externos. O esqueleto de um cílio, designado axonema, é determinado por um corpo basal, que consiste num centríolo maduro/mãe modificado necessário para a formação de centrossomas. Mutações em genes humanos que interfiram na função ou estrutura desses organelos causam doenças como infertilidade, ciliopatias e cancro. Em Drosophila melanogaster, os cílios estão presentes nos neurónios e no esperma. Defeitos nestas estruturas e/ou na sua função leva a fenótipos como desenvolvimento tardio, falta de coordenação e esterilidade. A CEP164, uma proteína centrossomal, localiza-se nos apêndices distais do centríolo maduro. Mutações no gene CEP164, em humanos, causam nefronoptise, uma ciliopatia que causa a degeneração dos órgãos.

O objectivo deste trabalho consiste no estudo da função da proteína CEP164 na ciliogenese e no papel dos cílios nos neurónios sensoriais e no esperma em D. melanogaster. Para tal, foram utilizadas duas estratégias: uma que envolve a depleção da proteína CEP164 em neurónios e no esperma utilizando o sistema Gal4/UAS, e a outra consistiu na criação de novos alelos de excisão de CEP164 para estudar o efeito desta proteína na mosca da fruta.

Nos neurónios olfativos de D. melanogaster, a CEP164 localiza-se na base dos cílios, numa estrutura equivalente á zona de transição dos axonemas. Observou-se através de depleção por RNAi que a CEP164 é importante para a coordenação apropriada na mosca. A estrutura dos cílios foi afectada nessas moscas, contudo a fertilidade dos machos não foi afectada.

keywords Cilia, centrioles, cilia biogenesis, neurogenesis, spermatogenesis, CEP164

abstract

Cilia/flagella are microtubule (MT)-based oraganelle emanating from the surface of many eukaryotic cells. They are involved in a variety of processes including cell motility, fluid flow, and sensing processes. The skeleton of cilium, called axoneme, is templated from the basal body, a modified mature centriole required for centrosome formation. Mutations in human genes disrupting the function or structure of these organelles cause several human disorders, including infertility, ciliopathies, and cancer. In Drosophila melanogaster, cilia are present in the neurons and in the sperm. Defects in these structures and/or in its functions lead to clear phenotypes, such as delayed development, un-coordination and sterility. CEP164, a centrosomal protein, localizes at the distal appendages of the mature centriole. Mutations in CEP164 gene, in humans, cause Nephronophthisis, a ciliopathy that causes organ degeneration.

The aim of this work consists of studying the CEP164 role in ciliogenesis and cilia function in sensory neurons and sperm in D. melanogaster. For that, we used two strategies: one involving knock down of CEP164 in neurons and sperm using the Gal4/UAS system, and other the generation of new excition alleles of CEP164 to study the effect of CEP164 in the fruit fly.

In D. melanogaster olfactory neurons, CEP164 localizes at the connceting cilia, a transition zone equivalent structure. We observed by RNAi knock down that CEP164 is important for adult fly proper coordination. The cilia structures were affected in those flies, however, the fertility of those males was not affected.

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List of Abbreviations

ATP	Adenosine Triphosphate
BB	Basal body
Bld10	Bold 10
CEP135	Centrosomal protein 135
CEP164	Centrosomal protein 164
CEP170	Centrosomal protein 170
CEP290	Centrosomal protein 290
Cyo	Curly wings (in *Drosophila melanogaster*)
D-PLP	*Drosophila* pericentrin-like protein
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
EB1	Ending-binding 1
FM	First chromosome (of *Drosophila melanogaster*)
GTP	Guanosine Triphosphate
H	Height
IFT	Intraflagellar transport
KIF17	Kinesin-like protein
MAP	Microtubule associated protein
MKS	Meckel–Gruber syndrome
MT	Microtubule
MTOC	Microtubule organizing center
NPC	Nuclear-pore complex
Odf2	Outer dense fiber protein 2
PC	Primary cilium
PCM	Pericentriolar material
Poc1	Protein of centriole 1
RNAi	RNA interference
SAS6	Spindle assembly 6
SM	Second chromosome (of *Drosophila melanogaster)*
TM	Third chromosome (of *Drosophila melanogaster)*
TZ	Transition zone
UAS	Upstream Activating Sequence
WT	Wild type

Chapter 1 - Literature Review

1.1. Centrioles, centrosomes and cilia

1.1.1. Microtubules, cytoskeleton and its function

The cell is a basic structural and functional unit of all living organisms. An eukaryotic cell is composed of several components such as plasma membrane, nucleus and cytoplasm. The cytoplasm harbors cytoskeleton and many organelles, such as Golgi-complex, endoplasmic reticulum, and ribosomes. The cytoskeleton plays a critical role in organelle organization within the cell and maintenance of cell shape. In all eukaryotic cells, the cytoskeleton is mainly built by three types of filaments: microtubules, actin and intermediate filaments [1].

Microtubules (MTs) are hollow cylinders structures, of ~25 nm diameter, composed of α- and β-tubulin heterodimers (Fig. 1). MTs usually consist of 13 protofilaments, however the number of protofilaments in a MT is variable and it can vary between 10 and 15. Each protofilament is formed by polymerization of α/β-tubulin heterodimers, induced by end-to-end binding of dimers. The cylindrical wall of MTs is formed by lateral binding of α- and β-tubulins [1, 2]. These tubes give rigidity to the cytoskeleton networks and are essential for cell shape. At the same time, MTs are dynamic structures and their lengths are actively regulated within the cell. This process is important for many intracellular processes, such as active transport of specific components, and cell morphogenesis [2, 3]. The variation in MT length is a consequence of the polymerization of α/β-tubulin dimers at the growing (+plus) end of the MT and depolymerization of α/β-tubulin dimers from the shrinking (–minus) end of the MT (Fig. 1) [1, 2].

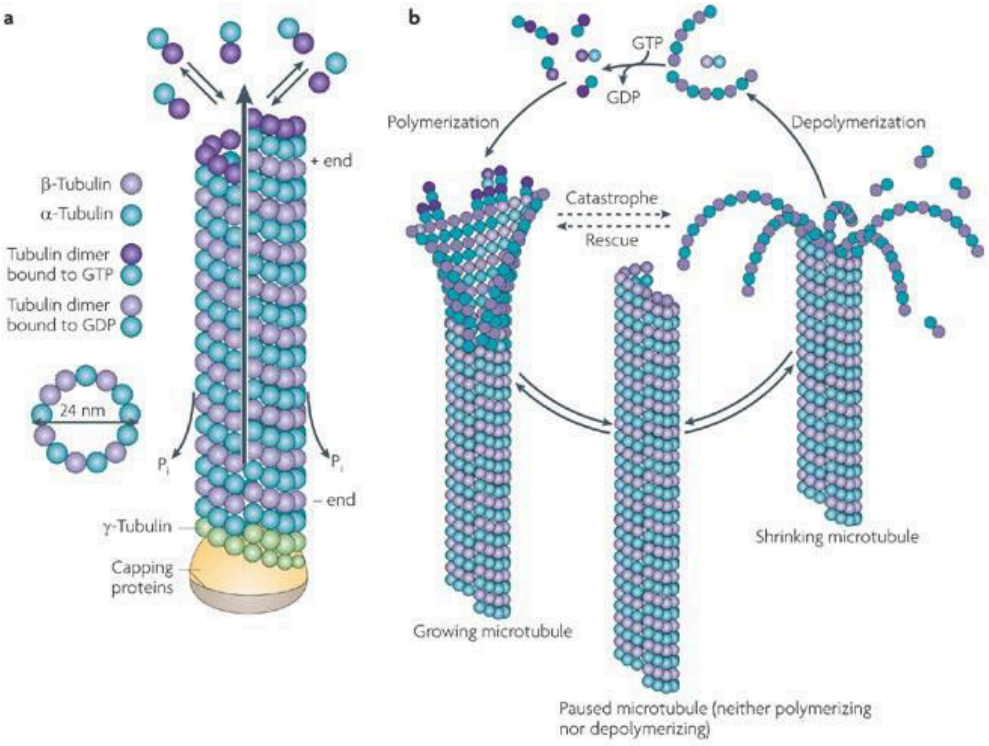

Figure 1 - MTs polymerization and depolymerization.

Polymerization occurs when α/β-tubulin dimers are added at the plus end of the microtubule. The polymerization of MTs is an active process regulated by GTP hydrolysis. The transition from polymerization to depolymerization process is called catastrophe. Figure adapted from [2].

Dynamics of MTs in the cell are regulated by γ-tubulin and microtubule associated proteins (MAPs). MAPs can be broadly classified into two types: motor proteins and non-motor proteins. Motor proteins are able to move along the surface of cytoskeleton filaments, such as MTs and actins [1]. They are powered by the hydrolysis of ATP and convert chemical energy into mechanical work. MT-based motors are, in general, of two types: kinesins and dyneins. The majority of the kinesins move towards the plus end of the MT and all dyneins move towards the minus end of the MT, thus helping to transport various components within the cell. Non-motor MAPs, promote the formation and maintenance of mitotic spindle through mechanisms such as nucleation, stabilization, and organization of MTs, influence motor proteins function, and regulation of cell cycle [3].

In dividing cells, MTs play predominant role in mitotic spindle formation and segregation of chromosomes while in non dividing cells, MTs are components of the cytoskeleton, centrioles and cilia [2, 3].

1.1.2. Centriole and centrosome: assembly and function

The centriole, a ~0.5 µm long MT-based cylinder, is required to form centrosomes [4, 5]. Centrosomes are composed of two centrioles, a mature (also called mother) and a daughter centriole orthogonally positioned, and pericentriolar material (PCM) [6]. They act as the major microtubule organizing center (MTOC) in cycling cells and play a critical role in determination of cell polarity [6, 7]. Each centriole has a radially symmetric arrangement of nine MT triplets. However, *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* (*D. melanogaster*) constitute exceptions to this structure. In *C. elegans*, the centriole is composed of singlet MTs, and in *Drosophila* embryo, the centrioles are composed of MT doublets [8, 9]. The radially symmetric MTs of the centriole emanate from the structure made of SAS6 and Bld10/CEP135, called cartwheel (Fig. 2). This structure, first described in *Paramecium* and *Chlamydomonas*, localizes at the proximal end of the daughter centriole (procentriole) and disappears from the mature centriole [10]. In the cartwheel, nine radial spokes act as linkers between the central hub and MT triplets (Fig. 2) [11, 12].

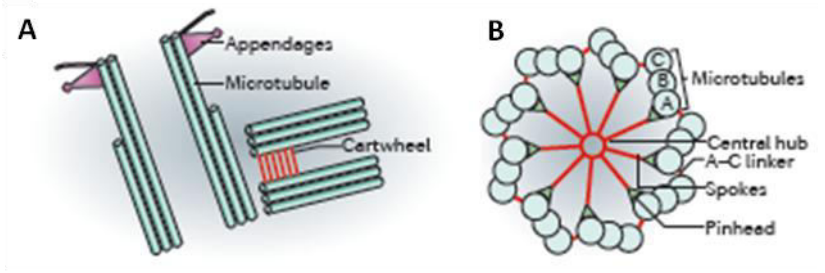

Figure 2 - Centrosome structure.

(A) Cartwheel structure present in red, microtubules in green and appendages in pink. The centrosomes are constituted by two centrioles orthogonally positioned. (B) Each one of the centrioles has 9 triplets of MTs (A, B and C) radially arranged. A-microtubule connects to the cartwheel and to the C-microtubule of the previous triplet. Figure adapted from [12].

The mother centriole acquires the sub-distal and distal appendages, which protrude out from the MT triplets. The sub-distal appendages function as anchoring microtubules and they are significantly variable in number, thickness and distribution (Fig. 3). The distal appendages display a constant 9-fold radially symmetrical organization, and those are important for the association of the mother centriole to the plasma membrane (Fig. 3) [13]. Many of the centriolar proteins show differential localization to the mature and daughter centrioles. While SAS6 and Centrobin localize at the daughter centriole, many others, such as Cep170, EB1, Ninein and Odf2, only localize at the mature centrioles [13]. Interestingly, Cep164 a centrosomal protein also localized at the distal

appendages of the mature centriole was recently reported as being involved in primary cilium formation in mammalian cells (Fig. 3) [14].

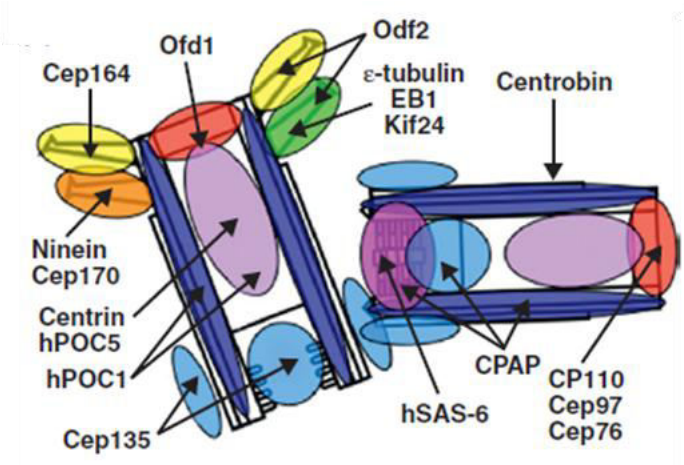

Figure 3 - Representation of a centrosome and some markers localized in it.

There are several proteins that are only localized at the mother centriole, such as Cep164, only localized in the distal appendages (yellow), Cep170, ε-tubulin, EB1, Ninein, only localized in the subdistal appendages, and Odf2,localized in both appendages. Sas6 is only localized to the daughter centriole as well as centrobin. Figure adapted from [10].

Besides making part of centrosomes, centrioles are also involved in cilia formation in animal cells. In ciliated cells, the basal body (BB), a structure derived from the mature centriole of the centrosomes, acts as a template for cilium/flagellum assembly [7, 15].

1.1.3. Structure of cilia

Cilia and flagella are MT-based organelles that emanate from the surface of eukaryotic cells. They are appendage structures that grow from a BB, a modified mother centriole, in animal cells. Cilia have amazing functional and structural diversities. They are involved in a variety of processes including cell motility, fluid flow, sensing, and sensory modulating processes [5, 15]. Notably, cilia can be motile or immotile, a property that reflects the MT arrangements of their skeleton, the axoneme [16]. Based on the MT arrangement in the axoneme, cilia can be classified in general in two structural classes: 9+2 and 9+0 [13, 16]. Generally, 9+2 structure (9 peripheral doublets of MT radially arranged and a central MT pair) is related to motility, while 9+0 structure (lacking the central MT pair) is associated to immotily. In 9+2 structure, there are also present radial spokes and dynein arms (Fig. 4). Cilia motility is due to the presence of dynein arms, which move towards the minus end of MTs bending cilia structure. However, there are exceptions to this general 9+2 structure associated with motility. For example, cilia in embryonic node cells in mouse, which establish the left and right symmetry in the body, lack the central MT pair. They have a 9+0 conformation but still they are motile. Another example, cilia of olfactory neurons, in *D. melanogaster*, have 9+2 structure but they are immotile [13].

A cilium consists of three morphologically distinct zones: basal body (BB), transition zone (TZ) and axoneme (Fig.4) [17].

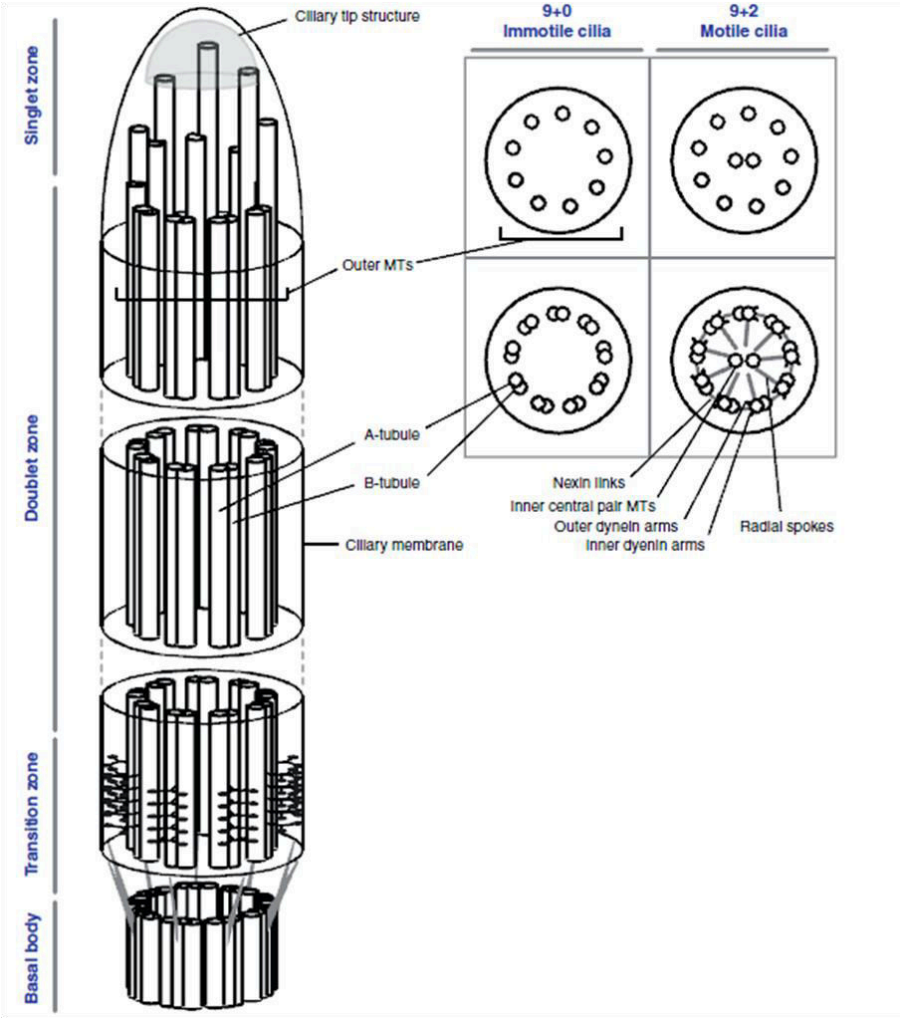

Figure 4- Cilia consist of three distinct zones: basal body, transition zone and axoneme.

BB localizes at the base of the cilia and templates the MT skeleton of cilia. Transition fibers (grey) are localized at the BB and along with Y-shaped linkers, localized in the TZ they constitute a ciliary gate from which molecules enter in the ciliary compartment. The axoneme is the ciliary skeleton, formed by the TZ and the doublet and singlet zones. It, generally, has two main structures: 9+0, mostly immotile, and 9+2, with dynein arms, radial spokes and nexin links, mostly motile. Figure adapted from [17].

The BB, a modified mother centriole, is a structure localized at the base of cilia/flagella and templates the axoneme, the MT skeleton of cilium. The TZ grows from the distal part of the modified mother centriole. This zone consists of transition fibers (TF) and Y-linkers, which might provide a filter for molecules that go inside cilium; and doublet MTs [9]. Thus, TZ bridges the triplet MTs of the BB and the doublet MTs of the axoneme (Fig.4) [17].

The TZ is proposed to regulate the entry of cytoplasmic components into the cilia. Some nuclear-pore complex (NPC) proteins are also located at the base of the cilia suggesting that the

mechanism of the trafficking at the ciliary base/TZ might be to some extent similar to that at the nuclear pore [18, 19]. Moreover, a recent study suggests that the ciliary base acts as a size-dependent barrier for diffusion of cytoplasmic molecules into ciliary compartment and larger molecules are trafficked into the cilia by active transport or diffusion through the membrane. Ciliary import systems use three of the molecules that regulate the entry of molecules in the nucleus: the importins, the RanGTPase system and nucleoporins (Fig.5) [19, 20].

KIF17, a kinesin-2 family anterograde motor, is involved in intraflagellar transport essential for primary cilia biogenesis, maintenance and function. KIF17 interacts with importin-beta2, a cofactor, and enters into the cilia through the ciliary gate and TZ (Fig.5). In cilia, RanGTP dissociates from the KIF17/importin complex, allowing KIF17 to proceed to intraflagellar transport along the axoneme. Thus, importin and RanGTP play crucial roles in transporting molecules and in controlling the entry of the molecules into the cilium, respectively [20].

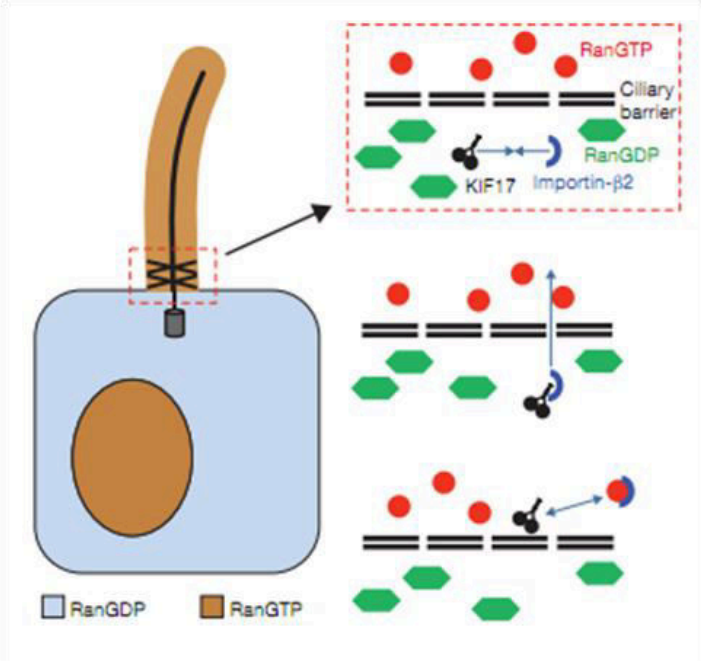

Figure 5 - Model structure of how KIF17 enters in ciliary compartment.

Model structure ciliated cell. RanGTP (red) dissociates from the KIF17 (black)/importin (blue) complex, allowing KIF17 to enter in the ciliary compartment. Figure adapted from [20].

1.1.4. Centrosomes and cilia: implications in human diseases

The mutations disrupting the function or structure of centrosome and cilia cause several human disorders, including infertility, ciliopathies, and cancer [5]. Comparative proteomics and genomics analysis showed that the cilium may require up to 1000 polypeptides for its proper function [7]. Cilia are present in several human organs and they are extremely important for human's health. Motile cilia are present in the human's respiratory airways, in the female/ male reproductive system and in embryo nodal cilia and they are important to remove the mucus from the body, move the reproductive cells to enable fertilization and establish left and right symmetry of human body, respectively. Immotile cilia have sensing functions. In humans, they are present in the kidney, in pancreas and in the photoreceptor cells in the eye. The first case of a human disease linked to a protein localized in cilia was reported in 2000 [7]. In the last two decades, a number of discoveries have led to a virtual explosion of research in the centriole and cilia field, highlighting the terrible consequences of absent or defective cilia in human health and development. Defects in the centriole and the cilia function and assembly were found to originate a huge range of human diseases. These diseases are collectively called 'ciliopathies' and they can be related to defects either in motile or non-motile cilia. Polycystic kidney disease, situs inversus, retinal degeneration, nephronophthisis, polydactyly, and obesity are some ciliopathies reported in humans (Fig.6) [7, 21].

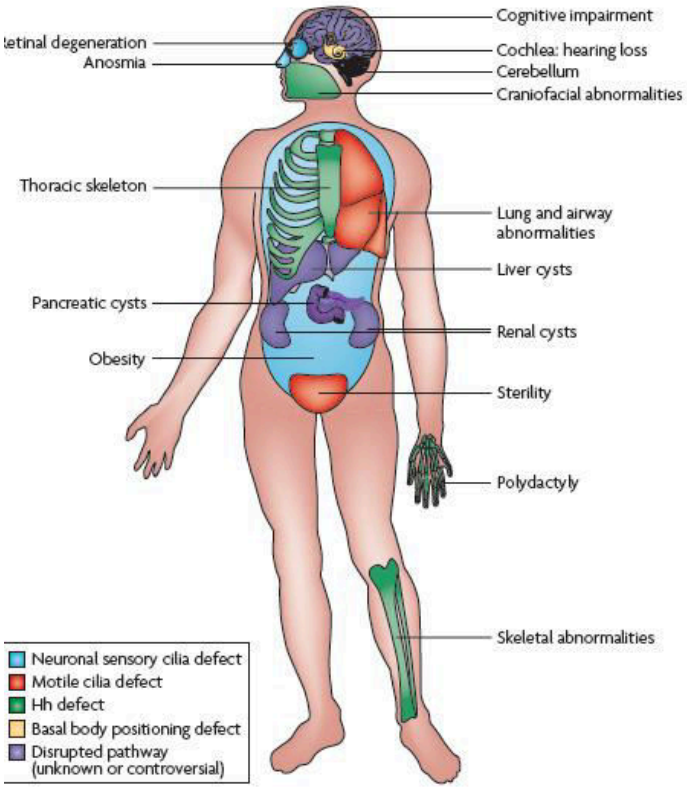

Figure 6 - Ciliopathies commonly associated with human organs.

The organs are represented in different colors, depending on the cilia defects reported to them. It is also represented the common ciliopathies associated to each organ. Figure adapted from [21].

1.2. Cilia Biogenesis

Ciliopathies are known to be associated with defects in ciliary structure and function, therefore the study of how cilia are assembled is of major importance. Cilia biogenesis (Fig.7) starts by the attaching of vesicles from the Golgi complex to the distal appendages of the mother centriole. Subsequently, the centrosome moves towards the cell membrane, process that is dependent on the actin cytoskeleton of the cell. During the process of the apical movement of centrosome, more vesicles from Golgi-complex fuse to the vesicle bound to the mother centriole. A TZ starts assembling from the distal end of the mother centriole covered by vesicular membrane. Later, the mother centriole docks through the distal appendages to the cell membrane, the vesicle fuses with the cell membrane and cilia grow [9, 22, 23].

The growth of cilia is predominately controlled by the intraflagellar transport (IFT), which mediates the transport of proteins from the cytoplasm until the ciliary tip [22, 23]. IFT was first observed in the alga *Chlamydomonas reinhardtii* (*C. reinhardtii*) as a bidirectional movement of particles (IFT particles) along the flagellum in between the ciliary membrane and the axoneme [24-26]. The movement of IFT particles from the base of the cilium to the tip is called anterograde movement; it is carried by kinesin-2 and it is associated to the axoneme MT. In the other hand, the retrograde movement, from the tip to the base to the cilium, is carried by cytoplasmic dynein-2 [24, 26].

In ciliated eukaryotic organisms, IFT proteins and motors are well conserved and defects affecting IFT cause a variety of developmental and functional defects. It was shown that defects in IFT affect cilia assembly in *C. reinhardtii*, in *Tetrahymena thermophila* (*T. thermophila*) and in mice. It was also shown that IFT is required for cilia elongation in sensory neurons but not in sperms, in *D. melanogaster* [8, 24].

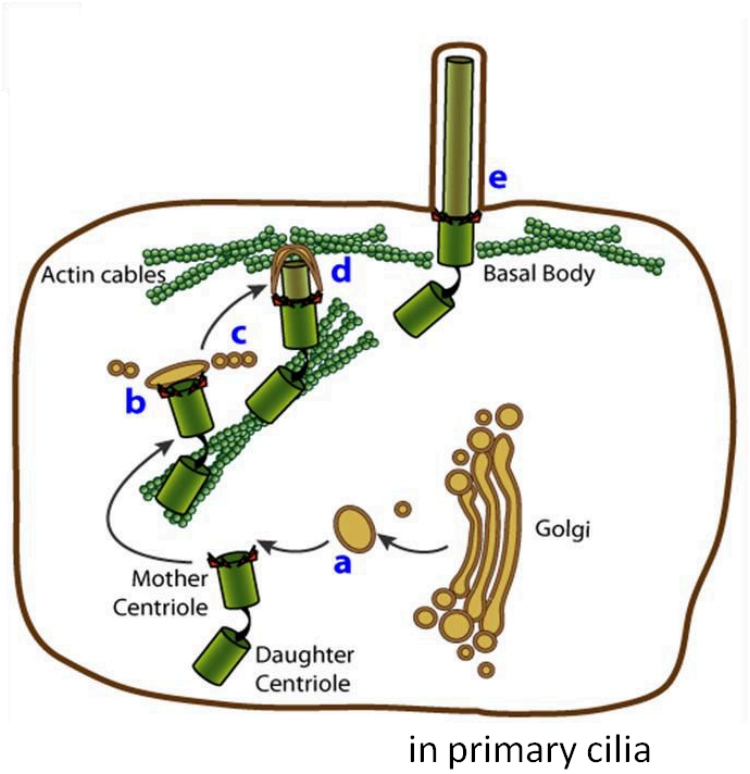

Figure 7 - Ciliogenesis schematic process.

A vesicle from the Golgi-complex binds to the distal appendages of mother centriole (a), more vesicles bind to the previous one (b) and the TZ starts growing (d). This system is transported to the cell membrane, through actin cytoskeleton, it docks to the membrane and the cilium elongates (e). Figure summarized based on [22]; model made by Swadhin Chandra Jana.

The process of ciliary growth is extensively investigated, however, the maturation of centrioles and its transformation to BB is poorly understood [27].

Many proteins causing ciliopathies, such as Odf2, MKS, Cep290 and Cep164, localize at the BB. However, the specific role of those molecules in cilia biogenesis and the interaction mechanism of those ciliopathy causing proteins are mostly unknown. Scientists have been using several model organisms to try to understand the possible role of these molecules in cilia assembly.

1.3. Model Organisms

The high conservation of ciliary structure enabled scientists to study cilia assembly and disassembly mechanisms using different model organisms, such as *C. reinhardtii, T. thermophila, D. melanogaster, C. elegans,* zebrafish, and mouse [28].

Some of those model organisms exclusively contain motile cilia used for its movement, such as *T. thermophila* and the green algae *C. reinhardtii,* both unicellular eukaryotic organisms. While *T. thermophila* has cilia distributed all over its surface, *C. reinhardtii* only has two flagella. In *C. elegans* all ciliated cells grow immotile cilia, which are important for the osmotic avoidance, mechano-sensation and chemotatic behavior [28].

Nevertheless, there are also other model organisms, such as *D. melanogaster* (the fruit fly), zebrafish and mouse, which contain motile and immotile cilia. For this reason, these organisms are appropriate for studies in which the main aim consists in studying both type of cilia structure and function [28].

Amongst these ones*, D. melanogaster* is the easiest model organism to work with for many reasons. At 25ºC, it has a short generation time, it takes nearly 10 days to obtain adult flies from fertilized eggs and it is easy to maintain. Its whole genome is sequenced, and >60% of human genes have functional homologues in it [29]. It is an excellent organism to do genetics because it only has four chromosomes and a wide variety of balancers with dominant phenotypic markers. This provide us a way to manipulate and trace individual chromosome [30]. Additionally they also have some homology with humans in terms of body structure. Taken together these characteristics make *D. melanogaster* an important model organism to study human diseases, such as neurodegenarative diseases, ciliopathies, inflammatory disorders and even cancer.

In *D. melanogaster* cilia are present only in type-I sensory neurons and in the sperm [7].

The antenna (Fig. 8) is a type-I sensory organ in the fruit fly and it localizes in the fly's head. It is composed by 3 segments (A1, A2 and A3) and the arista. Type-I sensory neurons, such as auditory, chordotonal and olfactory neurons localize in the fly's antenna and they are involved in audition, coordination and olfaction, respectively. The Johnson's organ, also localized in the antenna (Fig. 8), is the auditory organ. Its main roles are related to hearing, receiving the signals for the courtship song, to detect the wind current during flight/ walk and gravitactic behavior [7]. Chordotonal neurons are important for sensing the touch and for flies' proper movement execution [7].

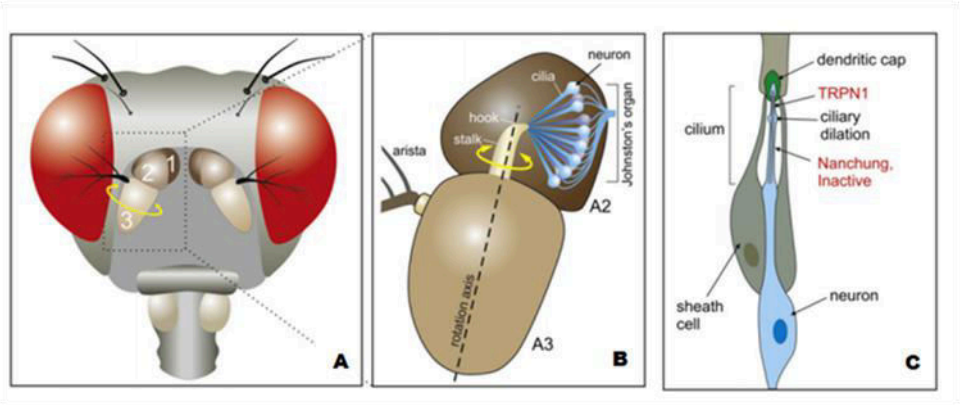

Figure 8 - Sensory neuron organs in adult fly.

A) Antenna localized in the fly head. It is composed by 3 segments (A1, A2 and A3) and the arista B) Detailed representation of A2 and A3 segments in the antenna. Johnston's organ, the auditory organ, is localized in the A2 segment and its vibration makes A3 segment rotate. C) Johnston's organ in which a neuron extends a cilium. Figure adapted from [31].

Sensory bristles and campaniform sensilla are other type-I sensory organs existent in the fruit fly. Sensory bristles are present in the head, in the thorax, in the wing and at the leg joint and they are essential for accurate movement execution. Campaniform sensilla are localized at the limb joints and along the wing blade and they act in response to local deformations of the cuticle such as wing bending [7].

As a consequence, defects in type-I sensory neurons are visible in *D. melanogaster* behavior, either in the larvae by an insensitivity to touch and smelling or in the adult fly by uncoordinated movement, anosmia and deafness [7].

In this model organism, motile cilia are found in spermatids and in chordotonal neurons, while immotile cilia are present in olfactory neurons and spermatocytes. Each sensory organ is constituted by one or more neurons, each of them with only one dendrite with a cilium at its tip, and several support cells [7]. For example, olfactory neurons are constituted by two neurons that together give rise to one dendrite and, consequently, to one cilium.

1.3.1. Drosophila melanogaster

D. melanogaster is an insect with about 3 mm long as an adult, which undergoes several stages during its development, like embryo, larvae, pupa and adult fly (Fig. 9) [32].

D. melanogaster life cycle starts when fertilization occurs. An embryo about 0,5mm long is formed 3 hours after fertilization and the larva (first instar) hatches about 24 hours after fertilization. Over feeding and growing process, the larva will pass through two more stages, called second and third instars. Imaginal dics of third instar larva, which are small sheets of epidermal cells derived from the cellular blastoderm, give rise to many body parts of adult fly upon metamorphosis. After the last instar, the larva becomes a pupa and metamorphosis into the adult stage takes place. The adult fly will emerge approximately 10 days after fertilization (Fig. 9) [32].

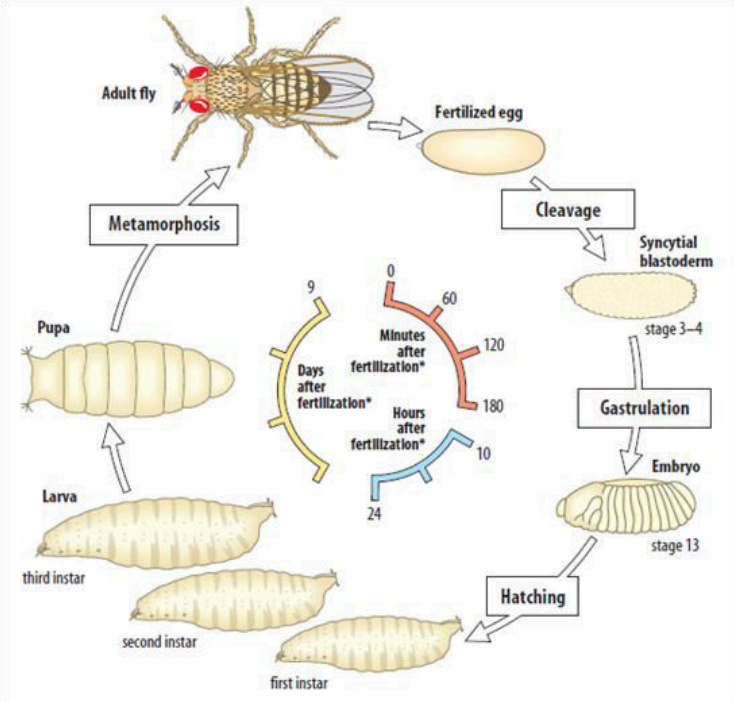

Figure 9 - *Drosophila melanogaster* life cycle.

After fertilization, the fertilized egg will develop and give rise to an embryo that will undergo 3 larva stages becoming a pupa approximately, 7 days after fertilization. In the 3rd instar larva stage imaginal discs are formed, they will develop and give rise to the fly organs. Then the metamorphosis happens and at the 10th day the fly eclodes. Figure adapted from [32].

During *D. melanogaster* life cycle ciliated cells are formed, such as neurons and sperms. Neurons are formed in a process called neurogenesis during embryonic development [33]. The sperm is formed through a process called spermatogenesis during testes development [34].

1.3.1.1. Neurogenesis

Neurogenesis starts when the precursor cell (pI) divides four times asymmetrically giving rise to a microchaeta composed of a socket and the shaft cells (support cells) and of the sheath and neuron cell (internal cells). The neuron cell is the last one to be formed and it derives of a neuronal precursor cell (pIIIb) (Fig. 10) [35].

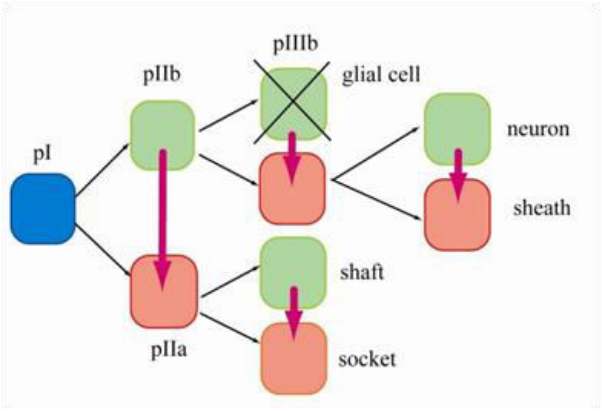

Figure 10 - Schematic process of a neuron biogenesis in *D. melanogaster*.
A precursor cell divides four times asymmetrically originating a neuron cell and the supporting cells: the shaft, the socket and the sheath cells. Figure adapted from [35].

When the neuron cell is formed the centrioles start migrating to the opposite side of the cell and the sprout is formed. Subsequently, a neurite elongates giving rise later to a dendrite and in the opposite side of the cell, the axon grows. The type-I sensory neurons maturation is followed by centriole docking to the membrane and cilia assembly (Fig. 11) [33].

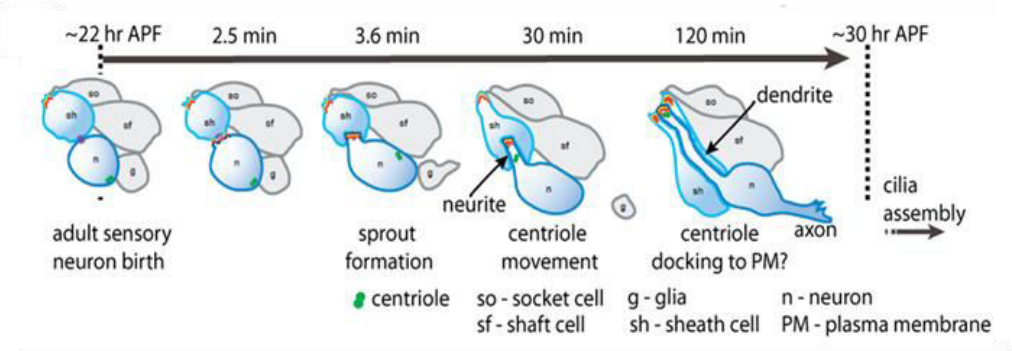

Figure 11 - Schematic process of neurogenesis in *Drosophila*.

After precursor cell divides 4 times asymmetrically, a neuron cell is formed. The centrioles in this cell migrate to one pole of the cell and a neurite forms. This neurite elongates forming the dendrite and in the other pole of the cell an axon grows. After this process cilia will assemble from the dendrite. Figure adapted from [33].

1.3.1.2. Spermatogenesis

Spermatogenesis starts when a stem cell gives rise to a gonial cell that will undergo 4 rounds of incomplete mitotic divisions, producing a cyst with 16 interconnected primary spermatocytes each one containing four centrioles. During primary spermatocyte maturation, the cells grow and the centrioles migrate towards the membrane and attach to it. After docking, each centriole gives rise to a small cilium involved by a membrane (Fig. 13). These structures are later internalized, with the membrane involving the small cilium, and the matured spermatocytes undertake two meiotic divisions giving rise to a cyst of 64 early spermatids. In this stage, the nucleus suffers some shape changes and each axoneme grows from one basal body (Fig. 12) [34].

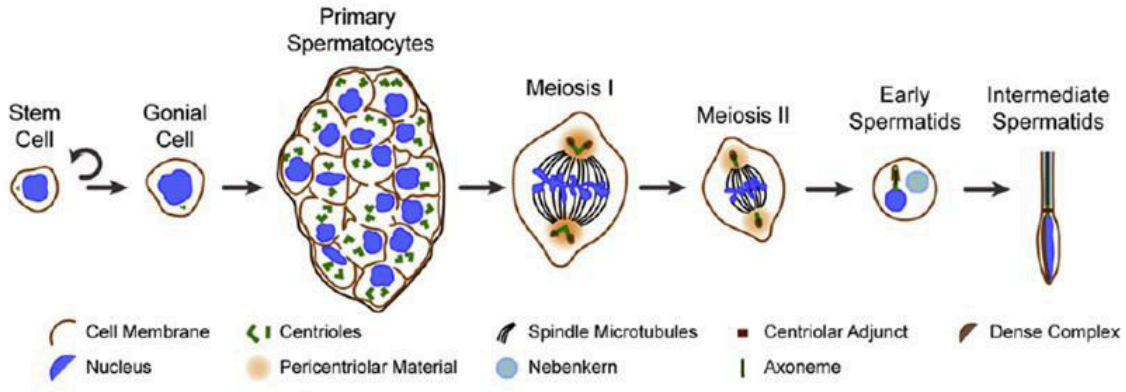

Figure 12 - Spermatogenesis process in *Drosophila*.

A stem cell grows and gives rise to a gonial cell that will undergo 4 mitotic divisions. These divisions are not complete and a cyst with 64 spermatocytes interconnected is formed. After this the cells undergo 2 meiosis and an early spermatid is formed. At this stage the spermatid already has a small cilium. Then the axoneme grows. Figure adapted from [34].

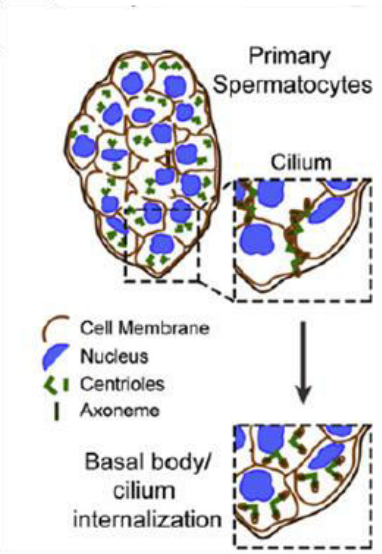

Figure 13 - Primary spermatocytes maturation.

In the primary spermatocytes cell stage the centrioles dock to the membrane and a small cilium grows. These structures are then internalized and the cells follow from the next stages of meiotic divisions. Figure adapted from [34].

1.4. CEP164

Centrosomal proteins are involved in cilia structure and function [15]. CEP164, a novel centrosomal protein, localizes at the distal appendages of mature centrioles, in U2OS and RPE cells. This was shown by immuno-electon microscopy, in U2OS cells, and in RPE cells, by co-localization with other distal appendages proteins, such as CEP83, and not co-localization with sub-distal appendages, such as ODF2, through super-resolution microscopy [14, 36].

Studies using stimulated emission depletion (STED) microscopy, in mouse tracheal epithelial cells (MTECs), showed that this protein localizes at the mother centriole in a 9-fold symmetry arrangement [37]. Also, using two nanoscopic techniques combined, such as photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) it was observed that CEP164 localizes specifically to the mother centriole in a ring-like structure surrounding the centriole, in HeLa cells [38].

CEP164 has been described as a protein of major importance to cilia assembly [14, 36, 39, 40]. CEP164 is implicated in primary cilium (PC) formation in mammalian cells [14]. PC is a MT-based structure, which protrudes from the surface of eukaryotic cells [23]. Interestingly, CEP164 also persists at the mature centriole during mitosis [39, 40]. Moreover, CEP164 is required for cilia membrane biogenesis by promoting vesicular docking to the mother centriole. This role is characterized by CEP164 interaction with Rabin8 and Rab8, essential components of the vesicular transport machinery (Fig. 14) [39]. This indicates that CEP164 may act as regulator and/or mediator of vesicular docking at the basal body and membrane incorporation to the cilia. However, this role has to be investigated.

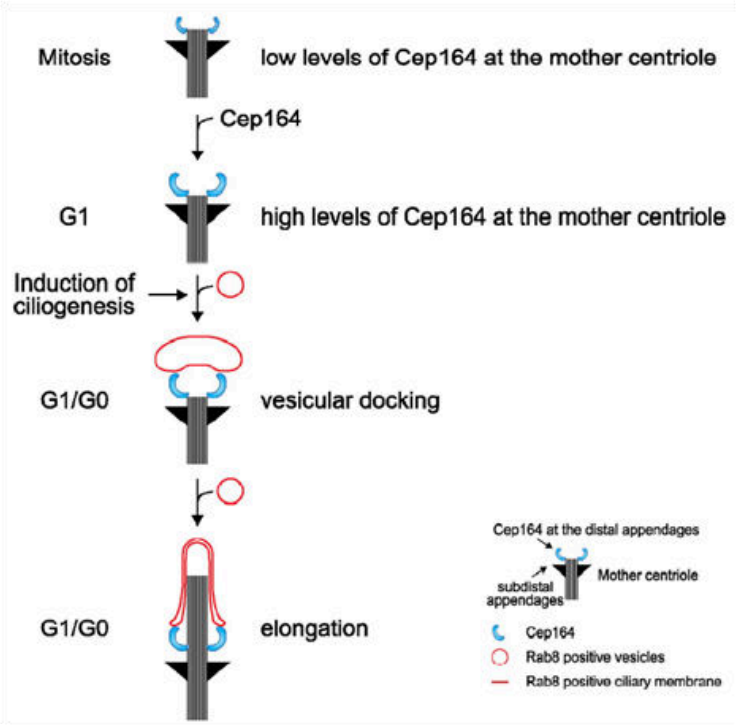

Figure 14 - Model of CEP164 role during cilia biogenesis, in human cells.

CEP164 is present specifically at the mother centriole during mitosis and cilia assembly. Ciliogenesis is induced when height levels of CEP164 are recruited to the distal appendages of the mother centriole. CEP164 promotes vesicular docking to the distal appendages via interaction with Rab8 and Rabbin8, components involved in vesicular transport. After vesicular docking to the distal appendages and dock to the cell membrane, the axoneme extends. Figure adapted from [39] .

Recent studies show that CEP164 is involved in two cellular processes, one related with cilia related diseases and the other related with genomic maintenance and stability. In zebrafish, mutation in *Cep164* was reported to cause nephronopthisis phenotype, a ciliopathy. Nephronopthisis is a recessive disease that affects several human organs like kidney, retina and brain through a degenerative process in the beginning of adulthood and by prenatal-onset dysplasia. In recent past, many mutations in CEP164 were reported to be present in several retinal ciliopathy patients [40]. In the other hand, mutation in CEP164 gene was also reported to cause deregulated DNA damage response (DDR), in the same organism [40]. This role in DNA damage response, for maintenance of genome stability and cell survival was also reported in Hela cells [41]. Notably, though CEP164 is shown to be involved in DNA damage response and ciliogenesis, the mechanism of the dual role of CEP164 in those processes is not well understood yet.

In RPE cells, CEP164 recruitment to the mature centriole seems to be regulated by the presence of other distal appendages proteins. Using an RNAi strategy, it was shown that CEP164 is crucial for ciliogenesis in these cultured cells [36].

Thus, CEP164 is now seen as an extremely important target to understand how cilia are formed. *D. melanogaster*, a model organism with relevant genomic similarity with human genome seems to be an excellent model organism to investigate the role of CEP164 in cilia assembly and it contribution to human diseases.

1.5. *Drosophila melanogaster* genetics

Fruit fly possesses two distinct types of cilia/flagellated cells: the sperm and the type-I sensory neurons, mainly used as chemo- and mechano-sensors. Mutations in genes that affect cilia/flagella assembly lead to clear phenotypes, such as delayed development, uncoordination and sterility in the fruit fly. This makes it an ideal system for studying the role of CEP164 in two different types of cilia assembly and function. As mentioned before the fruit fly has several advantages to do scientific research; especially the advantages in genetic and cell biological analysis [30].

The main genetic advantage consists in the chromosome balancers; this tool is what distinguishes flies genetics from the other organisms. Balancers are chromosomes whose sequence was modified using radiation [30]. This produced chromosomes with multiple inversions, which avoid recombination during meiosis. Recombination only happens in females; in males, meiotic recombination does not occur. Another advantage of these chromosomes is that they are usually marked with a dominant marker mutation and also recessive markers. Hence, these markers help to trace the flies with desired chromosomes. Many balancers exist for the first (X), second and third chromosomes: FM, SM and TM, respectively [30].

Marker mutations can be used to track chromosomes .There are numerous markers for the eye color, wing shape, bristle shape and others. For example, *Cyo* is a marker usually associated with the SM, in which flies present curly wings [30].

These markers associated with other genetics approaches are powerful tools for studying the role of a protein in a specific tissue or cell. There are some strategies commonly used by scientists to study the effect of a protein in a cell or tissue. Transposable elements and the Gal4/UAS system are two of the genetic approaches widely used in *D. melanogaster*.

1.5.1. Transposable elements strategy

Transposable elements are genomic segments that have the capacity to move from one place to another inside the genome of an organism. This movement can cause disruption of gene expression and/or produce different transcripts, thus having an effect on the phenotype.

Transposable elements are divided in two classes: retrotransposons, where the transposition intermediate is the RNA, and DNA transposons, where the transposition intermediate is the DNA. The transposition mechanism usually occurs in two ways: replicative and conservative. In the replicative mechanism the transposon copies itself (copy-and-paste) and moves to another place maintaining the original copy in the proper place inside the genome. This mechanism is used by retrotransposons [42]. DNA transposon does not copy itself (cut-and-paste), and the transposition mechanism is conservative [42].

P-transposable elements are DNA transposons, which act through a conservative mechanism [42]. They are considered as an important genetic tool used in D*rosophila* genetics, as they are artificial genetic elements engineered with a visible marker such as white^{+} gene (red eyes fly) [43]. They are composed of a central region flanked by inverted terminal repeats that are recognized by the transposases, which catalyze the “jump out” of the P-element of the genome and enables it to have mobility [42].

1.5.2. Gal4/UAS system

The Gal4/UAS system is a genetic tool that allows the expression of a gene in a specific cell or tissue. Gal4 is a transcription factor that encodes a protein responsible for regulating genes of the galactose metabolism, naturally present in yeast. This transcriptional factor was identified in *Saccharomyces cerevisiae*, and its DNA binding and transcriptional activations are separate [44]. Gal4 transcriptional factor originates a protein that will bind to the Upstream Activating Sequence (UAS) promoter, which is crucial for the transcription of the Gal4-regulated genes.

In 1993, the Gal4/UAS system was introduced in *D. melanogaster* by Brand and Perrimon for targeted gene expression [45]. It is one of the most widely used genetic tools. To activate the expression of the responder (gene of interest), responder lines are mated with flies expressing Gal4 in a specific pattern called driver (Fig. 15) [45]. Therefore, in the progeny the expression of the responder in a specific cell or tissue is determined by the driver (Fig. 16). In this system the driver and the responder are maintained as separate parental flies [46].

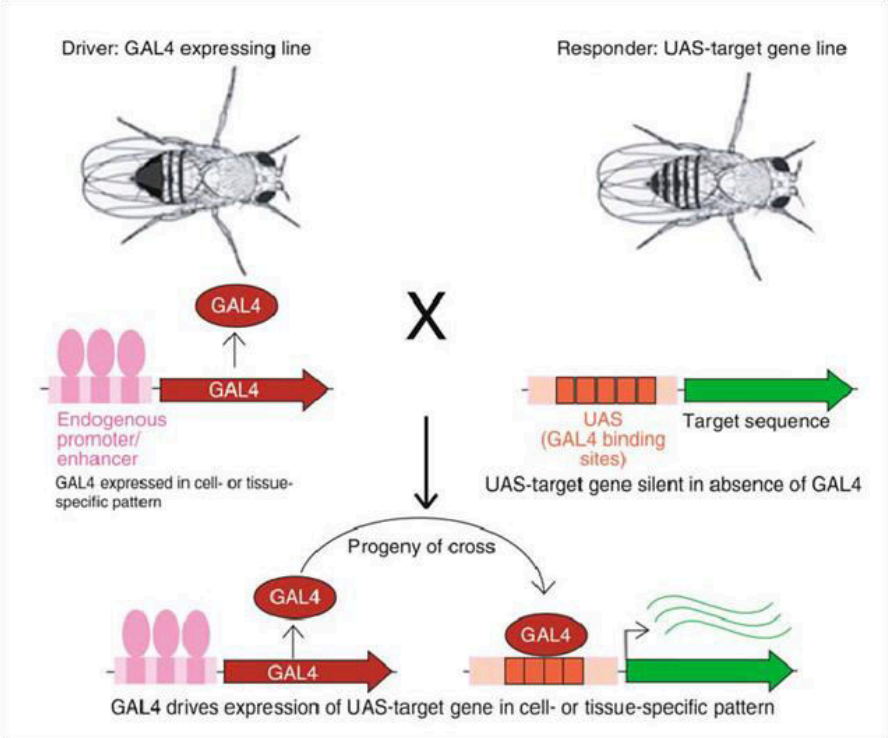

Figure 15 - Gal4/UAS schematic process.

Strategy commonly used by fly geneticists in which flies that express Gal4 in specific tissues are crossed with flies that have the promoter to which Gal4 will bind and then induce the expression of the target sequence. Figure adapted from [44].

1.6. Project objectives

The aim of this project is to study the role of CEP164 in *Drosophila melanogaster* ciliogenesis. To study this, two approaches will be taken: the first is to knockdown the protein expression in different tissues using Gal4/UAS-RNAi strategy; and the second is to create few *CEP164* mutant lines. Cilia are present only in Type-I sensory neurons and sperms in *D. melanogaster*, therefore, depletion of Cep164 was done expressing Cep164 RNAis using different driver lines reported to express in neurons and testes. The aim was to study the effect of knocked down in flies´ walking behavior, fertility and cilia morphology. A P-element mutant of Cep164 was available. However, the P-element is inserted at the 5´-end of the gene and does not cause any behavior defect. Therefore, the aim was to make few excision lines of *CEP164* gene.

Chapter 2 - Materials and Methods

2.1. Immunostaining of *D. melanogaster* embryo

To study the developmental profile of CEP164, the embryos of different ages were collected. The flies were synchronized and the eggs were collected for 2 hours on a petri dish containing normal fly food. The embryos were aged for different times (8, 12, 16 and 18) and methanol-fixed for immunostaining. They were washed with a 50% bleach solution for dechorionation and transferred to a solution containing heptane, formaldehyde and PBS. The embryos were then shaken vigorously and kept in the rotor for 20 minutes to permeabilize the vitaline membrane. Then, those were immersed in the methanol for dehydration and stored at 4°C. Previously stored embryos were rehydrated in PBS1x, followed by PBS 1×/0.1% Tween. Embryos were blocked in PBT (0.1 % Tween and 1% BSA in PBS1x) and were incubated with primary antibodies diluted in PBT at 4°C overnight. Samples were washed in PBT and incubated with secondary antibodies diluted in PBT for 2 hours at room temperature (RT). Embryos were washed in PTW (0.1 % Tween in PBS1x) and incubated with DAPI for 20 minutes at RT. The samples were then washed in PBS1x and mounted in Vectashield Mounting Media (Vector Labs; US). For my study, w; ChatGal4UASGFP; RFPPACT embryos were collected. Anti-CEP164 and anti-22c10 primary antibodies, and FITCanti-mouse and Cy5anti-Rabbit secondary antibodies were used for this study.

The images were collected using Leica TCS Sp5 confocal microscope using 60x PL APO CS objective. The images were analysed using ImageJ (NIH, US).

2.2. Knock down of CEP164 in ciliated cells

2.2.1. RNAi strategy

To study the depletion of CEP164 during cilia biogenesis in neurons and in sperm, Gal4/UAS-X-RNAi systems established in the fruit flies were used.

Two different RNAi lines were used to deplete CEP164 in different cell types by pre-selected drivers based on their expression pattern. The different drivers were selected to deplete the protein during the neuron and sperm developmental time (Fig. 16).

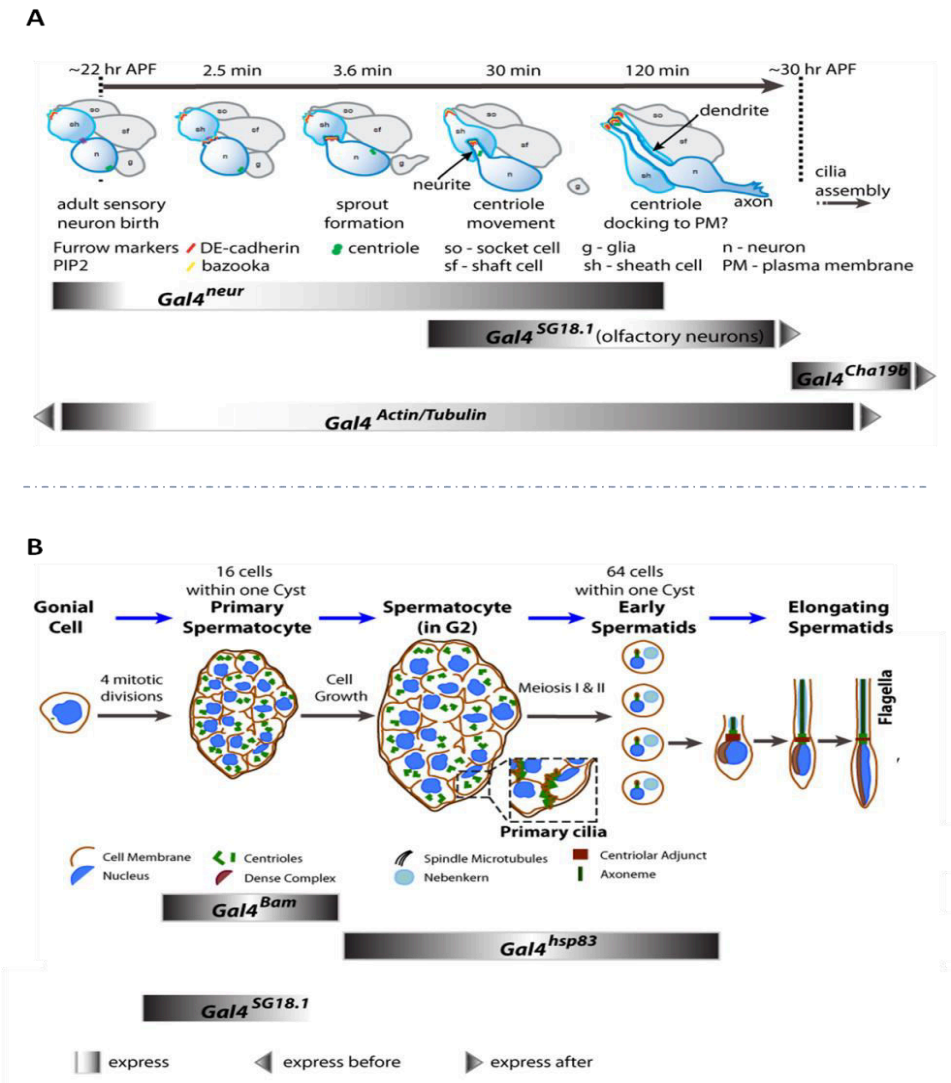

Figure 16 – Expression pattern of different Gal4 drivers.
(A) Expression profile of Gal4 drivers during neurogenesis. neur-Gal4 is expressed in pre-neuron stages, being crucial for neuron assembly, Cha19b-Gal4 is expressed after the neuron assembly, when cilia starts

growing, playing a role in maintenance and Sg18.1-Gal4 is expressed in olfactory neurons in the transition from assembly to maintenance. Actin-Gal4 and tubulin-Gal4 are expressed in all cells. (B) Expression profile of Gal4 drivers in spermatogenesis. Sg18.1 is expressed before and during primary spermatocytes stages, Bam-Gal4 is expressed in the primary spermatocyte stage. hsp83-Gal4 is expressed from spermatocyte to early spermatids stage [47-52]. Figure adapted from [33, 34]; models made by Swadhin Chandra Jana.

Depletion of CEP164 was done using UAS-CEP164 (1) RNAi (GD line) and UAS-CEP164 (2) RNAi (KK line). KK lines have a bigger depletion effect than GD lines [53, 54].

Since SAS6 is crucial for centriole assembly and centrioles are essential for cilia assembly, UAS-SAS6 RNAi was used as a positive control [55].

UAS-mCherry RNAi was used as negative control because mCherry protein does not endogenously express in *D. melanogaster*. The expressed interference RNA specific for mCherry does not interact with any *D. melanogaster* mRNA [56].

The flies were grown at two temperatures (25 and 29ºC) to investigate the effect of CEP164 depletion with the RNAis. It is known that depletions are stronger at higher temperatures using the Gal4/UAS system. At 29ºC Gal4 expression is more induced, so the effect of CEP164 depletion should be higher [46].

From these crosses the desired progenies were selected (Fig. 31) and their coordination and fertility were studied, as well as their cell morphology: neurons and sperm morphology.

2.2.1.1. Walking behavior of flies

Flies' walking behavior was tested using a bang assay [8]. This assay shows how depletion of CEP164 is affecting chordotonal neurons, the neurons responsible for flies' coordination.

In this assay, 10 flies were put in 100mL tubes (height (H)=18cm), the tubes were banged and the flies' climbing capacity was recorded during one minute. This assay was performed in triplicate to each sample of 10 flies of the same genotype, which means that each three videos for the same genotype represent a N number.

The videos were analyzed using ImageJ (NIH, US) and the number of flies was counted using a macro script written in the lab.

It was calculated the average of the number of flies that climbed above 9cm in the tube (50% of the tube) in the last second and it was investigated the tracks of the flies during 5 seconds

after banging the tube. The first demonstrate whether flies' climb capacity was affected through the depletion of CEP164 and the last reflect the flies' response to the stimuli submitted.

The average number of flies above half of the tube height in the last second was determined subtracting the average number of flies in the last second above half of the tube to the number of flies in t=0 seconds. Dividing the number the average number of flies in the last second above H/2 per number of flies at t=0seconds, the fraction of flies above H/2 is determined. The average of percentage of flies above H/2 was calculated, converting this fraction number to percentage number, and calculating the average of this percentage for all the N numbers from the same genotype.

The error bars represent the standard error of the mean (SEM), calculated dividing the standard deviation of the percentage of flies above H/2 per square root of the number of N videos analyzed.

2.2.1.2. Fertility of flies

Fertility tests were performed with the progenies arose from the crosses in which CEP164 as well as the controls were depleted using drivers expressed in testes: Sg18.1-Gal4; Bam-Gal4 and hsp83-Gal4 (Fig.16). The assay was done crossing 1 male of that progeny with three wild type virgin females in each tube [57]. Wild type (WT) crosses were considered as a negative as well as Gal4/UAS-mcherry RNAi control and Gal4/UAS-SAS6 RNAi as a positive control.

The number of pupa in the progeny was counted in the tenth day after the date in which the cross was sat. The average number of pupas counted was represented in graphs and the error bars represent the standard deviation of those numbers.

2.2.1.3. Cell morphology

To investigate the effect of CEP164 depletion in sensory neurons, we studied the effect of Cep164 knockdown in olfactory neurons' morphology.

The staining was made in neur-Gal4/CEP164 RNAis and actin-Gal4/CEP164 RNAis olfactory neuron because these were the progenies that showed a phenotype in walking behavior, meaning that chordotonal cilia is affected.

To study **neurons** morphology, the heads of the flies were cut and put in OCT and sections of 8μm were cut and attached to gelatinized/ poly-L-Lysine slides. The slides were fixed in 4% PFA during 30 minutes and then washed 4X10 minutes with 1X PBS at RT. Then they were blocked with 0.1% PBTX for 1 hour. Primary antibody was added in 0.1% PBTX plus 5% Bovine Serum Albumin for 2 hours at RT followed by washing in 0.1% PBTX plus 5% NGS at RT. The slides were incubated with the second antibody, washed in 0.1% PBTX and then incubated with DAPI for 20 minutes at RT. Finally the samples were washed in 1X PBS and mounted in Vectashield Mounting Media (Vector Labs; US).

anti-acetyl-tub and anti-D-PLP primary antibodies were used to mark cilia and peri-centriolar material, respectively. Anti-CEP164 antibody was used to check the efficiency of knockdown of Cep164. FITCanti-mouse, Cy5anti-Rabbit, Rodhaminanti-chicken secondary antibodies were used for this study.

The images were collected using Leica TCS Sp5 confocal microscope using 60x PL APO CS objective. The images were analysed using ImageJ (NIH, US).

To study **testes** morphology, RNAi progenies that have Gal4 expressing in testes were selected: Bam-Gal4.

Testes were dissected from the pharate adult that was still inside pupae. Males in pupae can be easily distinguished because they have black sex combs in their legs. Testes were dissected in a drop of 1X Testes buffer containing 1 mM EDTA (20 μL of a 0,5 M stock/ 10 mL of 1X Testis buffer) and transferred to a 4 μL drop of 1X Testis buffer placed in a poly-L-Lysine slide. The testes were random squashed with the forceps and covered with a 18X18 mm siliconized coverslip previously washed with ethanol. Then, the slides were put in liquid nitrogen and the coverslip removed. The slides were placed in dry ice cold methanol for 8 min, then transferred to dry ice cold acetone for 10 min and wash 3X 5 min each in PBS at RT. After this, the testes were blocked in PBS + 1% BSA for 1 h at room temperature and the slides were placed in a moist chamber pre-wetted with PBS and incubate with 1st antibody in PBS + 1% BSA o/n at 4ºC. Washed 4X 10 min each with PBS + 1% BSA at room temperature and incubated in the dark in a moist chamber pre-wetted with PBS and incubate with 2nd antibody 1:200 in PBS + 1% BSA for 2h at RT. Then washed 2X 15 min each with PBS + 1% BSA and then 1X 15 min with PBS at room temperature. In order to stain DNA, the slides were placed in a moist chamber pre-wetted with PBS and incubate with 1 μL DAPI /mL of PBS for 20 min at RT. Finally, washed with 1X 5 min with PBS and the slides mounted in Vectashield Mounting Medium for Fluorescence H-100).

PACT and UNC were used as markers for centrioles and cilia, respectively. DAPI were used as marker to nuclei and anti-CEP164 primary antibody was used to stain CEP164. Cy5anti-Rabbit secondary antibody was used in this study.

2.3.Generation of a CEP164 mutant

To excise out the P element inserted in the 5' UTR of the *Cep164* gene (CG9170) (Fig. 17), the EPgy2 line (Bloomington number 22524) was crossed with the flies containing a transposase (Fig. 18-Pcross). The male progenies (the jump starter fly) were out-crossed with Df(1)pn38,w/FM0 virgin females (Fig. 18-F1cross). It was screened for excised out flies in the following generations (Fig. 18-F2 and F3 crosses) and the effect of the excision in the progenies was analysed.

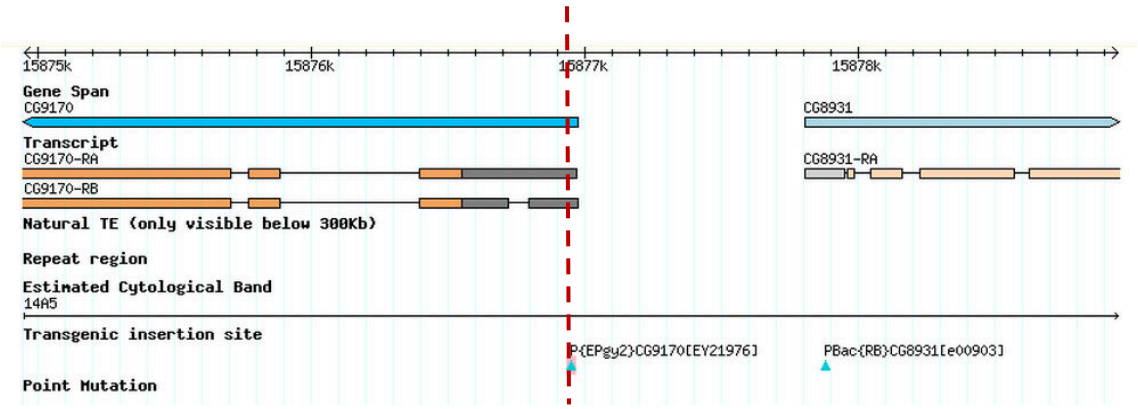

Figure 17 - Map showing the P element insertion in the CEP164 gene.

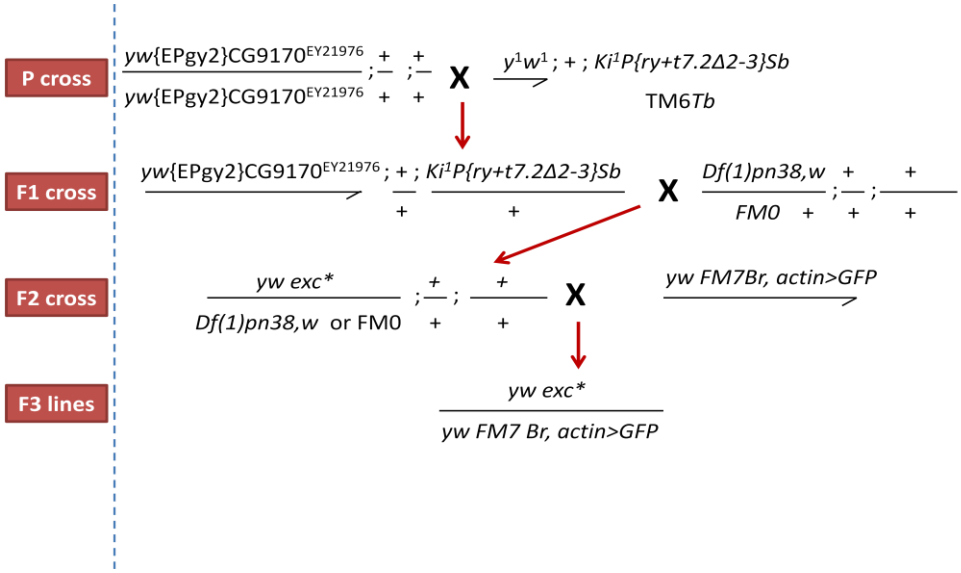

Figure 18 – Cross strategy followed to excise out the P element lines (EPgy2) and screen for the mutants of CEP164.

Chapter 3 - Results

3.1. CEP164 localization in tissues

Similar to all multicellular organisms, many proteins express to similar levels in all cells of fruit fly, while the expression patterns of diverse proteins are different in different tissues. For example, Actin5c expresses at similar level in most of the cells in *D. melanogaster*. Bld10, a centriolar protein required for centriole elongation, is more expressed in testes, which contains majorly sperms, than in the head, which contains majorly neurons. On the other hand, Cha, Choline acetyle transferase a synapse localizing protein, is more expressed in head than in testes. Interestingly, CEP164, a centriolar distal appendage localizing protein, is expressed in heighter amount in the head, but very low amount in testes (Table 1).

In *D. melanogaster*, cilia are present only in Type-I sensory neurons and in the sperm, present in the head and in testes, respectively. Therefore, we may correlate the tissue level expression with possible expressions in Type-I sensory neurons and sperms, which can be studied.

Table 1- Pattern of expression of CEP164 and other proteins in *Drosophila melanogaster* tissues.
Comparative pattern of expression of different proteins in D. melanogaster tissues. Cha show the same pattern of expression as CEP164. They are more expressed in neurons than in testes, while Bld10 has the opposite pattern of expression. Actin5C is similarly expressed in the fruit fly body (Information extracted from Flybase).

Gene Name/ No.	Brain	Head	Ovary	Testes
CG9170 (Cep164, BBS4)	35,6	**5**	0,20	**0,8**
CG12345 (Cha)	24	7	0,10	0,10
CG17081 (Bld10)	0,5	0,4	2	4,9
CG4027 (Actin5C)	0,90	0,7	0,9	0,6

To test whether the known higher level of mRNA expression of Cep164 in head is correlated to expression and localization of CEP164, we did immunostaining of CEP164 in the adult antenna and in testes in *D. melanogaster*. To understand the localization of CEP164 we co-immunostained with markers for cilia and for centrioles or pericentriolar material.

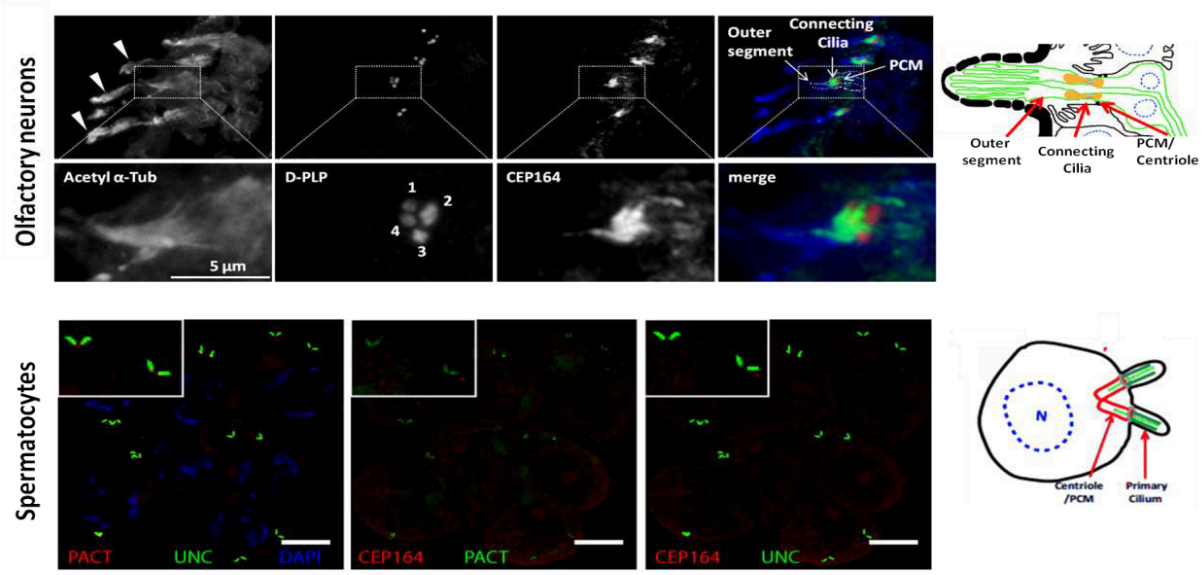

Figure 19 – CEP164 localization in sensory neurons (olfactory) and spermatocytes.
(A) Immunostaining in olfactory neurons of WT *Drosophila melanogaster*. Cilia (blue or arrowheads) was stained with acetyl alfa-tubulin, the pericentriolar material (PCM)(red) was stained with D-PLP and CEP164 (green) was stained with anti-CEP164. (B) Immunostaining in spermatocytes with CEP164 using UNC:GFP, RFP:PACT flies. Centrioles were marked with PACT, cilia were marked with UNC, nuclei were stained with DAPI and CEP164 (red) stained with anti-CEP164. (Images acquired by Swadhin Chandra Jana and model made by Swadhin Chandra Jana)

Interestingly, CEP164 localizes in olfactory neurons, which are external sensory neurons, and not in spermatocytes (Fig. 19), the cells that give rise to sperm. In olfactory neurons, CEP164 localizes at the beginning of cilia, distally to pericentriolar material. The beginning part of olfactory cilia is known as the connecting cilia, which display features of the ciliary transition zone. But, CEP164 does not localize in any part of the spermatocytes, which is in agreement with the low mRNA expression in testes.

This differential localization intrigued us to investigate the role of CEP164 in both sensory and sperm cilia assembly and maintenance. To investigate the phenotypes of loss of function of CEP164, two strategies were implemented. Firstly, the aim was to reduce the CEP164 expression by inducing expression of CEP164 RNAi in diverse cell types at specific times of development using different Gal4 drivers. The second aim was to generate CEP164 mutant lines.

3.2.RNAi strategy

3.2.1.Walking Behavior of flies

To test the effect of the CEP164 depletion in chordotonal cilia, responsible for proper coordination in flies, a bang assay was performed.

The flies have an anti-geotropic walking behavior, meaning that in this experiment they climb the tube after banging it. If chordotonal cilia are affected by CEP164 depletion it is expected to see defects in flies' capacity to climb the tube.

The results shown in the next figures represent the measurement in average of the percentage number of flies that climb above 9cm in the tube (50% of the tube) during the last second of recording (Figures A). The tracks of the flies during 5 seconds after banging the tube (Figures B and C) are also represented. The first figures (Figures A) demonstrate if the flies' climb capacity was affected through the depletion of CEP164 and the last figures (Figures B and C) reflect the flies' response to the stimuli submitted.

CEP164 knock down in sensory precursor and sensory neurons using neur-Gal4 cause uncoordination in the adult fly

Neur promoter induces the expression of Gal4 in neuronal precursor cells and sensory neurons, so at the neuron assembly stage. neur-Gal4/UAS-SAS6 RNAi flies do not eclode, while grown in both 25°C and 29°C , suggesting that SAS6 knock down severely affects the centriole, in turn affects cilia assembly. At 25ºC, the number of neur-Gal4/UAS-CEP164 (1) RNAi flies that climbed more than half of the tube height was approximately similar to the number of the negative control flies, suggesting they are coordinated. However, neur-Gal4/UAS-CEP164 (2) RNAi number of flies (40%) crossing half of the tube was very significantly reduced ($p<0,0001$) compared to negative control (82%) (Fig. 20-A). At 29ºC both neur-Gal4/UAS-CEP164 (1) RNAi and neur-Gal4/UAS-CEP164 (2) RNAi flies showed significant walking behavior defects comparing with the negative control line (Fig. 20-A). This suggests that CEP164 knock down using neur-Gal4 causes defects in the anti-geotropic walking behavior at the assembly stage of neurons.

Analysis of the initial parts of the anti-geotropic walk after the bang, suggests that the CEP164 knockdown flies are slow and sluggish compared to control (Fig. 20-B and C). At 25ºC,

neur-Gal4/UAS-CEP164 RNAi flies have a slower response compared to the negative control to the banging stimuli. This behavior was also clearly visible in flies grown at 29ºC (Fig. 20-B and C). Many dots or discontinuous lines in the projection of flies´ climbing grown at 29ºC were observed, which indicates the uncoordinated behavior of the flies. These results suggest that the flies partially lost the walking coordination and that they jump more frequently than the control (Fig. 20-Ci and Cii).

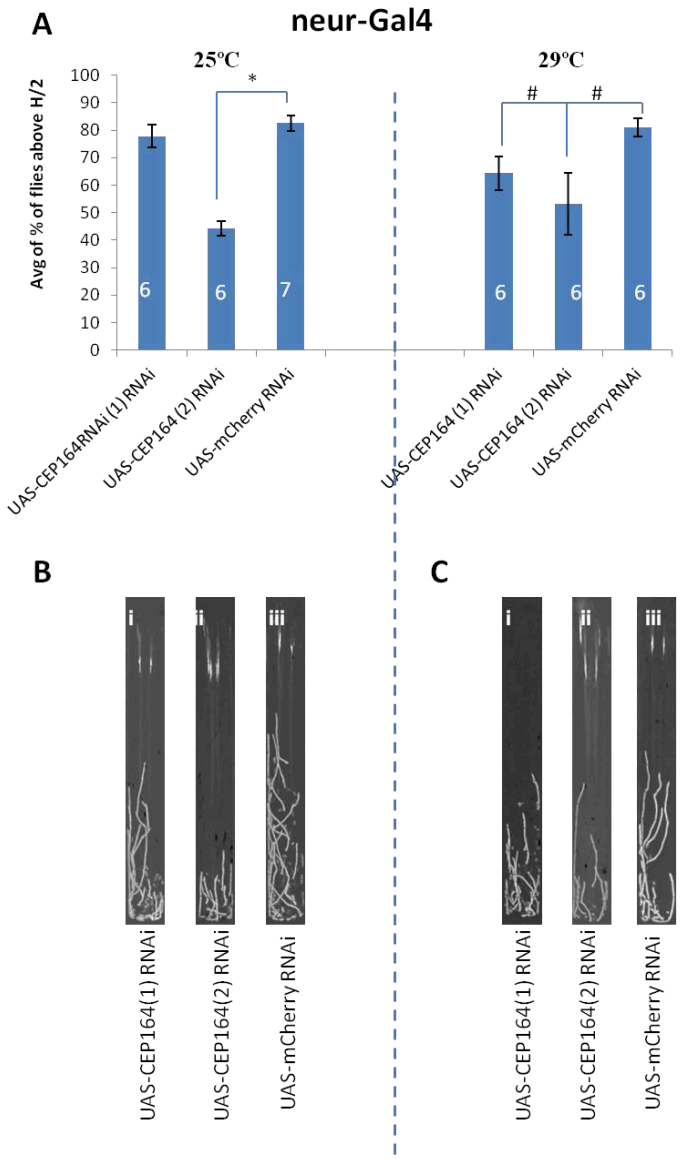

Figure 20 - CEP164 knock down in sensory neurons, at earlier stages, causes uncoordination in walking behavior in adult flies.
(A) Average of the percentage of the flies that climb above half of the tube of 18 cm height (H/2=9cm). Flies were grown at 25 and 29ºC. N≥6. * indicates p<0,0001 and # indicates p<0,03. (B-C) Representative images to display the tracks travelled by the flies within first 5 seconds, after the bang. At both 25ºC and 29ºC, neur-Gal4/UAS-CEP164 RNAi flies (B-i,ii and C-i,ii) have a slower response to the bang than the control flies (B-iii and C-iii).

Depletion of CEP164 at later stages in neurogenesis using Cha19b driver does not affect coordination

Cha19b promoter induces the expression of Gal4 when cilia start to assemble after dendrites of the neurons are completely formed. Comparing in the neuron developmental time, cha19b is expressed later than neur driver and it is related to the maintenance stage.

No significant defects were seen upon CEP164 knockdown using *Cha19bGal4*, at both 25ºC and 29ºC. Cha19b-Gal4/UAS-SAS6 RNAi flies do not show any walking behavior problems also (Fig.21-A), suggesting that *Cha19b*Gal4 expression starts after the centrioles are assembled.

These results are in accordance with flies' response in the first 5 seconds after the tube bang, meaning that flies have no climbing defects since the beginning of the assay compared to the control (Fig.21-B and C).

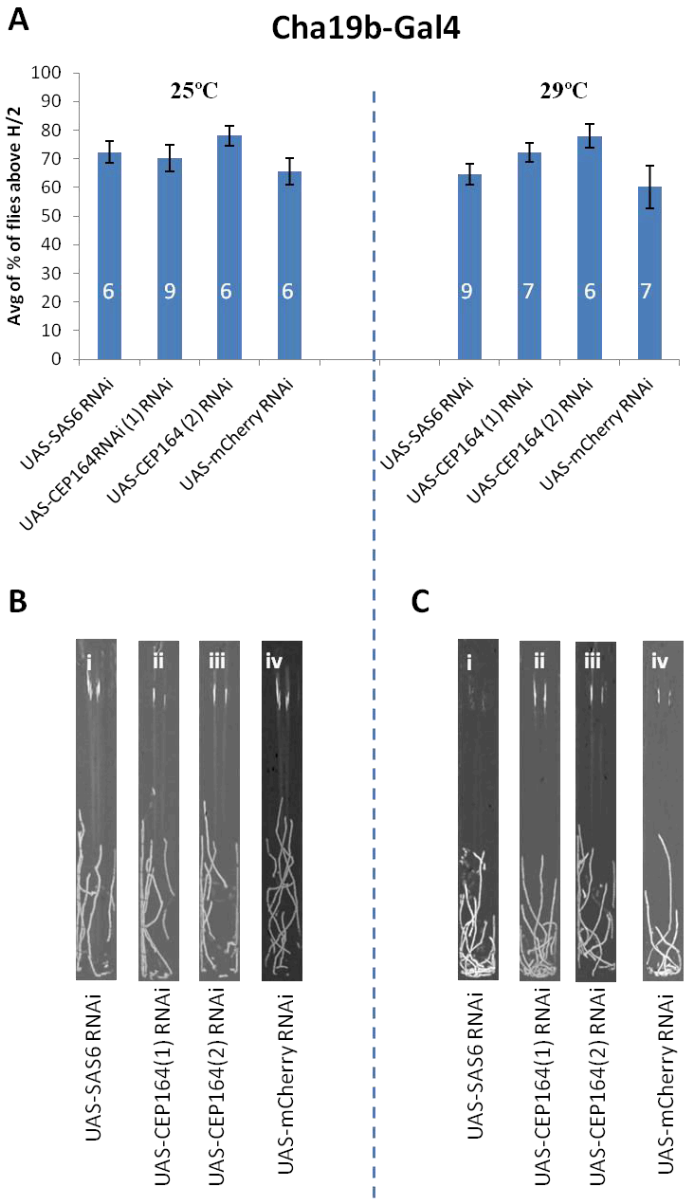

Figure 21 - CEP164 knock down in sensory neurons, after cilia assembly begins, does not cause uncoordination in walking behavior in adult flies.
(A) Average of the percentage of the flies that climb above half of the tube of 18 cm height (H/2=9cm). Flies were grown at 25 and 29ºC. N≥6. (B-C) Representative images displaying the tracks travelled by the flies in first 5 seconds, after the bang.

The results obtained with the knock down strategy in neurons using two different drivers, neur and Cha19b showed that CEP164 depletion causes behavioral defects when the protein is knocked down at earlier stages while at the maintenance stage it did not show any effect in flies' behavior. This raised the question when CEP164 should be starting to be expressed during chordotonal neuron development.

CEP164 localization in neurons dynamically changes

For that, embryos from different time points after egg laying were collected and stained with anti-22c10, to mark neurons and dendrites, and anti-CEP164 to mark CEP164. Centrioles were marked with PACT.

During chordotonal neuron maturation, in embryo, it is visible that CEP164 localization changes during the development and maturation of the neurons. CEP164 starts to be observed in the chordotonal neurons after 12hr of egg laying, and although it localizes distally to the centrioles, its localization changes during neuron maturation and cilia elongation. Although, CEP164 localization in the centriole is maintained, the localization along the cilia length changes. During cilia elongation, CEP164 moves from the centriole towards the distal tip of cilia (Fig.22).

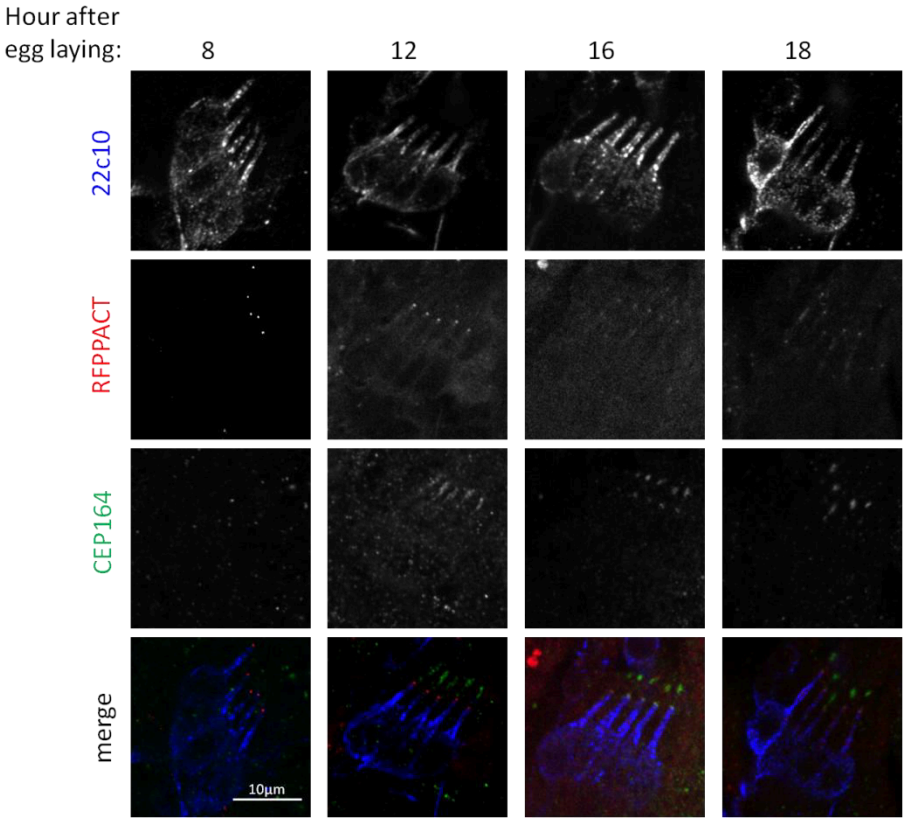

Figure 22 - CEP164 localization dynamically changes during neuron development.
Immunostaining from embryos collected 8, 12, 16 and 18hr after egg laying. Dendrite and neurons cell body stained with anti-22c10; centrioles are marked with PACT and CEP164 was stained with anti-CEP164.

CEP164 knock down in olfactory neurons and spermatocytes does not affect the coordination of the adult flies

Sg18.1 is only expressed in olfactory neurons and in early spermatocytes. It does not express in chordotonal neurons, responsible for flies' coordination, therefore, this driver was also used as a walking behavior assay control. These flies can be control flies to test whether RNAi knock down at different temperatures in flies cause any effect in their walking behavior.

Comparing the average number of all genotype flies above H/2 at both temperatures it can be observed they are similar to the negative control Sg18.1-Gal4/UAS-mCherry RNAi flies (Fig.23-A) suggesting that CEP164 knock down using Sg18.1 driver does not affect flies walking behavior. This is in agreement with our expectations based on the expression pattern.

The same results were obtained in the assays done with flies depleted for CEP164 in testes; those flies did not show defects in coordination (Fig. 32, 33 and 34).

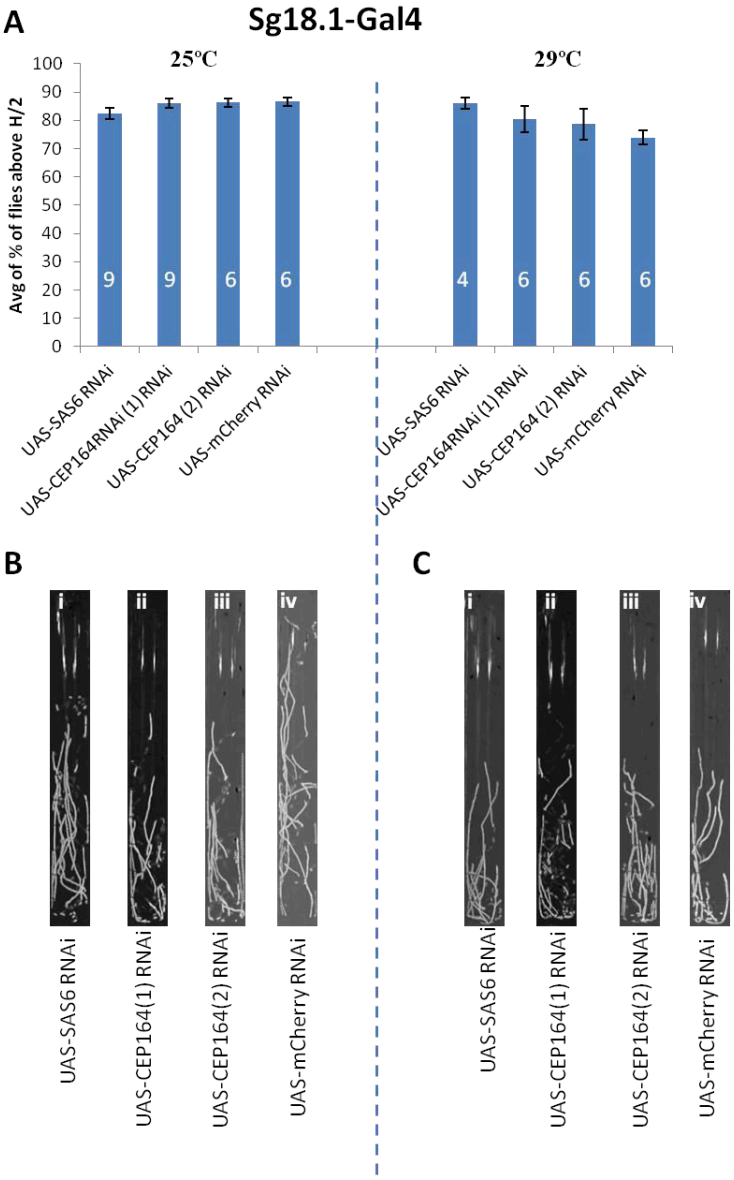

Figure 23 - CEP164 depletion in olfactory neurons and testes does not affect flies walking behavior.
(A) Average of the percentage of the flies that climb above half of the tube of 18 cm height (H/2=9cm). Flies were grown at 25 and 29ºC. N≥6. (B-C) Representative images displaying the tracks travelled by the flies in first 5 seconds, after the bang. Flies grown at 29ºC (C) have a slower response to the stimuli than the flies grown at 25ºC (B) but the average number of flies that climb more than half of the tube is similar at both temperatures.

CEP164 knock down using actin-Gal4 cause mild uncoordination in the adult flies.

Actin, as well as tubulin, induces the expression of Gal4 in all cell types. Actin-Gal4/UAS-SAS6 RNAi and tubulin-Gal4/UAS-SAS6 RNAi flies do not eclode, suggesting that knocking down SAS6 in most of the cells leads to lethality in larval stages.

At 25ºC, actin-Gal4/UAS-CEP164 (1) RNAi flies show more defects in walking behavior comparing to actin-Gal4/UAS-CEP164 (2) RNAi flies, however at 29ºC both knock down flies depleted for CEP164 show phenotype. The actin-Gal4/UAS-CEP164 (2) RNAi flies show very significant defective walking behavior compared to actin-Gal4/UAS-mCherry RNAi (Fig.24-A).

Concerning the flies' behavior in the first 5 seconds after banging the tube, it reflects that the walking behavior problems exist since the beginning of the recording (Fig.24-B).

It was observed that the frequency of the flies ecloded of actin-Gal4/ UAS-CEP164 (2) RNAi genotype was lower. Most of the actin-Gal4/ UAS-CEP164 (2) RNAi larvae fail to pupate and die.

The average number of tubulin-Gal4/UAS-CEP164 RNAi flies above H/2 are significantly reduced depleting CEP164 with the KK line at 29ºC, with only 50% of the flies crossing half of the tube height (Fig.35). Similarly to actin, it was also seen that most of tubulin-Gal4/UAS-CEP164 (2) RNAi flies do not eclode. Therefore, the effect of knock down of CEP164 is even bigger than what is observed in walking behavior graph.

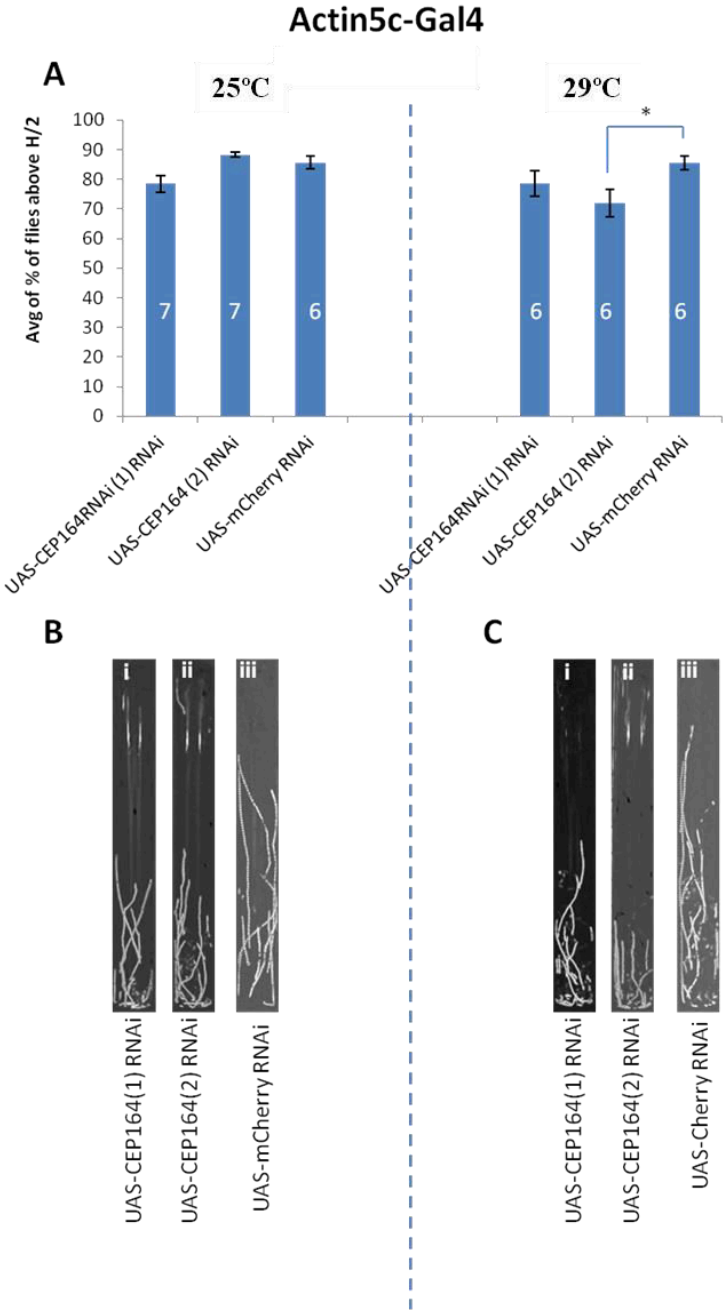

Figure 24 - CEP164 depletion using actin-Gal4 driver at 29°C causes mild defects in adult flies coordination at 29°C.

(A) Average of the percentage of the flies that climb above half of the tube of 18 cm height (H/2=9cm). Flies were grown at 25 and 29°C. N≥6. actin-Gal4/UAS-SAS6 RNAi flies do not eclode. * indicates p< 0,0012. (B-C) Representative images displaying the tracks travelled by the flies in first 5 seconds, after the bang. At 25°C, actin-Gal4/UAS-CEP164 (1) RNAi flies show mild defects in coordination while at 29°C that effect is stronger.

3.2.2.Cell morphology

To investigate the effect of CEP164 depletion in sensory neurons, we studied the effect of Cep164 knockdown in olfactory neurons' morphology.

In olfactory neurons, two neuron cells give rise to only one dendrite and, consequently, to one cilium. At the distal part of each neuron cell and at the beginning of cilia, in the connecting cilia, two centrioles are present in each cell (Fig. 25-B and 26-B). D-PLP is a marker for *D. melanogaster* pericentriolar material/ centrioles, so at the base of each cilium in olfactory neurons it is expected to observe two or four dots of D-PLP, depending on the two neuron cells orientation.

In olfactory neurons present in the antenna in WT adult flies CEP164 is localized distally to the basal body and at the connecting cilia region (Fig.19-A).

The staining was made in olfactory neurns expressing neur-Gal4/CEP164 RNAis and actin-Gal4/CEP164 RNAis; in CEP164 maximum intensity graph the measurements are presented for the two genotypes comparing to the wild type control. However, in the immunostaining images of the genotype which showed a walking behavior phenotype are presented.

Cilia of olfactory neurons are affected in neur-Gal4/UAS-CEP164 (2) RNAi flies

Immunostaining images reveal that neur-Gal4/UAS-CEP164 (2) RNAi flies have cilia defects, cilia in the CEP164 knocked down flies are slender compared to wild type (Fig. 25-A and Fig. 25-C). Although, cilia seem to be affected, we observe that cilia are present.

D-PLP localization is not affected by CEP164 depletion and CEP164 localization does not change. Similarly to wild type control CEP164 is localized distally to the D-PLP staining, so distally to PCM or centrioles and at the base of cilia in the connecting cilia (Fig.25-C).

Regarding the CEP164 maximum intensity in the staining, it is significantly lower in both neur-Gal4/UAS-CEP164 RNAi lines than in the control (Fig. 25-D), suggesting that the protein was knocked down, but the knock down was not complete.

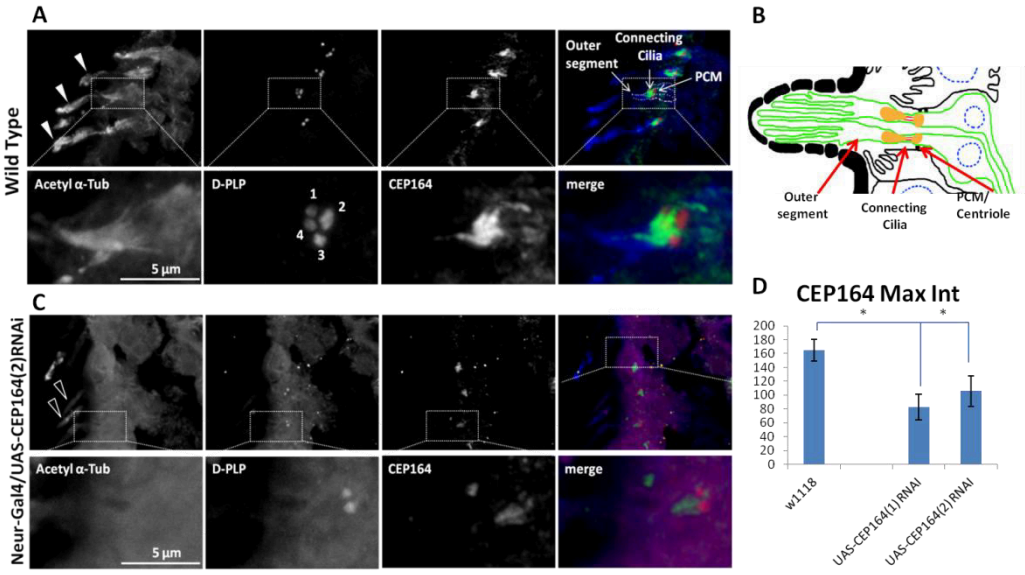

Figure 25 - Cilia of olfactory neurons is affected in neur-Gal4/CEP164 (2) RNAi flies.
(A and C) Immunostaining images acquired from wild type (negative control) and neur-Gal4/UAS-CEP164 (2) RNAi flies both grown at 29ºC. Cilia (blue or arrowheads) was stained with acetyl alfa-tubulin, the pericentriolar material (PCM)(red) was stained with D-PLP and CEP164 (green) was stained with anti-CEP164. (A) CEP164 localization in olfactory neurons in *Drosophila* wild type. (B) Model of olfactory cilia. Two neurons cells, give rise to one cilium. (made by Swadhin Chandra Jana). (D) Maximum intensity of CEP164 staining in w1118 (negative control) and in the neur-Gal4/ UAS-CEP164 RNAi flies. N≥11. * indicates p<0,0001. (C and D) Defects on the cilia assembly and the CEP164 maximum intensity.

Depletion of CEP164 in all cells using actin driver affects cilia assembly

Immunostaining images from actin-Gal4/UAS-CEP164 (2) RNAi flies show that cilia assembly is severely reduced. As well as neur-Gal4/UAS-CEP164 (2) RNAi flies, in actin-Gal4/UAS-CEP164 (2) RNAi flies CEP164 localization do not change; it is present in the connecting cilia (Fig.26-C). D-PLP is part of the PCM and its localization suggest that the BB structure and pericentriolar protein localization is not visibly affected in actin-Gal4/CEP164(2)RNAi flies (Fig.26-C).

Looking to CEP164 maximum intensity measurements, only actin-Gal4/UAS-CEP164 (2) RNAi flies show significant reduction of protein intensity (Fig.26-D). This shows that CEP164 depletion leads to reduction in the intensity levels, but not to complete knock down.

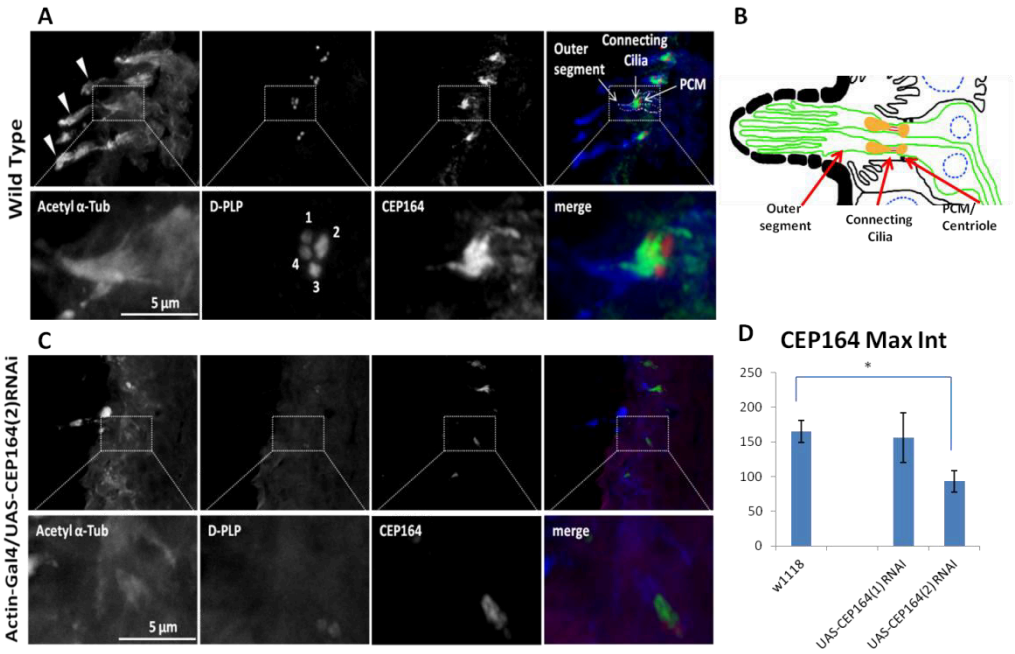

Figure 26 - Cilia assembly of olfactory neurons in actin-Gal4/CEP164 (2) RNAi flies is severely affected.
(A and C) Immunostaining images acquired from wild type (negative control) and actin-Gal4/UAS-CEP164 (2) RNAi flies both grown at 29ºC. Cilia (blue or arrowheads) was stained with acetyl alfa-tubulin, the pericentriolar material (PCM)(red) was stained with D-PLP and CEP164 (green) was stained with anti-CEP164. (A) CEP164 localization in olfactory neurons in *Drosophila* wild type. (B) Model of olfactory cilia (made by Swadhin Chandra Jana). (D) Maximum intensity of CEP164 staining in w1118 (negative control) and in the actin-Gal4/ UAS-CEP164 RNAi flies. N≥11. * indicates p<0,0001. (C and D) Severe defects in cilia assembly and CEP164 maximum intensity is reduced.

Centriole length is not affected and the intensity of pericentriolar material is affected in ActinGal4/CEP164 RNAi flies

Looking to D-PLP length and maximum intensity measurements, we observe that in neur-Gal4/UAS-CEP164 RNAi flies D-PLP length and intensity are not affected (Fig. 27). In actin-Gal4/CEP164 (2) RNAi flies, though the BB length is not affected, staining with D-PLP shows that depletion of CEP164 significantly reduced the intensity of D-PLP (Fig. 27).

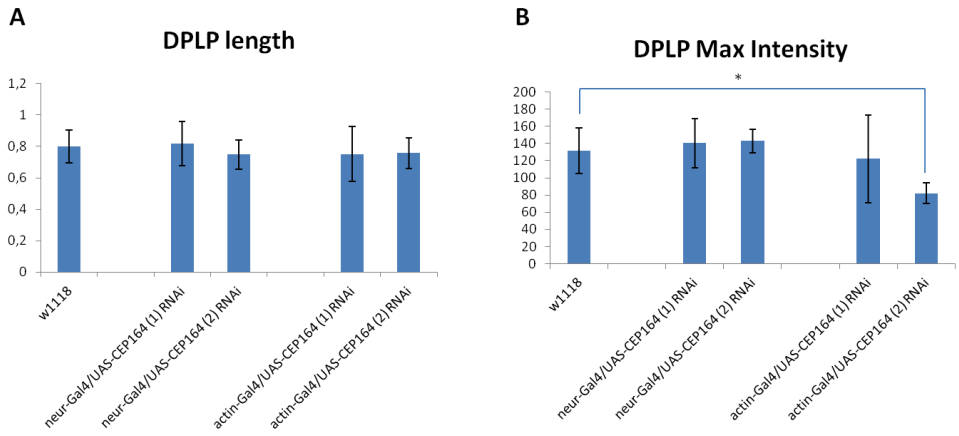

Figure 27 - D-PLP length is not affected in olfactory neurons.
(A) DPLP length measurement corresponding to pericentriolar material length in wild type (negative control) and in the Gal4/UAS-CEP164 RNAi flies grown at 29ºC. (B) Maximum intensity of DPLP staining in w1118 and in the CEP164 knocked down flies. Measurements were done using the ImageJ program. N≥12. * indicates p<0,0001. Centrioles length in knocked down flies is not affected but the intensity of pericentriolar material is affected in actin-Gal4/UAS-CEP164 RNAi flies.

Depletion of CEP164, at early stages, induces basal body problems in olfactory neurons

As neur-Gal4/UAS-CEP164 (2) showed cilia defects in immunostaining images, it was investigated, using electron microscopy (EM), whether it had problems in the ultra-structure.

In all images acquired the proximal basal body is missing and the distal basal body has severe defects which could be observed through longitudinal and cross sections. In cross sections it is observed the loss of 9-fold symmetry of MT doublets (Fig. 28). This suggests that CEP164 might be needed to maintain BB at the base of cilia.

Though, distal basal bodies show structural defects when CEP164 is depleted at earlier stages, EM images show that cilia still are present in olfactory neurons. This is consistent with the immunostaining images. In the images acquired, we do not see defects in axoneme's structure; however, more images should be obtained.

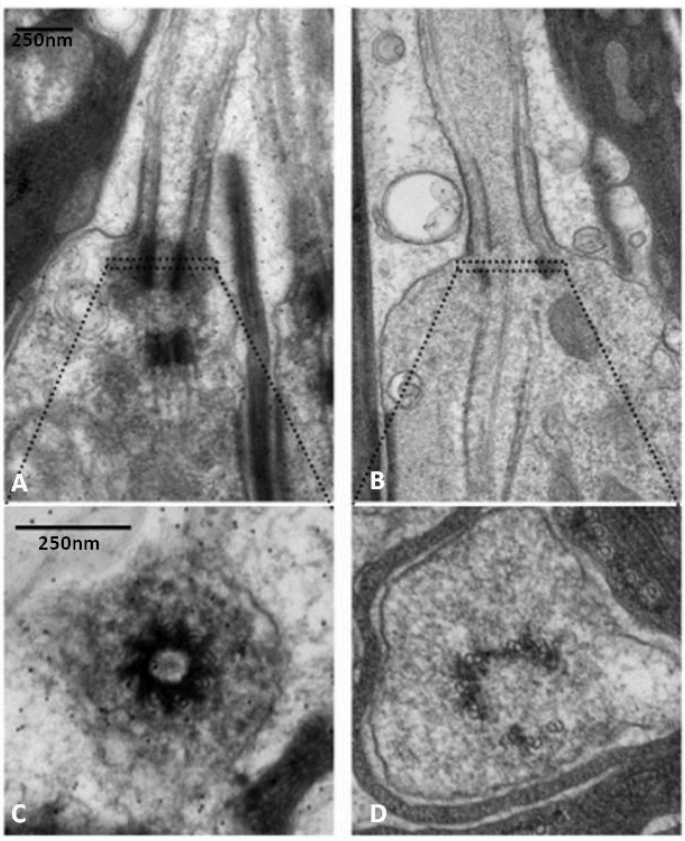

Figure 28 - Proximal BB is missing and distal BB in neur-Gal4/UAS-CEP164 (2) RNAi flies is defective.
Electron microscopy images of adult flies' olfactory neurons. (A) Longitudinal section of WT olfactory neuron. (B) Longitudinal section of neur-Gal4/UAS-CEP164 (2) RNAi olfactory neuron. Proximal BB missing and distal BB structure affected. (C) Cross section of WT distal BB. (D) Cross section of neur-Gal4/UAS-CEP164 (2) RNAi distal BB. 9-fold symmetry affected. (Samples prepared and cut by Pedro Machado. Images acquired by Pedro Machado)

3.2.3.Fertility of flies

Even though, the pattern of CEP164 expression reveals that this protein is expressed at lower levels expressed in testes, it was important to know whether a small amount of undetectable protein could cause any effect in fertility.

At 29ºC, using the Sg18.1 and hsp83 drivers, the average number of progenies per male is reduced compared to 25ºC; this is due to the effect from the temperature induction in the GAL4/UAS system (Fig. 29).

At 25ºC, fertility is not affected using both Sg18.1 and hsp83 drivers to deplete CEP164 in testes.

At 29ºC, using Sg18.1 driver to deplete CEP164 do not show affected fertility phenotype. Also, at 29ºC using this driver, UAS SAS6 RNAi do not show a fertility phenotype, we observe that the average number of progenies is variable compared do UAS- mCherry RNAi. However, using hsp83 driver, UAS SAS6 RNAi show reduced average number of progenies when compared to the negative control. Using this driver at this temperature, CEP164 depletion does not have effect on fertility results. This is consistent with the results obtained from the localization perspective.

Knocked down flies with mCherry (negative control) and WT present the same average number of progenies per male, which proves the effectiveness of this assay.

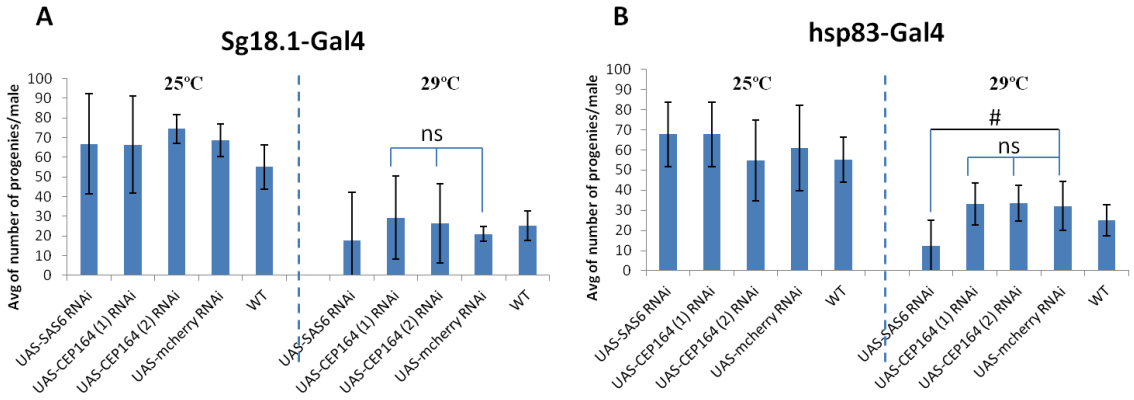

Figure 29 – Fertility is not affected by CEP164 knock down using Sg18.1 and hsp83 drivers.
(A and B) Average number of progenies per male at 25 and 29ºC. Males used to test fertility were SAS6 depleted (+ control), CEP164 depleted and mcherry depleted (- control). WT is also used a negative control. N≥5 (A) Fertility results using Sg18.1 driver. CEP164 depletion does not affect fertility in Sg18.1-Gal4/ UAS-CEP164 RNAi flies. (B) Fertility results using hsp83 driver. CEP164 depletion does not show effect in fertility in hsp83-Gal4/ UAS-CEP164 RNAi flies. # indicates p<0,01.

3.3.Generation of a CEP164 mutant

P element excision strategy was used to generate null or loss of function mutants. The aim with this strategy includes the excision of P element that was inserted close to CEP164 locus, in 5' UTR of the *CEP164 gene.* When the P element excise out it can be either a precise excision, in which only the P element is excised, or an imprecise excision in which when the P element excise out it also removes some part of the gene where it is inserted, in this case *CEP164* gene.

The aim consisted in obtaining excision lines and in the future map the excision and characterize the mutant lines obtained.

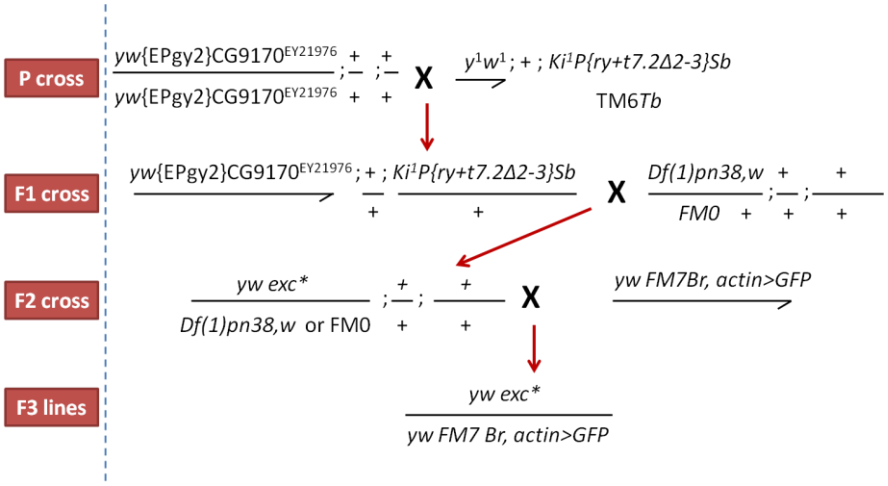

Figure 30 – Strategy followed to obtain a strong CEP164 mutant.
In the P cross virgin females with the P element insertion were crossed with males with the transposase. Male progenies were selected from that cross and crossed with wFM0 virgins. Then a F2 cross was sat up crossing females with the excision with ywFM7Br males with actin tagged with GFP. F3 lines with the excision were obtained.

The generation of a CEP164 mutant was started by crossing virgin females with transposase males (Fig. 30). These virgins with the P element insertion have red eyes and brown body in a background of white eyes and yellow body. The transposase males have a marker in the third chromosome that is *Sb*, stubble, and these flies have also red eyes and brown body.

From this cross, jump starter males with mosaic eye, meaning that they have a cell mixture of different excisions, and *Sb* were selected. Males were crossed with FM0 virgins with white eyes and bean eyes. It was observed that FM0Br homozygous females are sterile; these flies have *Br*, barr, eyes because they have two copies of the marker Br. When the flies are

heterozygous (wDef/wFM0Br) the eyes are bean and these were the virgin females used to set the F1 cross.

Then virgin females from the last cross were selected, with white eyes and non *Sb*, which means that the transposase is not in the flies' chromosome. Flies either with normal eyes (yw exc*/ wDef) or with bean eyes (yw exc*/ wFM0Br) were selected and crossed with a male FM7Br actin<GFP>. These males had red and barr eyes. The lines were obtained and marked with actin-GFP. Therefore, those individual excised files were converted to excision lines. Until now we have selected 52 lines where the excision was obtained.

Chapter 4 - Discussion

CEP164 localizes distally to the basal bodies in sensory neurons

In U2OS (human osteosarcoma) cell lines, and in RPE (retinal pigment epithelium cells), CEP164 localize specifically in the distal appendage of the mother centriole in cycling stages and at the base of the cilia [14, 36]. We found that in adult olfactory receptor neurons in *D. melanogaster*, CEP164 localizes at the distal part of the basal bodies. In fact, it localizes to the connecting cilia (also considered as TZ) of these neurons. These observations very well correlate with the known high mRNA expression profile of *Cep164* gene in head, which majorly contains sensory neurons in flies. Moreover, this localization at the distal part of the centrioles or at the base of the cilia across species suggests that the localization of CEP164 is conserved across different organisms during evolution.

CEP164 does not localize in spermatocytes and does not affect fertility

Interestingly, CEP164 does not localize to the centrioles or cilia in spermatocytes. Furthermore, the fertility results show that CEP164 RNAi knockdown using two different drivers, such as Sg18.1-Gal4 and hsp83-Gal4 has no effect in fertility. These results are in agreement with the known very low mRNA expression profile of CEP164 in testes. These results, suggest that unlike its role in sensory neurons, CEP164 does not play any role in cilia assembly or maintenance in testes. It also suggests that i) the expression of ciliary proteins may vary across different cell types in an organism, ii) the molecules involved in centriole and cilia biogenesis might be different in different ciliated cell types in the fly. Therefore, the mechanisms of cilia assembly during spermatogenesis are different from that in sensory neurons. Indeed, while the cilia/flagella assembly in *D. melanogaster* testes is not IFT dependent, the assembly of sensory neuron cilia is IFT-dependent [8, 24]. These results raise the hypothesis that CEP164 could be an IFT related protein, playing a role in cilia assembly in neurons but not in testes.

CEP164 is required at the assembly of cilia but not their maintenance in neurons

Knockdown of CEP164 in sensory neurons cause uncoordination in walking behavior of flies, suggesting CEP164 role in proper coordination of the adult flies. In flies, chordotonal neurons are responsible for the proper walking behavior. Depletion of CEP164 during the maturation of the neuron and assembly of cilia, using neur driver, causes uncoordination in adult

flies. However, when we depleted CEP164 in these neurons after the centriole conversion to basal body, using Cha19b driver, the flies' walking behavior is not affected. In general, walking behavior assays are used to access the sensory neurons functions, especially the chordotonal neurons containing motile sensory cilia. Therefore, these results suggest that CEP164 is involved in proper assembly of the sensory neurons, but not their maintenance. To further understand the reasons underlying the malfunction of sensory neurons, we studied the morphology of ciliated Type-I sensory neurons, such as olfactory receptor neurons. Interestingly, we observed that CEP164 knock down leads to a severe defect in the immotile olfactory cilia morphology and ultra-structure defects. These findings show that CEP164 plays a role in cilia assembly. EM images show that CEP164 depletion leads to loss of proximal basal body and partial loss of 9-fold symmetry in distal basal body in olfactory neurons. These results are in agreement with studies in ciliated mammalian cell lines, such as RPE and IMCD3 cells [14]. However, these novel observations raise several possible new roles of CEP164 in ciliated sensory neurons, which need to be investigated in the future. CEP164 might be either involved in promoting basal body docking to the membrane in order to assemble the cilia or involved in basal body maintenance after its differentiation.

All together, these results indicate that CEP164 plays a key role in both motile and immotile cilia assembly, present in chordotonal and olfactory neurons, respectively.

CEP164 localization dynamically changes

Intrigued by the cilia and basal body morphology defects observed in the sensory neurons upon Cep164 knock down during neurogenesis, we studied the localization of CEP164 protein at different stages of neuron development. CEP164 depletion affects the cilia assembly, but does not affect cilia maintenance. This raised the question on when is CEP164 being recruited to the neuron. CEP164 localization was first observed in the chordotonal neurons after 12hr of egg laying, when ciliogenesis begins in larval chordotonal neurons. Although CEP164 localized distally to the centrioles at early hours of neurogenesis, the localization pattern changes during neuron maturation, ie, cilia elongation. Although, CEP164 localization at the centriole was maintained, the localization along the length of the cilia changed during neuron maturation. Initially CEP164 localizes long the entire length of the cilia, and then it was only observed at the distal tip of the cilia. This suggests that CEP164 may move from the TZ to the distal tip of the cilia, during the cilia elongation and maturation in larval chordotonal neurons.

Chapter 5 - Conclusion

Even though CEP164 was discovered as a centriolar distal appendage protein in last decade [14], only in very recent past the mechanistic descriptions of CEP164 roles have been investigated [39]. It is known that CEP164 localizes at the mature centriole appendages contributing to cilia assembly, its absence causing disease in humans [40]. This also raised the interest to understand better the mechanism of action and interactors of CEP164 indispensable for formation of cilia.

This work provides novel clues to the role of CEP164 in ciliogenesis during neuron assembly. CEP164 revealed to be important mainly at the assembly stages of cilia but not to their maintenance in neurons. Its knock down led to a reduction in cilia number. Most interestingly, there was a clear problem in centriole structure, a phenotype that had never been described before. It will be important to follow centriole markers during development after depletion using RNAi or in the mutant to understand how that phenotype arises. It is possible that CEP164 has a role in centriole stability.

Using a model organism to do scientific research is very important, because it can enable scientists to study a correlation between behavior of an organism and its cellular morphology.

In future, it would be interesting to study the morphology of motile cilia in the chordotonal neurons of CEP164 knockdown flies. This would help us complement walking behavior results and lead us to know CEP164 role in these neurons. It may also help us to understand whether CEP164 knockdown has a different effect on centriole/ BB in chordotonal neurons from that of olfactory neurons. Furthermore, it will be important to do a smelling assay [58], to study the flies' response to a stimuli regarding odor sensing to complement the morphological defects we observed in the olfactory cilia. Finally, will be very interesting to study the localization of CEP164, in *D. melanogaster*, using immuno-EM techniques to fine map where it localizes to.

Though we have isolated several *Cep164* excision lines, the mapping of the excision loci was incomplete. It will be very important to map the location of the excision in CEP164 locus and characterize them in respect to uncoordination behavior, fertility and cell morphology of neurons and testes. Null alleles will make the description of the phenotype simpler as compared with the knock downs described in this thesis. In the future, it will also be extremely interesting to study the interactors of CEP164 in the centriole and cilia biogenesis, in *D. melanogaster*.

Chapter 6 - References

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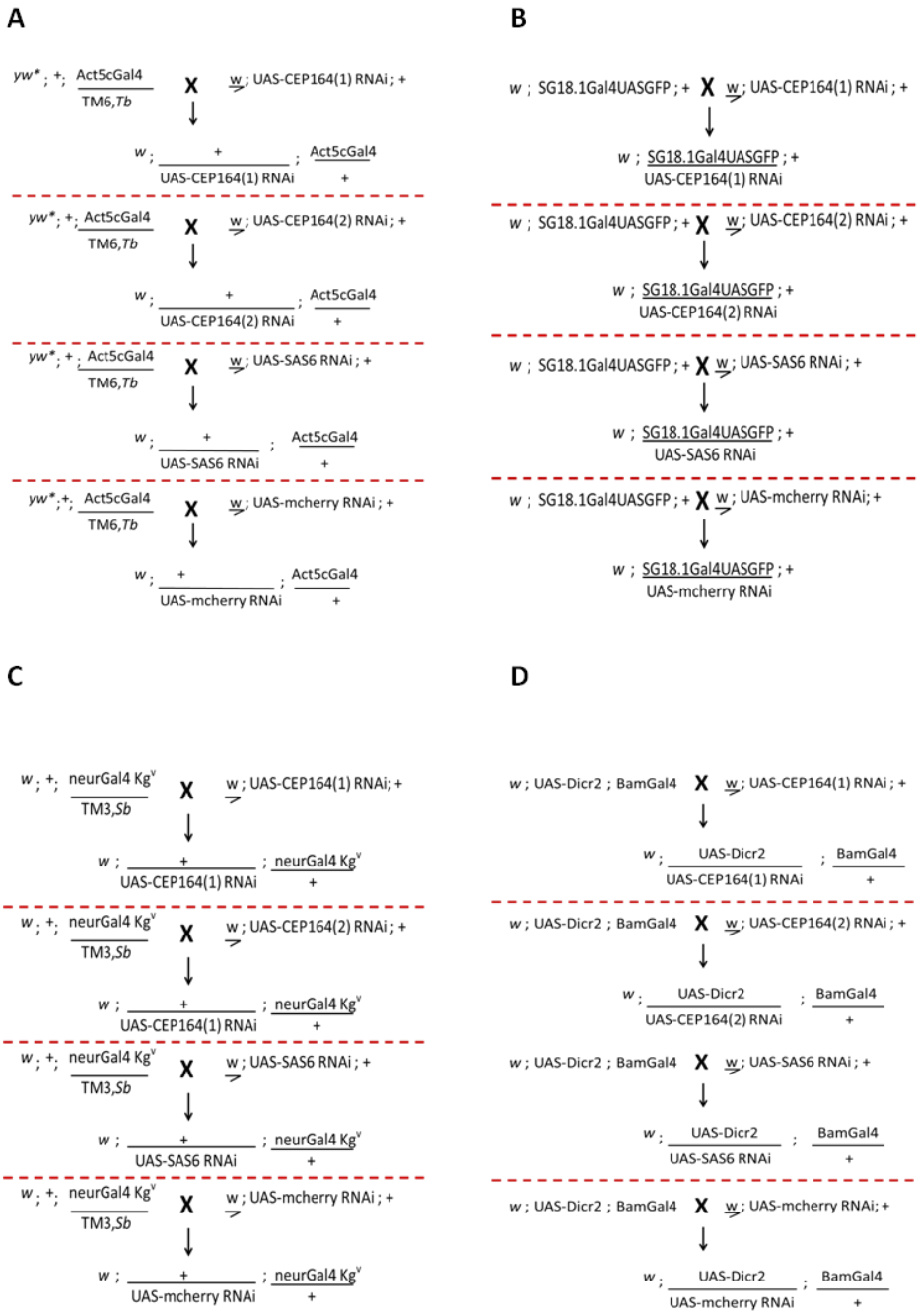
Appendix A


A
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w ; + / UAS-CEP164(1) RNAi ; Act5cGal4 / +
yw* ; + ; Act5cGal4 / TM6,Tb X w ; UAS-CEP164(2) RNAi ; +
w ; + / UAS-CEP164(2) RNAi ; Act5cGal4 / +
yw* ; + ; Act5cGal4 / TM6,Tb X w ; UAS-SAS6 RNAi ; +
w ; + / UAS-SAS6 RNAi ; Act5cGal4 / +
yw* ; + ; Act5cGal4 / TM6,Tb X w ; UAS-mcherry RNAi ; +
w ; + / UAS-mcherry RNAi ; Act5cGal4 / +
B
w ; SG18.1Gal4UASGFP ; + X w ; UAS-CEP164(1) RNAi ; +
w ; SG18.1Gal4UASGFP ; + / UAS-CEP164(1) RNAi
w ; SG18.1Gal4UASGFP ; + X w ; UAS-CEP164(2) RNAi ; +
w ; SG18.1Gal4UASGFP ; + / UAS-CEP164(2) RNAi
w ; SG18.1Gal4UASGFP ; + X w ; UAS-SAS6 RNAi ; +
w ; SG18.1Gal4UASGFP ; + / UAS-SAS6 RNAi
w ; SG18.1Gal4UASGFP ; + X w ; UAS-mcherry RNAi ; +
w ; SG18.1Gal4UASGFP ; + / UAS-mcherry RNAi
C
w ; + ; neurGal4 Kg^v / TM3,Sb X w ; UAS-CEP164(1) RNAi ; +
w ; + / UAS-CEP164(1) RNAi ; neurGal4 Kg^v / +
w ; + ; neurGal4 Kg^v / TM3,Sb X w ; UAS-CEP164(2) RNAi ; +
w ; + / UAS-CEP164(1) RNAi ; neurGal4 Kg^v / +
w ; + ; neurGal4 Kg^v / TM3,Sb X w ; UAS-SAS6 RNAi ; +
w ; + / UAS-SAS6 RNAi ; neurGal4 Kg^v / +
w ; + ; neurGal4 Kg^v / TM3,Sb X w ; UAS-mcherry RNAi ; +
w ; + / UAS-mcherry RNAi ; neurGal4 Kg^v / +
D
w ; UAS-Dicr2 ; BamGal4 X w ; UAS-CEP164(1) RNAi ; +
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w ; UAS-Dicr2 ; BamGal4 X w ; UAS-CEP164(2) RNAi ; +
w ; UAS-Dicr2 / UAS-CEP164(2) RNAi ; BamGal4 / +
w ; UAS-Dicr2 ; BamGal4 X w ; UAS-SAS6 RNAi ; +
w ; UAS-Dicr2 / UAS-SAS6 RNAi ; BamGal4 / +
w ; UAS-Dicr2 ; BamGal4 X w ; UAS-mcherry RNAi ; +
w ; UAS-Dicr2 / UAS-mcherry RNAi ; BamGal4 / +

Appendix A

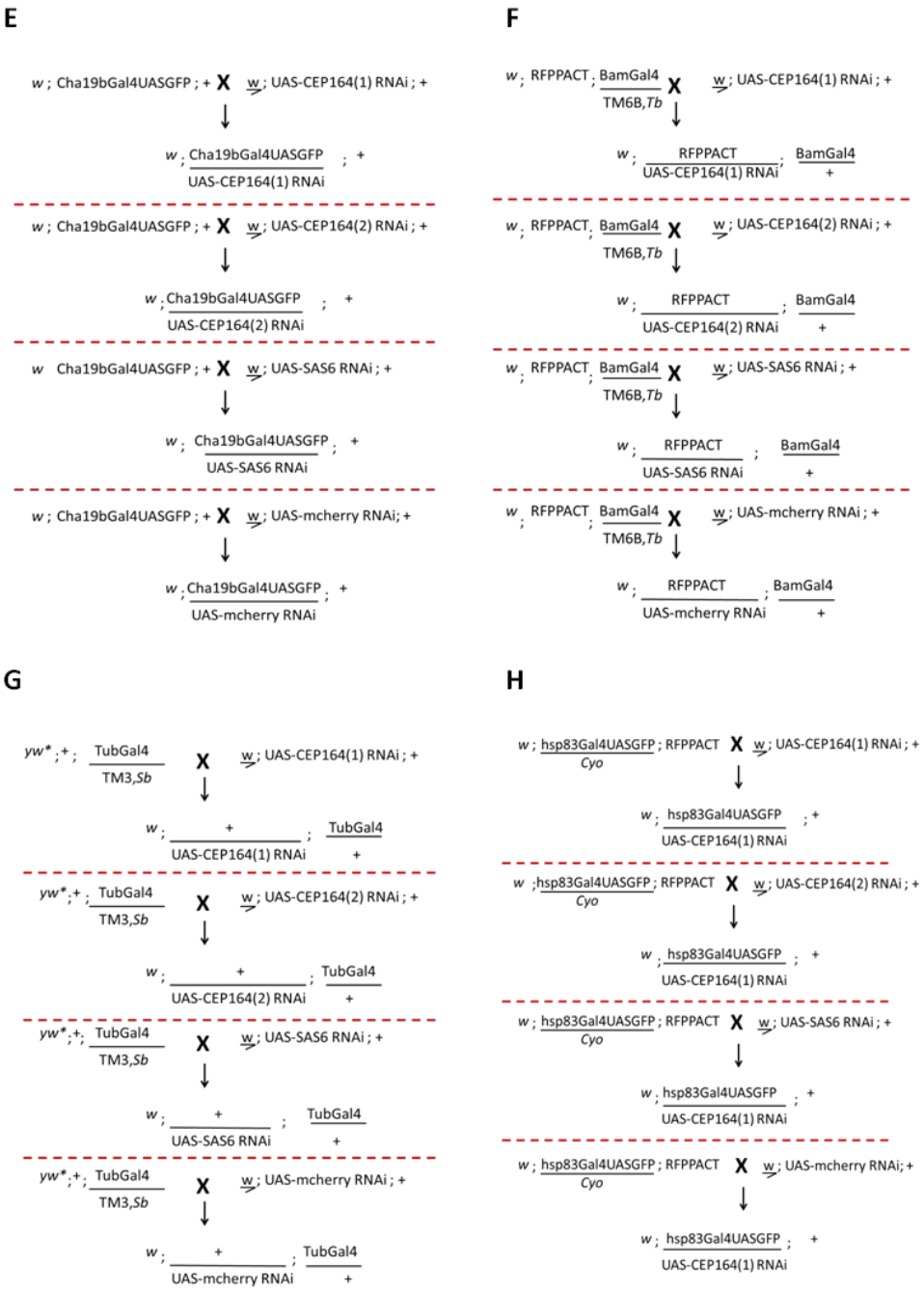

Figure 31 – Crosses set between virgin females Gal4 driven and males RNAis.
(A-H) Crosses to actin, Sg18.1, neur, Dicr-Bam, Cha19b, RFPPACT-Bam, tubulin and hsp83 drivers, respectively.

Appendix B

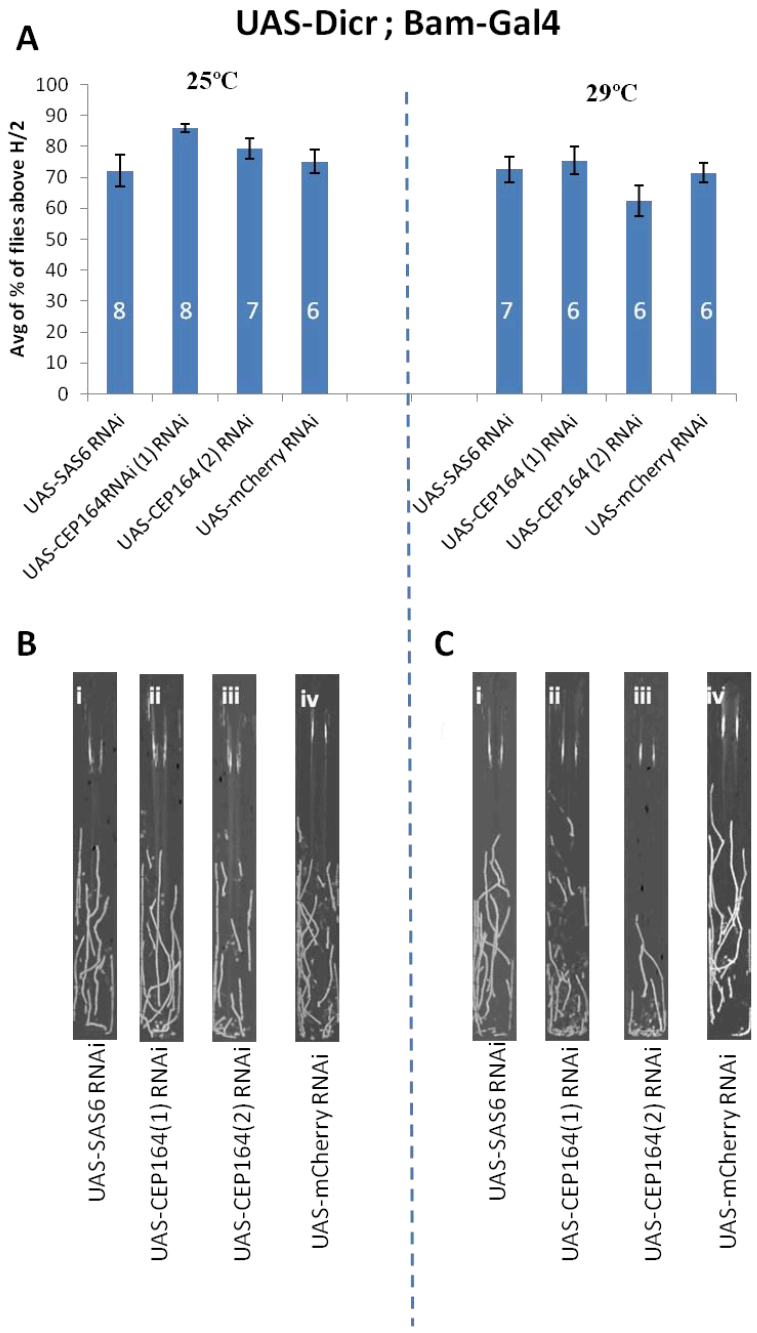

Figure 32 – Knock down of CEP164 in spermatocytes using Dicr;Bam drivers does not affect coordination in flies.

(A) Average of the percentage of the flies that climb above half of the tube of 18 cm height (H/2=9cm). Flies were grown at 25 and 29ºC. N≥6. (B-C) Representative images displaying the tracks travelled by the flies in first 5 seconds, after the bang. At 29ºC, Bam-Gal4/UAS-CEP164 RNAi flies (C-ii,iii) have a slower response to the stimuli than the control flies (C-i,iii), still these flies do not show defects in climbing ability .

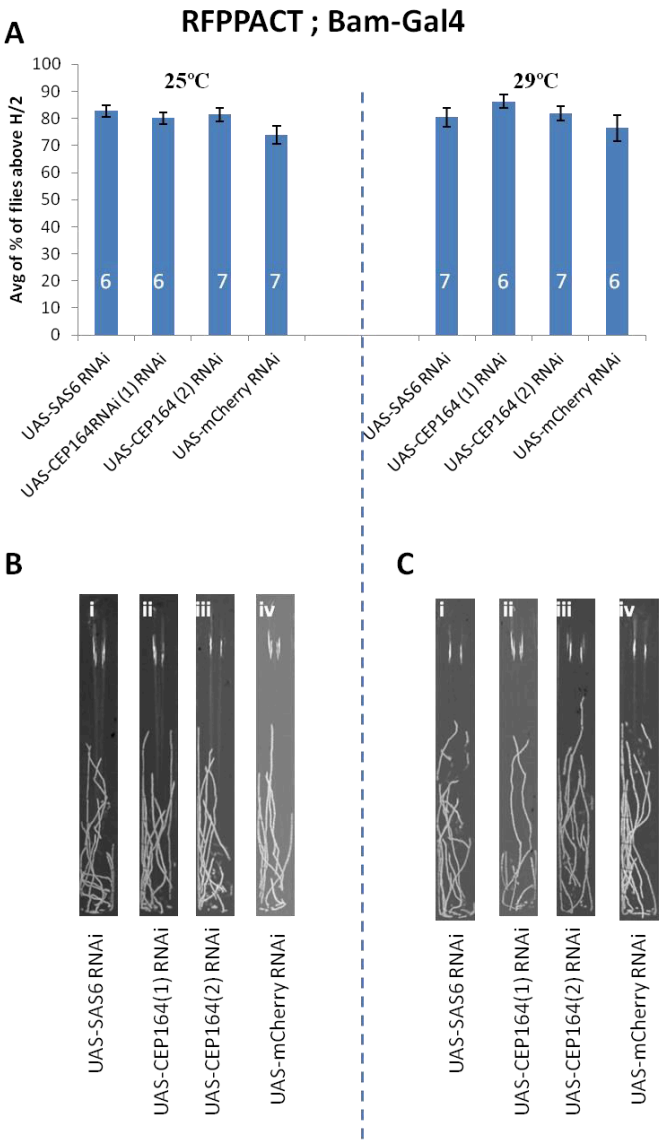

Figure 33 - CEP164 knock down in primary spermatocyte does not affect coordination in adult flies.

(A) Average of the percentage of the flies that climb above half of the tube of 18 cm height (H/2=9cm). Flies were grown at 25 and 29ºC. N≥6. (B-C) Representative images displaying the tracks travelled by the flies in first 5 seconds, after the bang. Knocked down flies do not have walking behavior problems.

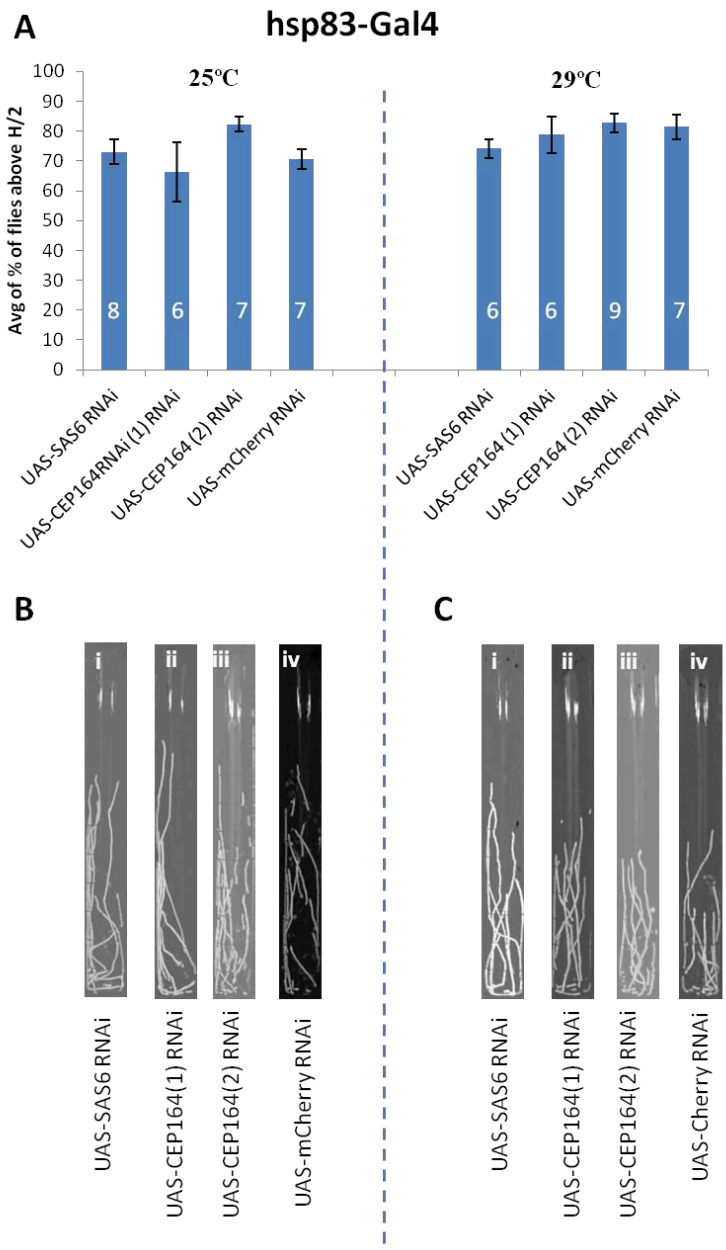

Figure 34 – Depletion of CEP164 in spermatids using hsp83 driver does not affect walking behavior in adult flies

(A) Average of the percentage of the flies that climb above half of the tube of 18 cm height (H/2=9cm). Flies were grown at 25 and 29°C. N≥6. (B-C) Representative images displaying the tracks travelled by the flies in first 5 seconds, after the bang. At 29°C, hsp83/UAS-CEP164 RNAi flies (C-ii,iii) have a slower response to the stimuli than the control flies (C-i,iii), however in general walking behavior is not affected.

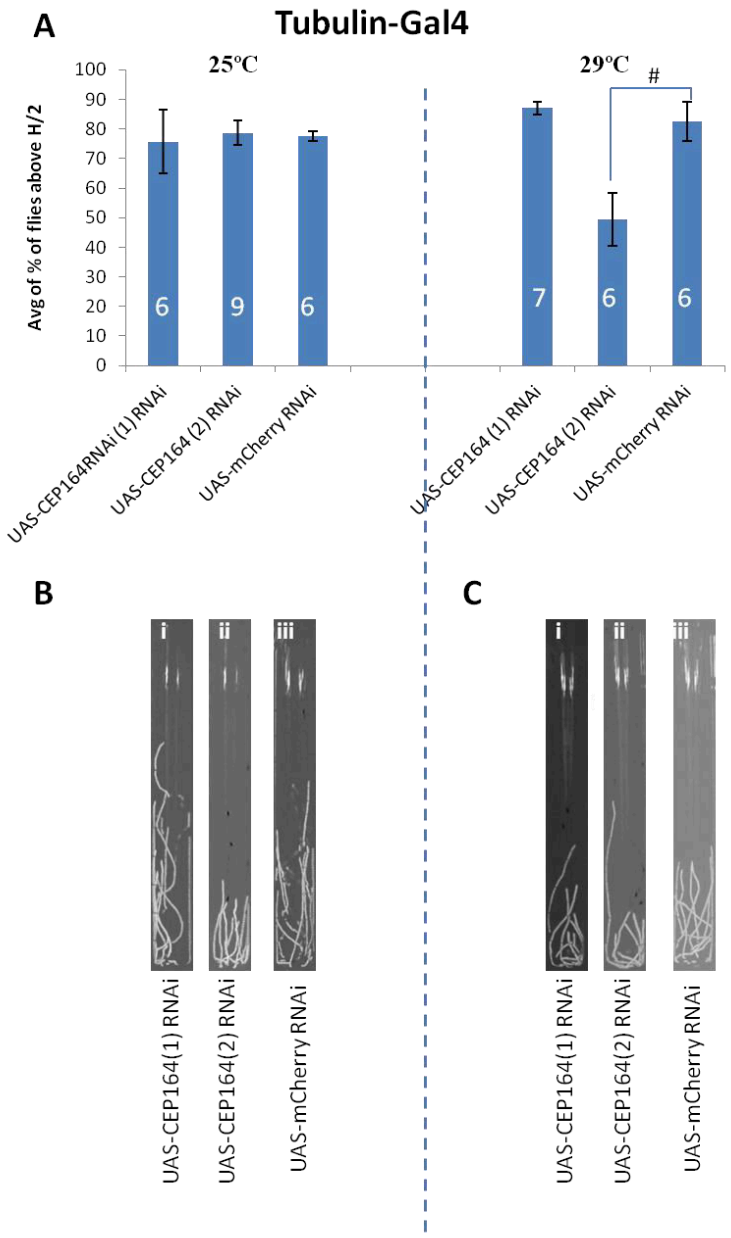

Figure 35 - CEP164 depletion using Tubulin-Gal4 driver at 29ºC causes defects in adult flies coordination at 29ºC.

(A) Average of the percentage of the flies that climb above half of the tube of 18 cm height (H/2=9cm). Flies were grown at 25 and 29ºC. N≥6. Tubulin-Gal4/UAS-SAS6 RNAi flies do not eclode. (B-C) Representative images displaying the tracks travelled by the flies in first 5 seconds, after the bang. At both 25ºC and 29ºC, Tubulin-Gal4/UAS-CEP164 (2) RNAi flies (B-ii and C-ii) have a slower response to the stimuli than the control flies (B-iii and C-iii) and than the Tubulin-Gal4/UAS-CEP164 (1) RNAi flies.