

FERHAN YENİSERT

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INVESTIGATION OF NEW CILIOPATHY GENES

A THESIS
SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
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ABSTRACT

INVESTIGATION OF NEW CILIOPATHY GENES

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May 2021

Cilia consist of microtubules in its internal structure and evolutionarily conserved an antenna-like organelle. The disease caused by defects in the cilia structure is called ciliopathy and Joubert syndrome is one of the ciliopathies. Patients display a range of symptoms, such as delayed intellectual and language development, hypotonia, ataxia, mental retardation, liver cyst, retinal defect/degeneration, genital defect, and cystic kidney. As a result of recent studies, 38 different genes have been associated with Joubert syndrome. In 2012, CEP41, an evolutionarily conserved gene, was associated with JS, one of the diseases of ciliopathy, but that study did not investigate the molecular mechanism of CEP41. In this study, the effect of ceph-41 mutation on the structure and function of cilia was investigated by using *C. elegans*, which is widely used as a model system in cilia studies.

Keywords: CEP41, Joubert syndrome, Ciliopathy,

ÖZET

YENİ SİLYOPATİ GENLERİNİN ARAŞTIRILIMASI

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Mayıs 2021

Kirpikler, iç yapısında mikrotübüllerden oluşur ve evrimsel olarak anten benzeri bir organel korunmuştur. Kirpik yapısındaki bozuklukların neden olduğu hastalığa silyopati adı verilir ve Joubert sendromu silyopatilerden biridir. Hastalar, gecikmiş zihinsel ve dil gelişimi, hipotoni, ataksi, zeka geriliği, karaciğer kisti, retina kusuru / dejenerasyonu, genital kusur ve kistik böbrek gibi bir dizi semptom gösterir. Son çalışmaların bir sonucu olarak, 38 farklı gen Joubert sendromu ile ilişkilendirilmiştir. 2012 yılında, evrimsel olarak korunmuş bir gen olan CEP41, silyopati hastalıklarından biri olan JS ile ilişkilendirildi, ancak bu çalışma CEP41'in moleküler mekanizmasını araştırılmadı. Bu çalışmada, cilia çalışmalarında yaygın olarak model sistem olarak kullanılan *C. elegans* kullanılarak *ceph-41* mutasyonunun kirpiklerin yapısı ve işlevi üzerindeki etkisi araştırılmıştır.

Anahtar Kelimeler: CEP41, Joubert sendromu, Silyopati

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List of Abbreviations

BBS	Bardet-Biedl syndrome
BB	Basal Body
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
DS	Distal Segment
IFT	Intraflagellar Transport
JS	Joubert syndrome
MKS	Meckel-Gruber syndrome
MS	Middle Segment
PKD	Polycystic kidney disease
TZ	Transition Zone

To Kaplan lab member and my family

Chapter 1

1. Introduction

1.1 Cilia and Flagella

Projecting as the cellular antenna into the extracellular space, cilia and flagella are present in unicellular species such as *Chlamydomonas reinhardtii* and *Tetrahymena thermophila*, as well as multicellular organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals, such as mouse and human. The discovery of cilia was dated back to the 17th century because Anthony van Leeuwenhoek described them as “little feet” in 1674. Otto Muller named these “little feet” as “cilium” (eyelash in Latin) in 1786 [1,5]. Scientists used electron microscopy to examine the ultrastructure of cilia and flagella, which revealed a 9+0 or 9+2 microtubule doublet in a ring form surrounded by the plasma membrane [4,6].

As globular α -tubulin and β -tubulin molecules form α -/ β -tubulin heterodimers that undergo polymerization to build a protofilament, 13 protofilaments unite to form a complete ring known as A tubule, while the 10 protofilaments form an incomplete ring known as B tubule (Figure 1.1) [7]. Since protofilaments are intrinsically polarized, the plus end of the microtubule is anchored to the ciliary tip, while the minus end is rooted to the basal body, a modified mother centriole at the cilium's base [9]. Depending on the presence of centrally located microtubule-based doublets in the cilia, cilia are divided into two classes: motile and non-motile cilia (primary cilia). Primary cilium has nine pairs of microtubule organization (9 + 0), while motile cilia have two additional microtubule doublets (9+2) in the center of cilia (Figure 1) [8,10].

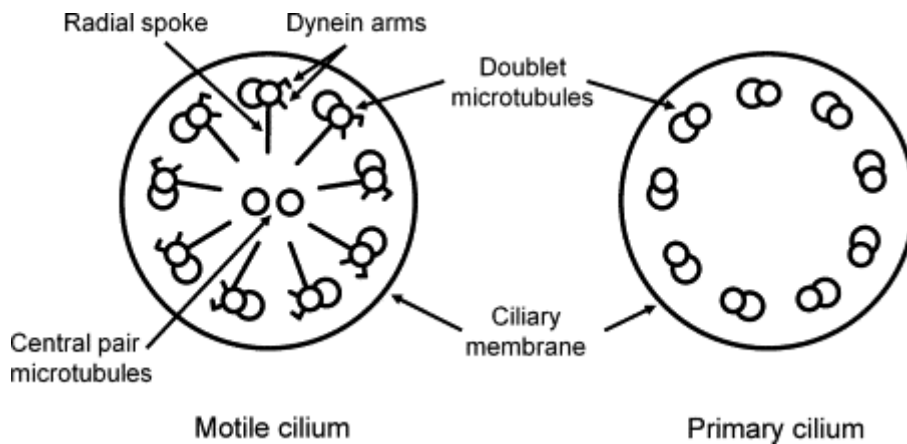


Figure 1.1 A difference between the structure of motile cilia and primary cilia.

The cross-sections of motile and primary cilia are illustrated in the diagrams [3].

Despite the fact that motile cilia were discovered in the 17th century, scientists such as Paul Langerhans, Alexander Ecker, Aleksandr Kowalevsky, Albert Kolliker, and Karl Zimmermann were the first to recognize primary cilium (non-motile cilia) in a large variety of vertebrate cells in the 19th century. The primary cilium is unable to move, which may be due to the absence of centrally located microtubule doublets, as research in the unicellular organism *Chlamydomonas reinhardtii* revealed that mutants without the central pair of microtubules are unable to move [7,12,13]. Furthermore, the molecular motor dynein, which is needed for cilia movement, does not appear to be present in the primary cilium.

Furthermore, accumulating evidence over the years proved that motile and primary cilia have distinct functions. For example, mucus removal is mediated by motile cilia on the surfaces of cells in the respiratory tract and the middle ear, and sperm cell movement is facilitated by motile cilia [14]. Interestingly, the correct left-right asymmetry in embryo development needs the nodal cilia (motile cilia) in the mammalian embryo. Primary cilium, on the other hand, is located on the cell surfaces of most mammalian cells, including olfactory epithelium, brain cells, fibroblasts, mesenchymal cells, and retinal photoreceptors, where they perform a variety of sensory roles such as mechanosensory and chemosensory. The primary cilium of retinal pigment cells, for example, contains rhodopsin molecules, which are required for vision [11,14]. Primary cilium on the cells surrounding the kidney nephron is needed for detection of the flow of fluid; however, if the fluid flow sensing is disrupted, cysts form in the kidneys, resulting

in polycystic kidney disease (PKD). PKD was the first disease that was linked to primary cilium [7].

In vertebrates, the primary cilium acts as a signaling organelle that transmits a variety of signaling pathways that regulate embryo development, such as Hh, Wnt [16,17], Notch [19], Hippo [20] Platelet-Derived Growth Factor Subunit A (PDGFa)[18], and mTOR [21,22] pathways[15]. Furthermore, cilia are implicated in neuronal migration [23,24] and axonal pathway development in differentiated neurons [25].

1.1.1 Basal Body

Cilia are divided into several subcompartments, such as basal body (BB), transition zone (TZ). BB, the modified mother centriole, is a barrel of nine three-fold microtubules, sub-distal extensions, and nine strut-like structures known as distal extensions or transition fibers, attached to the membrane at the base of the cilium [26,27]. During cilia formation, the mother centriole is connected to the ciliary vesicle or plasma membrane through distal extensions, which then become transitional fibers (Williams CL). Protein entry into cilia and departure from cilia are thought to be regulated by BB and TZ at the ciliary base, and there is a concept of a diffuse barrier or gate that prevents unspecified membrane protein movement into and out of the cilium [28].

1.1.2 Transition Zone

The transition zone (TZ) arises at the BB's distal end and consists of a collection of transition fibers that extend from the B-tube to the C-end tubes and are embedded inside the periciliary plasma membrane. In the main body of the TZ, there are multiple rows of Y-shaped connectors projecting from the outer pairs and connecting to the ciliary membrane [29]. The TZ layout is then formed by nine outer microtubular pairs with a 9-bladed propeller base structure [30]. TZ ends distally with the final Y-linker chain. As mentioned above, TZ works as a gatekeeper by blocking the entry of non-ciliary proteins into cilia and assists by working on vesicle trafficking of other TZ proteins such as CC2D2A [31].

1.2 Ciliopathy

The motile cilia were first related to a human pathology named primary ciliary dyskinesia (PCD) in 1976 [32], but later years proved that structural and functional defects in the primary cilium, in addition to motile cilia, contribute to a human disorder [33]. Since then, the list of cilia-related diseases has expanded to include Joubert syndrome (JBTS) [34], Senior-Loken syndrome (SLS), Nephronophthisis (NPHP)[36], Bardet-Biedl syndrome (BBS)[37], Meckel-Gruber syndrome (MKS) [35], Alström syndrome (AS), autosomal dominant polycystic kidney disease (ADPKD) [33], Orofaciodigital syndrome type I (OFD), and Cranioectodermal dysplasia (CED), they are collectively called ciliopathy. While Cornillie FJ and colleagues first-named ciliopathy in 1984, it gained popularity after Ansley and colleagues discovered that a mutation in BBS8 caused BBS in 2003 [38,39]. Ciliopathy shares a considerable number of clinical symptoms, including mental retardation, liver cyst, retinal defect/degeneration, sterility or genital defect, cystic kidney, hydrocephalus, craniofacial defect, rib/thoracic defect, polydactyly, pelvic bone defect, pancreatic cyst, lung or airway defect, cardiac defect, but not all symptoms are present in the patient (Figure 1.2).

Our lab has collected the list of genes causing ciliopathy (unpublished data) and the number of ciliopathy-causing genes has reached over 200. Most proteins encoded by ciliopathy-causing genes localize to cilia and cilia sub-compartments, while mutations in genes encoding non-ciliary proteins can cause a ciliopathy.

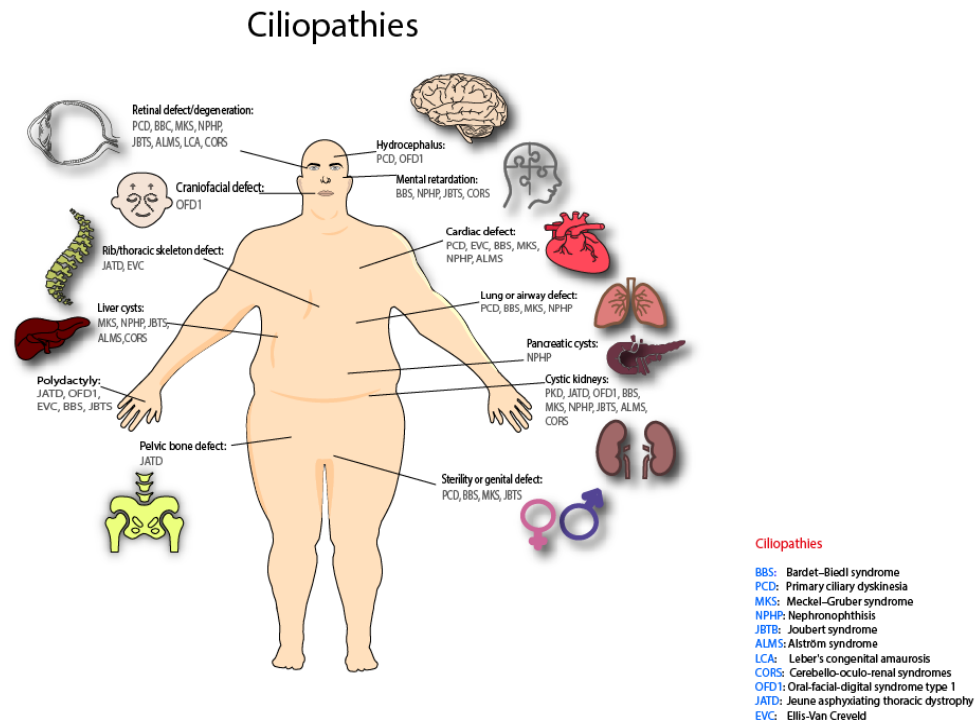


Figure 1.2. Demonstration of ciliopathies and affected part of human body.

1.2.1 Polycystic kidney disease (PKD)

Polycystic kidney disease (PKD) was first identified as kidney disease in the 16th century, with the first recorded case being Polish king Stefan Bathory, who experienced weakness (fatigue) and chest pain. They found that the king's kidney is "as big as those of a bull, with an uneven and bumpy appearance" after he died [40]. After 350 years, Krakow historians and clinicians concluded that the cause of death was most likely uremia and autosomal dominant PKD. The polycystic term was firstly used in 1888.

The name "PKD" refers to two different forms of the disease, each with varying degrees of phenotypic difference and genetic cause: autosomal recessive polycystic kidney disease (ARPKD) and autosomal dominant polycystic kidney disease (ADPKD) [41]. ADPKD is a genetically heterogeneous disorder in which two genes, PKD1 (16p13.3) and PKD2 (16p13.3), have been involved [42]. On the other hand, ARPKD is the less frequent of the two forms of PKD related to the PKHD1 gene [43]. These three genes encode proteins that localize within cilia. Pkd displays a range of symptoms including urine blood, elevated blood pressure, headache, stomach pain, and frequent urination.

1.2.2 Joubert syndrome

Joubert syndrome (JBTS; OMIM 213300) is a rare ciliopathy disease affecting the cerebellum, which regulates balance and coordination. The following are the signs and symptoms of Joubert syndrome: abnormal eye movements, fast breathing, delayed intellectual and language development, hypotonia, ataxia, mental retardation, liver cyst, retinal defect/degeneration, genital defect, and cystic kidney [44]. Number of genes implicated in JBTS have increased and there are currently 38 genes that cause Joubert syndrome and related cerebellar diseases, and the identified genes are : TMEM216 [45], AHI1 [46], NPHP1 [47], CEP290 [48], TMEM67 [49], RPGRIP1L [50], ARL13B [51], CC2D42A [52], CXORF5 [53], TTC21B[54], KIF7 [55], TCTN1 [56], TMEM237[57], CEP41[58], TMEM138 [59], C5ORF42 [60], TCTN3 [61], ZNF423[62], TMEM231 [63], CSPP1 [64], PDE6D [65], KIAA0586[66], TCTN2 [67], CEP104 [68], KIAA0556 [69], B9D1[70], MKS1[70], TMEM107[71], ARMC9[72], CEP120[73], SUFU[74], PIBF1[75], B9D2[76], ARL3[77], FAM149B1[78], TOGARAM1[79], and IFT74[80] (Table 1). Accumulating evidence revealed that these JBTS genes encode proteins that are found in the cilia or basal body or transition zone. Cilia formation and/or function are impaired as a result of mutations in either of these JBTS genes. In this thesis, we investigated the function of CEP41 in the nematode *Caenorhabditis elegans*.

Human Gene Name	OMIM ID	<i>C. elegans</i> Orthologous Gene Name	Wormbase ID	References
TMEM216	613277	<i>mks-2</i>	WBGene00194710	[45]
AHI1	608894	NA	NA	[46]
NPHP1	607100	<i>nphp-1</i>	WBGene00010898	[47]
CEP290	610142	<i>cep-290</i>	WBGene00012121	[48]
TMEM67	609884	<i>mks-3</i>	WBGene00018042	[49]
RPGRIP1L	610937	<i>mks-5</i>	WBGene00007490	[50]
ARL13B	608922	<i>arl-13</i>	WBGene00021349	[51]
CC2D2A	612013	<i>mks-6</i>	WBGene00010642	[52]
CXORF5	300170	NA	NA	[53]
TTC21B	612014	<i>ift-139</i>	WBGene00022696	[54]
KIF7	611254	<i>kfp-12</i>	WBGene00002223	[55]

TCTN1	609863	<i>tctn-1</i>	WBGene00017120	[56]
TMEM237	614423	<i>tmem-237</i>	NA	[57]
CEP41	610523	<i>ceph-41</i>	WBGene00249817	[58]
TMEM138	614459	<i>tmem-138</i>	WBGene00008643	[59]
C5ORF42	614571	<i>hpo-40</i>	WBGene00014113	[60]
TCTN3	613847	<i>tctn-1</i>	WBGene00017120	[61]
ZNF423	604577	<i>lin-13</i>	WBGene00003002	[62]
TMEM231	614949	<i>tmem-231</i>	WBGene00020825	[63]
CSPP1	611654	NA	NA	[64]
PDE6D	602676	<i>pdl-1</i>	WBGene00003966	[65]
KIAA0586	610178	<i>talp-3</i>	NA	[66]
TCTN2	613846	NA	NA	[67]
CEP104	616690	<i>c40h1.3</i>	WBGene00008039	[68]
KATNIP	616650	<i>k04f10.2</i>	NA	[69]
B9D1	614144	<i>mksr-1</i>	WBGene00019364	[70]
MKS1	609883	<i>mksr-2</i>	WBGene00021416	[70]
TMEM107	616183	<i>tmem-107</i>	NA	[71]
ARMC9	617612	<i>F59G1.4</i>	WBGene00019128	[72]
CEP120	613446	NA	NA	[73]
SUFU	607035	NA	NA	[74]
PIBF1	607532	NA	NA	[75]
B9D2	611951	<i>mksr-2</i>	WBGene00021416	[76]
ARL3	604695	<i>arl-3</i>	WBGene00000188	[77]
FAM149B1	618413	NA	NA	[78]
TOGARAM1	617618	<i>che-12</i>	WBGene00000491	[79]
IFT74		<i>ift74</i>	WBGene00016005	[80]

Table 1.1. Orthologous variant of 38 genes associated with Joubert syndrome.

38 JS gene and *C. elegans* orthologous of these genes are shown in the table.

1.3 Intraflagellar transport (IFT)

Joel Rosenbaum and his lab discovered that intraflagellar transport (IFT) undergoes bidirectional motility along the cilia of *Chlamydomonas reinhardtii*, a unicellular organism, in 1993, and two years later, his lab biochemically isolated the multi-protein IFT complex [81]. The multi-protein IFT complex consists of three essential components; (1) IFT proteins divided into two sub-complexes: IFT complex B and IFT complex A, (2) anterograde motor protein heterotrimeric Kinesin II, and (3) retrograde motor protein cytoplasmic Dynein. Genes in these two complexes named according to their molecular weights are as follows: IFT A complex IFT144, IFT140, IFT139, IFT122, IFT121, and IFT43 and IFT B complex IFT172, IFT88, IFT81, IFT80, IFT74, IFT72, IFT57, IFT52, IFT46, IFT20, IFT27[82], IFT70/Dyf-1 [83], IFT25 [84] IFT54/Elipsa [85] and IFT22/IFTA-2/Rab5 [86]. In later years, it was discovered that IFT is necessary for cilia formation and flagella maintenance. However, IFT is responsible for the transport of ciliary constituents and signal molecules, such as PKD and Smoothen receptor SMO. Depending on the organism, the heterodimeric kinesin-II motor protein or the heterodimeric kinesin-II motor protein in combination with the homodimeric OSM-3 (Human KIF17) kinesin motor protein transports the IFT complex from the basal body to the cilia end (anterograde) [86].

Another protein complex that undergoes IFT is the BBSome made up of eight distinct proteins, including BBS1, BBS2, BBS4, BBS5, BBS8, BBS7, BBS9, and BBS18/BBIP10. There are several different functions attributed to *bbs* genes. For example, work from *C. elegans* revealed that the BBSome keeps IFT motors and IFT sub-complexes A and B together. The BBSome assembly requires the BBS/CCT complex activity that includes the three chaperonin-like BBS proteins (BBS10, BBS6, and BBS12) and the chaperonin containing TCP-1 (CCT) / T-complex protein-1 ring complex (TriC) family chaperonins [87].

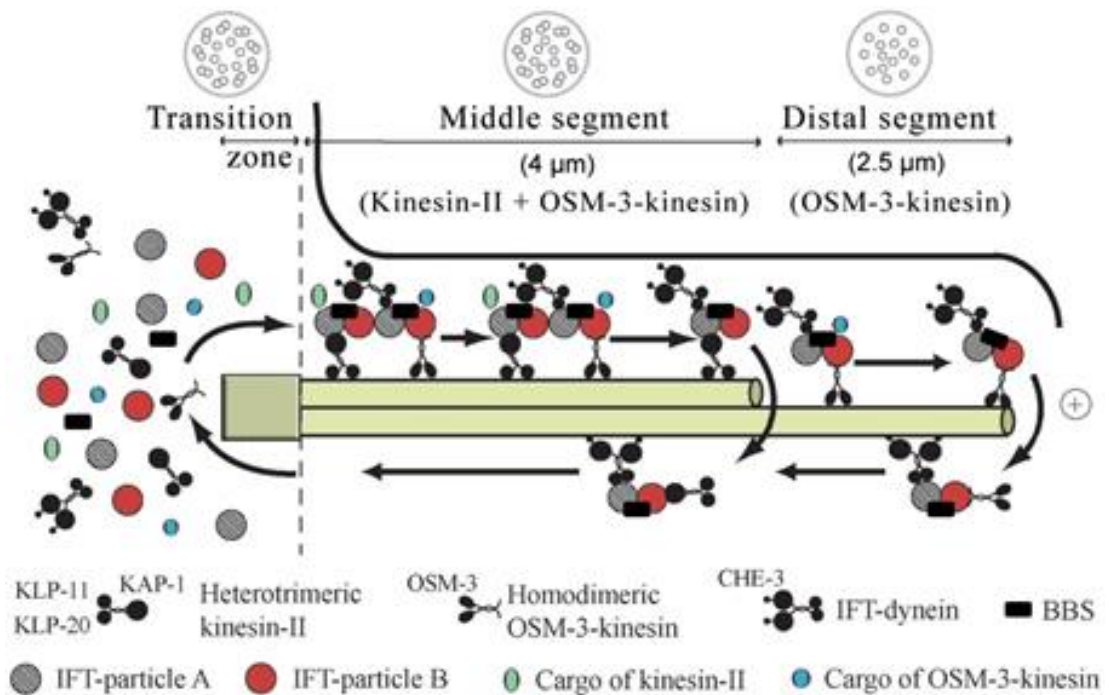


Figure 1.3 Intraflagellar transport in *C. elegans*. Intraflagellar transport in *C. elegans*. Components of the IFT machinery and ciliary cargo assemble at or near the transition zone (basal body), and heterotrimeric kinesin-II, and homodimeric OSM-3-kinesin, separately bind IFT particle subcomplexes A and B in order and transport them along the middle segment in the anterograde (+) direction with IFT-dynein and cargo. OSM-3-kinesin carries IFT particles and dynein/cargo alone in the distal segment. The BBS proteins work to keep the motors and IFT particle subcomplexes A and B together. The IFT-dynein molecular motor recycles components of the IFT machinery, possibly other ciliary molecules, back to the cilium's foundation. In conjunction with the microtubular schematics of the cross-specifications, the lengths of Transition Zone (1 mc), Medium Section (4 mc), and Distal (2,5 mc) areas are presented (for cilia amphid); (on top) [98].

IFT proteins travel at a certain speed along the cilia (Figure 1.3). The first IFT speed study was performed in *Chlamydomonas*, which revealed that the heterodimeric kinesin II protein carries the anterograde complex IFT at a speed of 2.5 μm/s, whereas the dynein protein carries the retrograde IFT at a speed of 4 μm/s [84]. In human cell culture, the situation is somewhat different. Human cells have a slower IFT motility than *Chlamydomonas*. In mammals, the IFT complex is transported at speeds ranging from 0.3 to 0.7 μm/s in both anterograde and retrograde directions [85]. In this regard, the IFT velocity of *Caenorhabditis elegans* might be closer to the slower IFT speed of a mammal than that of *Chlamydomonas*. In *C. elegans*, there are two kinesin motor proteins heterodimeric Kinesin II and homodimeric kinesin OSM-3 (human KIF17). These two kinesin motors transport the IFT/BBSome complex along the middle segment in an anterograde direction at 0.7 m/s, while the homodimeric kinesin motor OSM-3/KIF17

drives them along the distal segment at 1.2 m/s. The return of the IFT/BBSome complex from the ciliary tip/middle part of the cilia is achieved by the retrograde cytoplasmic dynein motor protein that moves at a rate of 1.3 $\mu\text{m/s}$.

1.4 Model Organism: *Caenorhabditis elegans*

Caenorhabditis elegans is a free-living nematode approximately 1 mm long and 65 μm thick. *Caenorhabditis elegans* have 2 sex: hermaphrodite has XX chromosomes and male has one X chromosome. Hermaphrodites are living things that can reproduce their eggs and sperm. It can produce 300 eggs in 3-4 days. There is a noticeably short generation span, like 2-3 days. Therefore, this gives the chance to see the phenotype desired to be looked after in the next generation in a noticeably short time. A short lifetime provides a great advantage especially in studies such as embryogenesis and development (Figure 1.4). Each adult hermaphrodite always contains exactly 959, and each adult male contains 1031 somatic cell nuclei. The first multicellular organ with a full genome sequence was *C. elegans* [88].

C. elegans is widely used in cilia research because of its ease of use and functional similarity with human studies [89]. Many evolutionarily conserved ciliary genes were first identified as model organisms using *C. elegans*. The cilia functions of IFTA-1 (IFT

A

sub-complex

gene),

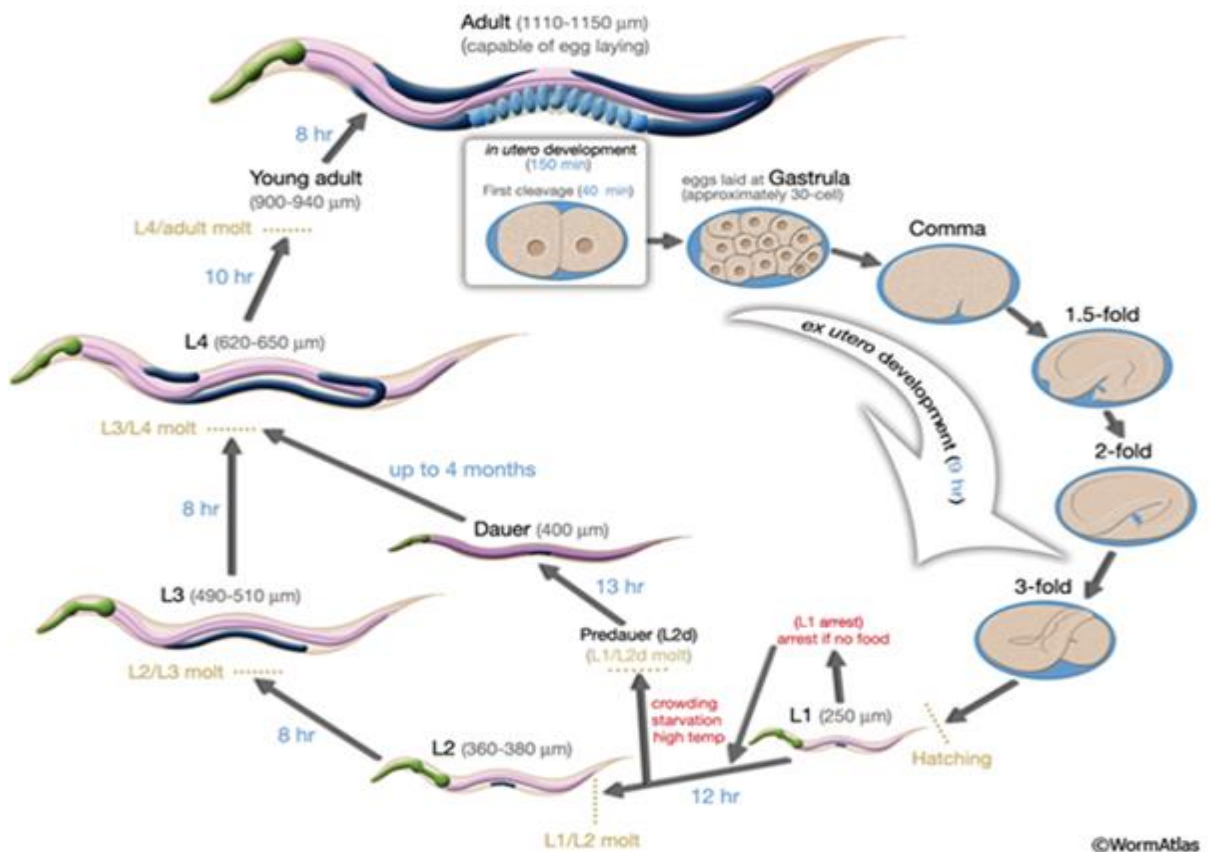


Figure 1.4 The life cycle of *C. elegans* at 22°C.

It is fertilized for 0 minutes. The blue numbers on the arrows indicate the time the animal spends at a certain stage. The primitive ends about 40 divisions after fertilization. The eggs are released approximately 150 minutes after fertilization and gastrulation are glazed. The length of the animal in each stage is marked in micrometers (μm) next to the stage name [99].

DYF-1, DYF-2, DYF-13 (IFT B sub-complex genes) were first identified in *C. elegans* and later it has been found that these genes are involved in forming the cilia structure in other animals, including humans [90]. Cilia functions of the evolutionarily conserved gene *K04F10.2* were first demonstrated in *C. elegans* [91]. DYF-19 (FBF1), RAB-28, AP-2 complex, AP-1 are examples of genes whose functions have been shown for the first time in *C. elegans* [70]. The cilia association of the Joubert syndrome gene *ARL13B* was first shown in mice in 2008 [63]. *ARL13B* study showed that *ARL13B* was localized in cilia and found that its deficiency caused structural defects in microtubule pairs in cilia [63]. Later, the role of this gene was characterized using *C. elegans* [89]. The study carried out in *C. elegans* has demonstrated that *ARL13B* is localized in cilia as well as structural defects in microtubules of cilia in an *ARL-13* mutant study. In addition

to mammalian studies, in 2010 a study found that ARL13b is localized in the cilia membrane and regulates IFT by using *C. elegans* [89]. This all proves that *C. elegans* may be used as an excellent model system for studies of cilia.

The usage advantages/features of *C. elegans*, one of the most preferred model systems by scientists, are listed.

It is easy and cheap. They live in the laboratory on agar curtains containing *E. coli*. Millions of worms can live in a petri dish. *C. elegans* can be rapidly frozen and stored in a refrigerator (- 80 degrees).

C. elegans in Japan (<http://www.shigen.nig.ac.jp/c.elegans/index.jsp>) and America (<http://www.cbs.umn.edu/CGC/>) there are mutant centers. These centers have more than 40% mutants of all genes. Requesting scientists can obtain mutants for a small fee.

Since *C. elegans* is a multicellular organism, it undergoes complex developmental processes such as embryogenesis, morphogenesis, and reaching maturity, as is the case with higher organisms. All cells (neuron, muscle, and intestine, etc.) starting from the first cell until adulthood have been characterized by scientists. Specific markers are available for these cells.

Many molecular studies, which are very laborious, time-consuming, and expensive, can be easily performed in *C. elegans*. For example, programmed cell division (apoptosis) was first found in *C. elegans*. However, studies such as gene expression, studying the molecular bases of axon and dendrite, cell division, and cell differentiation can be easily performed in *C. elegans*. In addition to all these, *C. elegans* is a widely used model organism in lifespan studies. Functional studies of genes associated with the disease can be done.

The fundamental structure of non-motile cilia is at the dendritic ends of neuronal cells (60 of 302 neurons). These primary cilia found in *C. elegans* form a grouped structure called sensilla (amphid and phasmid sensilla), which has a sensory task. *C. elegans* is surrounded by cuticles (the structure composed of collagen surrounding *C. elegans*). Amphid and phasmid cilia open to the environment through two pores and communicate with the environment. These are the only structures that communicate with the environment. In this project, using the advantages of *C. elegans*, the role of CEP41, which

is the Joubert Syndrome gene, in cilia will be investigated for the first time in this organism.

1.5 *CEP41*, a Joubert syndrome gene, regulates cilia biogenesis and polyglutamylation

It was first identified by Joseph G. Gleeson that *CEP41*, encoding a protein of 41-kilodalton (kDa), was mutated in patients with Joubert syndrome in 2012 [Lee, J.E]. Since Joubert syndrome is a disease associated with cilia, it was assumed that *CEP41* may have a role in cilia. In the 2012 study, *CEP41* localization was examined, which revealed that it localized in cilia and centrioles in mouse and human cells [58]. In mRNA expression studies performed in zebrafish, it was found that *CEP41* was expressed in the ciliated organs Kupffer's vesicles, ear, heart, nerves, and kidneys [58]. When the *CEP41* gene was silenced with morpholino (antisense oligonucleotides), phenotypes associated with ciliopathy (hydrocephalus, abnormal ear otolith formation, peripheral heart edema, tail defects) were also repeated in zebrafish [58]. Likewise, when *CEP41* was mutated in mice, cilia-related abnormalities were observed, and the mice died before completing embryo development, so their functional studies were limited to these studies [58].

Another important function of *CEP41* is its involvement in tubulin glutamylation. In the 2012 stud., it was found that tubulin glutamylation was reduced in fibroblast cells obtained from patients with Joubert syndrome with *CEP41* gene mutations [Lee, J.E]. Simultaneously, the protein complex immunoprecipitation (Co-IP) assay showed that *CEP41* is precipitated by TTLL enzymes [58]. Although the 2012 study states that *CEP41* is a part of TTLL enzymes, *CEP41* does not have a ligase-like enzyme activity in its protein structure. The main role of *CEP41* in tubulin polyglutamate is currently unknown. Although only the 2012 article showed that the mutation of *CEP41* leads to phenotypes associated with ciliopathy, it is not yet known how the deficiency of this gene leads to these phenotypes. My thesis aimed to define the role of *CEP41* in *C. elegans*.

Chapter 2

2. Materials and Methods

2.1 Materials

2.1.1 Strains

The strains used in this work:

N2

N2; [*Pstr1*::ODR10::GFP]

N2; [*Pstr1promoter*::GFP]

N2; *ntIs1* [*gcy-5p*::GFP + *lin-15(+)*] V; integration of *adEx1262* [*gcy-5p*::GFP + *lin-15(+)*].

N2; *gmls13* [*srb-6p*::GFP+pRF4]

N2; [OSM-3::GFP+PRF4]

N2; *Is* [OSM6::GFP]

N2; *casIs586*(KAP-1::GFP)

N2; *jhuEx* [CHE-11::GFP+pRF4]

The following strains were generated in this work:

cep41 (*tur001*)

cep41 (*tur001*) ;;[*Pstr1*::ODR10::GFP]

cep41 (*tur001*) ;;[*Pstr1promoter*::GFP]

cep41 (*tur001*); *ntIs1* [*gcy-5p*::GFP + *lin-15(+)*] V; integration of *adEx1262* [*gcy-5p*::GFP + *lin-15(+)*].

cep41 (*tur001*); *gmls13* [*srb-6p*::GFP+pRF4]

cep41 (*tur001*); *Is* [OSM6::GFP]

bbs-5 [OSM-6::GFP]

cep-41(tur001); bbs-5 [OSM-6::GFP]
cep41 (tur001); jhuEx [CHE-11::GFP+pRF4]
cep41 (tur001); casIs586(KAP-1::GFP)
cep-41(tur001) IV;Ex[OSM-3::GFP++pRF4]
cep41 (tur001); Ex [CHE-11::GFP +pRF4]

him-5(e1490) V; myIs1 [PKD-2::GFP + Punc-122::GFP] IV.; cep-41promoter_CEP41(F42G8.19)_wrmscarlet (25 ng)
him-5(e1490) V; myIs1 [PKD-2::GFP + Punc-122::GFP] IV.; cep-41promoter:CEP41(F42G8.19)::-wrmscarlet
N2 [cep-41promoter_CEP41(F42G8.19)::wrmscarlet ;str-1p::GFP]
N2 [cep-41promoter: CEP41(F42G8.19)::wrmscarlet ;str-1p::GFP]

2.1.2 Primers and Plasmids

F42G8.19_sgRNA1_for TCTTGTC AATATCAATAGCCGAATG
F42G8.19_sgRNA1_rev AAACCATTCGGCTATTGATATTGAC
F42G8.19_sgRNA2_for TCTTGCTCAATCCGAGCAAACAAG
F42G8.19_sgRNA2_rev AAACCTTGTTTGCTCGGATTGAGC
F42G8.19_sgRNA3_for TCTTGCTGTATTTGGAGGTCGTCTG
F42G8.19_sgRNA3_rev AAACCAGACGACCTCCAAATACAGC

pRB1017 was a gift from Andrew Fire (Addgene plasmid # 59936;
<http://n2t.net/addgene:59936>; RRID:Addgene_59936).

The following primers were used in polymer chain reaction (PCR).

F42G8.19_CRISPR_for GCTTCCTACGACTTTCTCTG
F42G8.19_CRISPR_rev GTTCCTAGATTGGCTCGTTG

2.2. Methods

2.2.1 Dye Filling Assay

Dye filling tests are an efficient and straightforward way of testing the morphological durability of cilia and sensory neurons [93]. When live worms are put into a solution with fluorescent dyes including the FITC, DiI, DiO, and DiD, exposed ciliate ends will fulfill the head of the amphibian sensory neurons and phasmid sensory neurons in the tail. DiI will distinguish amphid neurons ASI, ADL, ASK, AWB, ASH, and ASJ, and phasmid neurons PHA and PHB as one of the carbocyanine dyes [94]. Normally when worms are exposed to fluorescence dye like Dil, wild-type worms take up to amphid and phasmid cilia. If there is a defect in cilia that cannot take dye in cilia is called dyf (Dye-filling defective).

C. elegans in nematode growth medium (NGM) were collected with M9 solution. *C. elegans* were washed with the prepared M9 solution from the petri dish to the Eppendorf tube and rotated for 1 minute at 2000-3000 rpm in the tabletop centrifuge. After centrifugation, the supernatant portion of the fluid was discarded and 1 ml of new M9 is added on top of the bottomed-out *C. elegans* to transfer worms from Petri to Eppendorf. This process is repeated till removing bacteria. After that M9 was removed as possible to achieve a 200: 1 ratio of M9 - dye solution. The lipophilic fluorescent dye is briefly prepared as follows: 199 μ l M9 solution is added to 1.5 ml autoclaved Eppendorf tube and then 1 μ l of lipophilic fluorescent dye is applied on the top (200: 1 ratio) and the mixture is vortexed. This mixture is added to the washed *C. elegans* and it remains at room temperature for 45 minutes. After this procedure, *C. elegans* is washed twice with M9 to remove the non-specific lipophilic dye. After washing, it is analyzed under a microscope (Leica DM6000 and Andor EMCCD Camera system) in a red filter at 20X.

2.2.2 Cross system

The crossing is done to add another mutant and/or transgenic to a mutant or transgenic according to the Mendelian principles. Hermaphrodite *C. elegans*, which can produce offspring by itself, ensures the continuity of the cross product. In this project, we generally produced single mutant *ceph-41(tur001)* with transgenic and double mutant with *ceph-41(tur001)* transgenic. The single mutant cep 41 with transgenic marker production is as follows. Firstly, male worms' mate with hermaphrodites expressing fluorescent markers. Next, males containing fluorescent markers pair with hermaphrodites containing the *ceph-41(tur001)* mutant. At the end of mating, fluorescent F1 hermaphrodites will be present/absent (+ / -) for the *ceph-41 (tur001)* mutant, and

these hermaphrodites will be left to produce offspring on their own. After 3 - 4 days at 20°C, 16 F2 worms containing fluorescence markers from these offspring are transferred to individual agar plates. After 2 days, the hermaphrodite worms in each Petri are tested by Polymerase Chain Reaction (PCR) to look at the genomic status of the *ceph-41(tur001)* mutant. The expected genomic probabilities for the *ceph-41(tur001)* mutant at step F2 are: +/+, +/-, -/-. The desired crossing product for *ceph-41(tur001)* is obtained by selecting the hermaphrodites with -/-.

2.2.3 CRISPR/Cas9

CRISPR (Clustered regularly spaced short palindromic repeat DNA sequences), was found by Dr. Nakata et al. in 1987 as a defense mechanism in bacteria in *E. coli* [95]. In 2013, Feng Zhang and colleagues developed the CRISPR-Cas9 technology to achieve double-strand breakage in the genome of mouse and human cells [96]. CRISPR-Cas9 is engineered with guide RNA, which has a complementary RNA in the target DNA sequence to attach and break to a particular double-strand DNA in the genome. The Cas9 enzyme follows the guide RNA to the relevant location in the DNA sequence and cuts both strands of DNA. At this stage, the cell realizes that the DNA has been damaged and tries to repair it. It can make changes in one or more of the genomes of the cell using the DNA repair mechanism.

The unique 3 different single guide RNAs (sgRNA) for knockout of specific genes in the genome were designed using the SYNTHOGO CRISPR tool (design.synthego.com) and ordered from Macrogen. In this experiment, an empty vector with a Kanamycin selection site and gRNA scaffold was selected for cloning the sgRNA (Addgene: # 59936). In the cloning process, the Golden Gate assembly technique has been applied to the clone. First, forward and reverse primers (1 µl each) were combined in a mixture of 2 µl 10x T4 ligase buffer (Thermo) and 6 µl ddH₂O; It is incubated at 95°C for 5 minutes, then allowed to cool slowly to room temperature using Thermo Scientific Thermocycler. 0.5 µl BsaI restriction enzyme, 0.5 µl T4 DNA ligase, 2 µl T4 DNA ligase buffer, 1 µg pRB1017 vector, and ddH₂O were added to the annealed primers until a total of 20 µl and this mixture was incubated for 1 hour at 37°C, 5 minutes at 50°C and 20 minutes at 65°C. The cloned plasmid was transferred to dh5 alpha strain of *E. coli* subjected to CaCl₂ treatment to induce competence to express. Competent cell and ligation products were incubated on ice for 30 minutes. Then the bacterias were incubated in a water bath at

42°C for 2 minutes and then incubated on ice for 2 minutes. The competent cell was then grown for 30 minutes at 37°C to express the protein. Finally, bacteria were transferred to agar plates containing kanamycin and incubated overnight at 37°C. With the colony PCR method, single colonies are checked whether sgRNA has been successfully added to the plasmid. selected single colonies are grown in LB broth at 37°C overnight. Plasmids containing SgRNAs were isolated using the TransGen EasyPure Plasmid MiniPrep kit.

2.2.4 Microscope analysis

For microscope analysis, 2% agarose solution is melted in water and its continuity is maintained in a 65 ° C water bath. 2% agarose is dripped onto the slide with a pipette, and a slide is covered on it. Gently press to avoid bubbles and leave to freeze. *C. elegans* transfer into a drop of anesthetic solution such as 1-2 ul 50 mM sodium azide or 1-2 ul 50/100 mM levamisole depends on the experiment. Then the cover slide is gently closed on it. Anesthetized worms stop moving after 1-2 minutes. These worms are placed on the microscope and are pictured with the fluorescent as a Z stack or movie.

Chapter 3

3. Result

3.1. *CEP41* is an evolutionarily conserved ciliary gene

The orthologs of *CEP41*, one of the 38 μ b genes, were found to be F42G8.19 in *C. elegans* by the blast. We also checked if *CEP41* is evolutionarily conserved in unicellular and multicellular organisms, by using multiple sequence alignment (MSA) for *cep41* gen with *H. sapiens*, *C. lupus*, *D. rerio*, *C. elegans*, *P. troglodytes*, *B taurus*, *M. mulatta*, *M musculus*, *R norvegicus*, *G. gallus*, *X. tropicalis*,. The protein sequence of human *CEP41* retrieved from NCBI detected the orthologues of this gene in the following species by BLASTp: *H. sapiens*, *G. gallus*, *P. troglodytes*, *M. mulatta*, *C. lupus*, *B taurus*, *M musculus*, *X. tropicalis*, *D. rerio*, *S.pharaonia*, *R norvegicus*, *C. elegans*. Here are orthologous protein sequences: *C_elegans* F42G8.19(NP_001294206.1), *H_sapiens* CEP41 (NP_061188.1), *P_troglodytes* TSGA14 (XP_001157373.1), *M_mulatta* TSGA14 (NP_001253767.1), *C_familiaris* CEP41 (XP_005628432.1), *B_taurus* CEP41 (NP_001179050.1), *M_musculus* Cep41 (NP_114387.1), *R_norvegicus* Cep41 (NP_001020941.1), *G_gallus* CEP41 (NP_001186322.1), *X_tropicalis* cep41 (NP_001016937.1), *D_rerio* cep41 (NP_001002194.1) We saw that *CEP41* is evolutionarily conserved in unicellular and multicellular organisms and has Rhodanese Like Domain, which is known as intracellular transport and regulatory pathway(Figure 3.1 A) [97].

We created a phylogenetic tree to calculate similarities for the *CEP41* gene between these organisms. Firstly, all protein sequences are loaded into the Geneious software (Geneious version 2020.2) to generate the consensus phylogenetic tree and remove it with Geneious Alignment, free-end gaps alignment type, and global alignment selection with other options. After the alignment was created, the Geneious Tree Builder tool was selected with Neighbor-Combine as the tree generation model and Jukes-Cantor as the genetic distance model with all default options (Figure 3.1 B).

Then the consensus support (%) to create the consensus tree, showing the percentage of the node inside. The scale bar represents the length of its branches in consensus as 0.125. To find conserved regions in all these orthologous sequences, the language classification tool InterPro (Version 5.46-81.0) for protein families is used. All protein sequences are individually loaded into the scanner to see predictable domain types and positioned sequences in the gene of interest. These data used in the Figure show similarity. Jalview version 2.11.1.3 is used to generate multiple sequence alignment (MSA) by using Clustal Omega (Figure 3.1 B). Consequently, the organism that most closely resembles the human CEP41 gene is TSGA14 gene in *P. troglodytes* and the most distant ones are *ceph-41* in *C. elegans* and CEP41 in *S. pharonis*

Since *CEP41* is one of the ciliopathy genes, it has been estimated that *CEP41* may have a role in cilia. The Xbox sequence is an evolutionarily conserved sequence found in all cilia genes. To prove the evolutionarily conserved *CEP41*'s relationship with cilia, we used known cilia genes, including *che-2*, *bbs-1*, *bbs-5*, *xbx-1*, *osm-5*, *osm-6*, to see if *cep41* had the Xbox sequence. We find that the *CEP41* is an Xbox sequence, as with other known cilia genes (Figure 3.1 C).

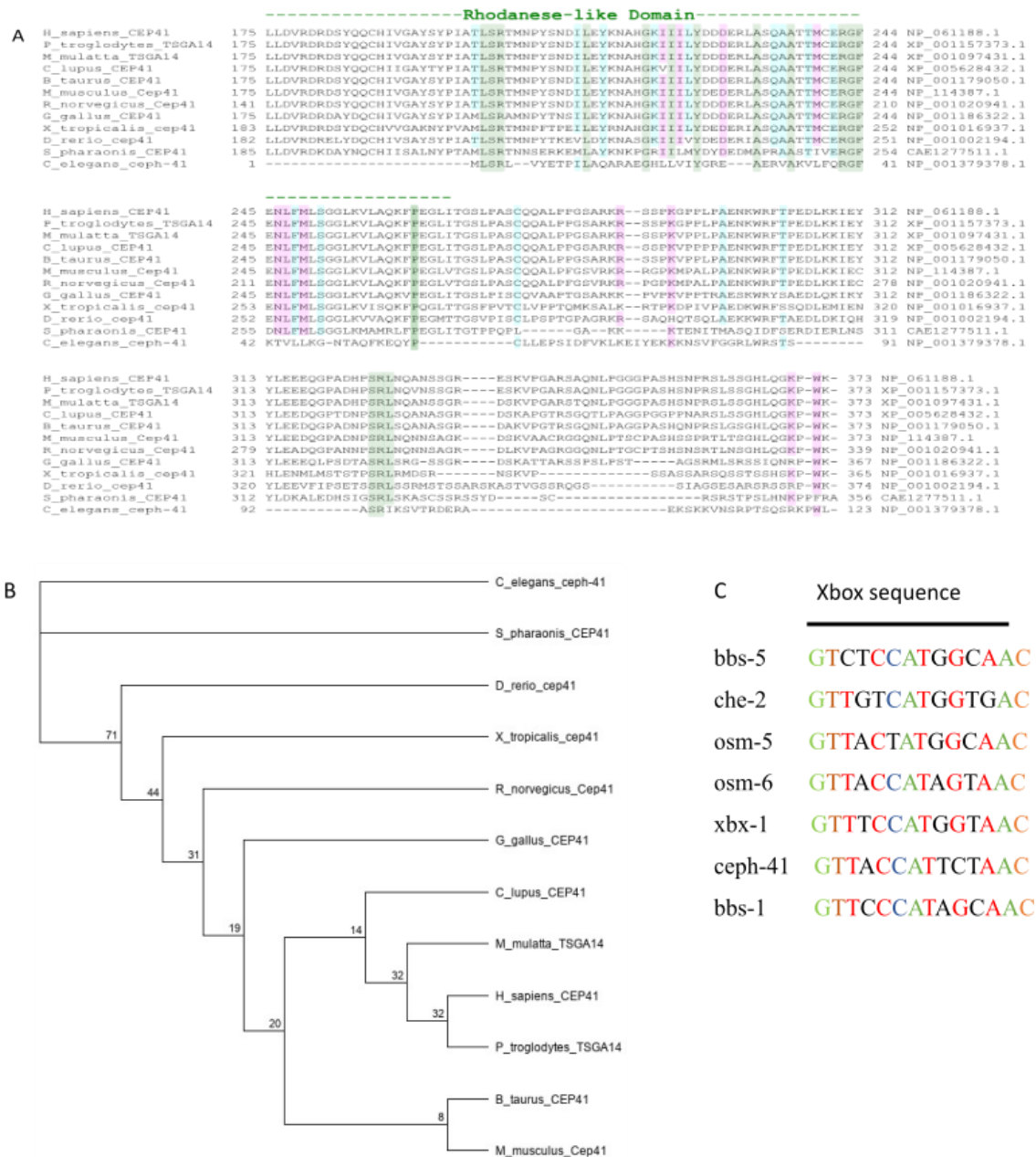


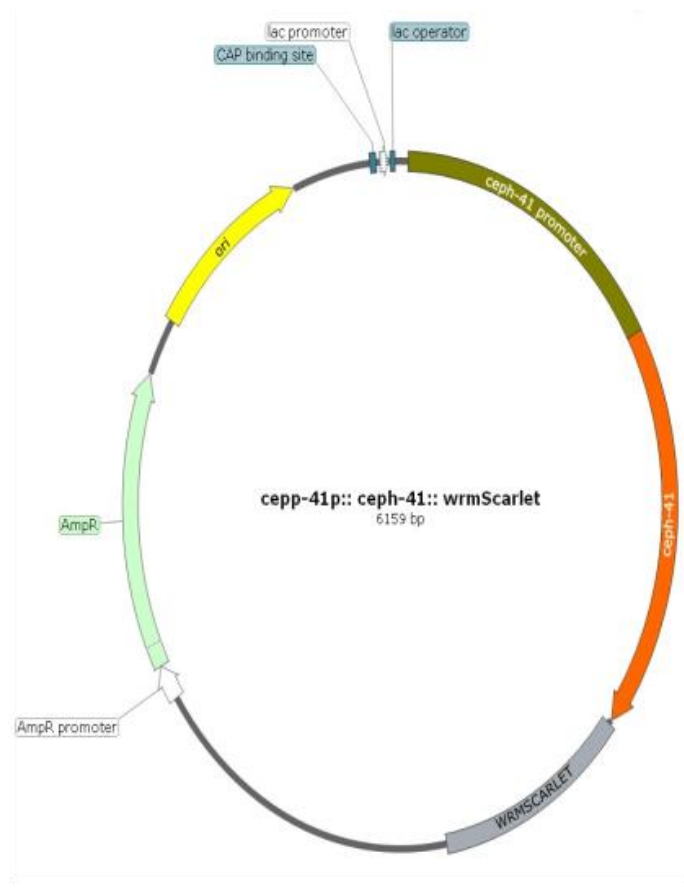
Figure 3.1 *CEP41* gene is evolutionarily conserved in unicellular and multicellular organisms.

A) Multiple sequence alignment (MSA) shows conserved amino acids colored according to the 50% conservation level (green represents fully preserved, pink represents 9 points preserved and blue represents 8 points). B) This data indicate similarity between organism for *CEP41* gene. C) Comparison of the Xbox sequence conserved in cilia genes with *ceph-41*.

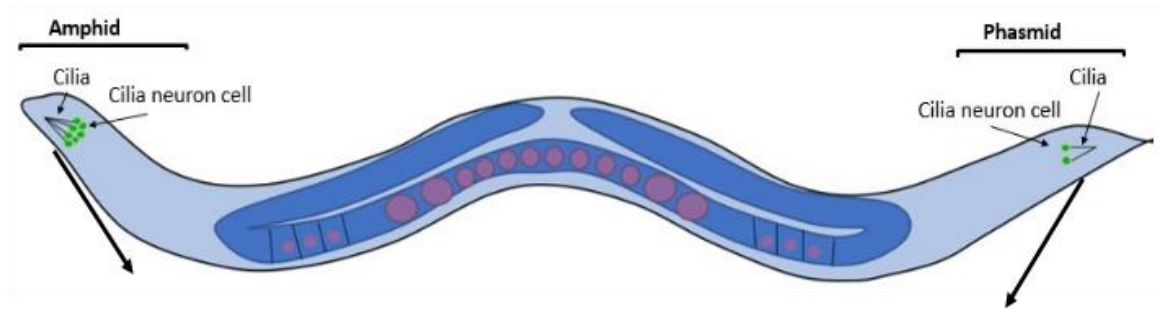
3.2. *CEPH-41* specifically localize to the middle segment of cilia in *C. elegans*

We generated a plasmid that contains the 1000 bp promoter of *ceph-41* and exons and introns of CEPH-41, *wrmScarlet* was added to the C terminus of CEPH-41 (Figure 3.2 A). The resulting plasmid *ceph-41p::CEPH-41::wrmScarlet* was then injected at 1 n/g into *C. elegans* to examine whether CEPH-41 is expressed in the ciliated sensory neurons. A number of independent transgenic strains expressing *ceph-41p::CEPH-41::wrmScarlet* were obtained, followed by confocal microscope analysis. Our confocal microscopy images revealed that CEPH-41 is exclusively expressed in the ciliated sensory neurons and it localizes to the cilia, but it does not localize to the entire cilium, instead it does not enter into the distal part of cilium (Figure 3.2 D). Similar to mammals, our study revealed that *C. elegans* CEPH-41 localizes to the cilium, indicating localization of CEPH-41 is evolutionary conserved.

A



B



C



D



Figure 3.2 *CEP41* in *C. elegans* is localized middle segment of cilia.

A) Schem of *ceph-41p::CEPH-41::wrmScarlet* plasmid. B) Schem of an adult hermafrodite *C. elegans*. C) Schem of amphid and phasmid cilia. D) *ceph-41p::CEPH-41::wrmScarlet* localized middle segment of cilia.

As a further investigation of localization of *CEPH-41* in cilia, we co-injected wrmScarlet tagged *CEPH-41* plasmid with GFP tagged *TMEM-145* plasmid. *TMEM-145* is a new ciliary protein discovered by our lab (unpublished data). Our data from our lab suggests that *TMEM-145* is a membrane protein that localizes in the middle segment of cilia in *C. elegans*. Therefore, we wanted to compare the exact localization of *CEPH-41* with *TMEM-145* gene. We generated two independent transgenic strains expressing [*ceph-41pCEPH-41::wrmScarlet* + *tmem-145::TMEM-145::GFP*], and we imaged both of them with the confocal microscope. Our confocal microscopy images revealed that the localization of *CEPH-41* is restricted to the middle segment of cilia (Figure 3.3).

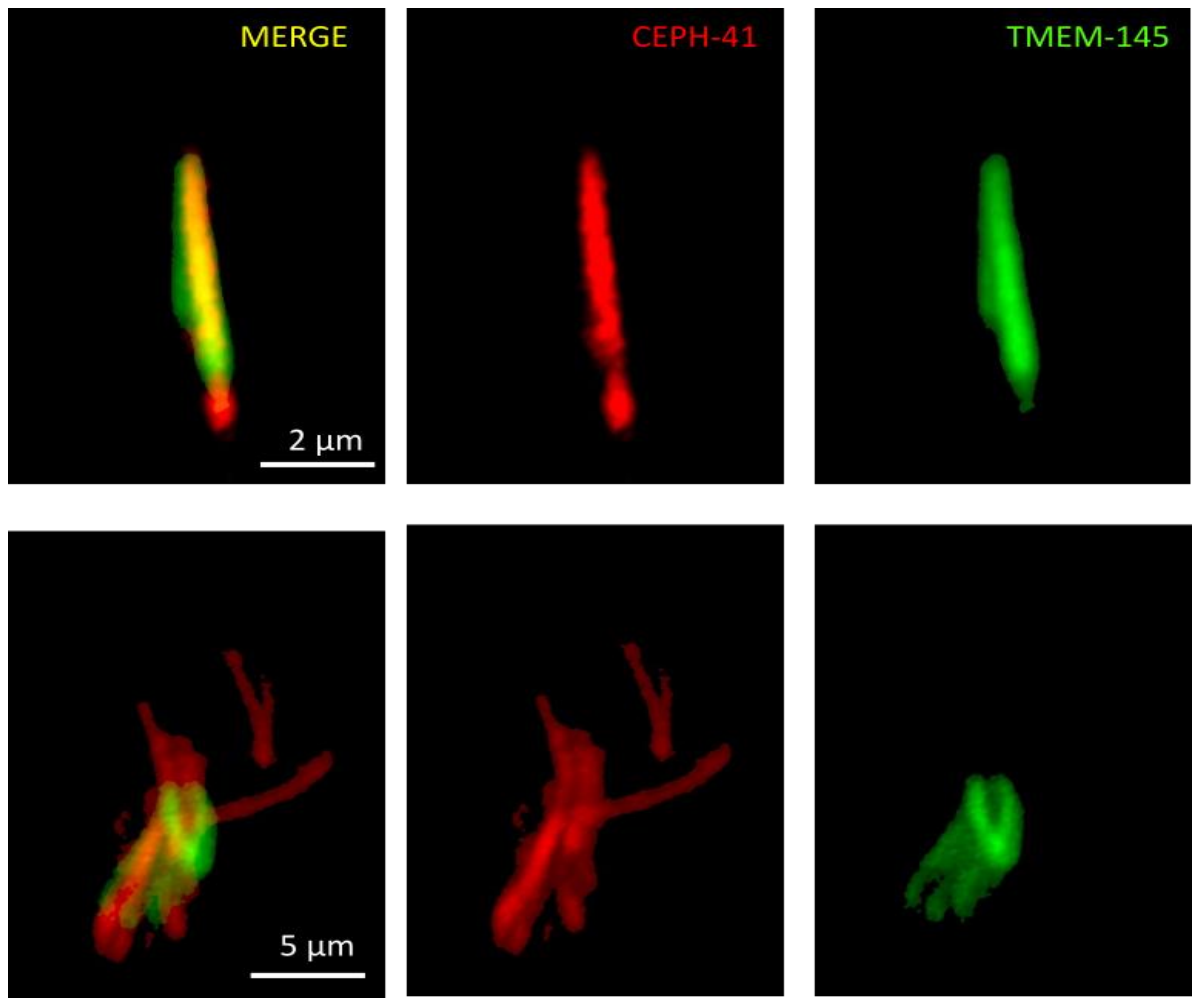


Figure 3.3 [*ceph-41pCEPH-41::wrmScarlet* + *tmem-145::TMEM-145::GFP*] colocalization in middle segment of cilia

Localization of [*ceph-41pCEPH-41::wrmScarlet* + *tmem-145::TMEM-145::GFP*] in amphid and phasmid cilia.

3.3. The null *ceph-41* mutant does not possess the ciliary structure defect

Having established that CEPH-41 is a ciliary protein in *C. elegans*, we next wanted to investigate the role of *ceph-41* gene in the ciliogenesis initiation and defining cilia morphology. Before I joined the Kaplan lab, the lab already generated a null *ceph-41* mutant, which removes the entire gene including all exons and introns (1173bp), using CRISPR/Cas9 (Figure 3.4 A).

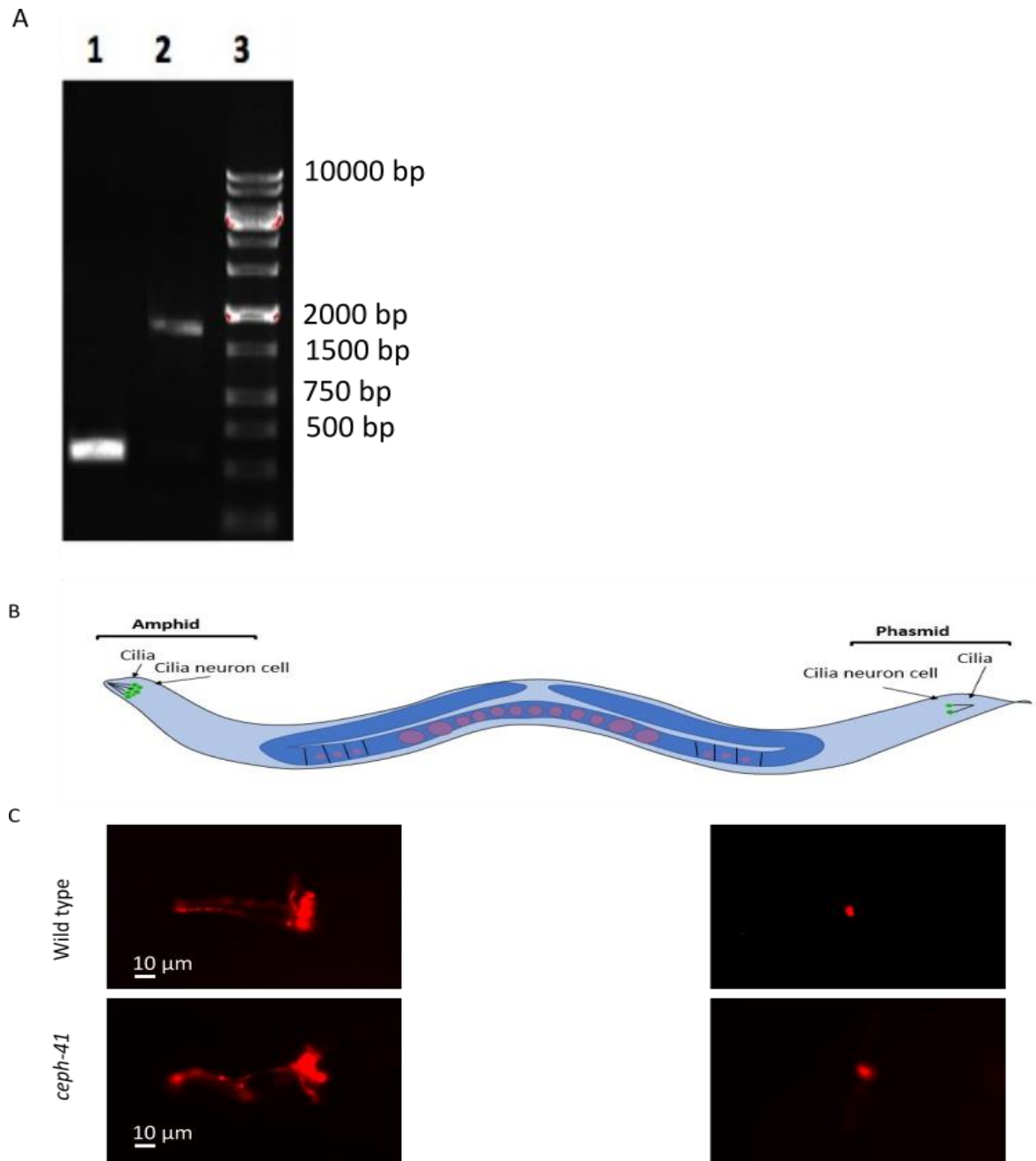


Figure 3.4 *ceph-41* did not affect sutructure of cilia.

A) A non-allelic mutant of *ceph-41* has a deletion of 1790 bp. B) Shape of *C. elegans*. C) Dye fill test was examined for wild type and *ceph-41*.

With this null mutant, we next examined whether the *ceph-41(tur001)* influenced the structure of the cilia. We performed the dye filling assay, and this assay is one of most basic techniques that can be used to assess the cilia structure. In the dye filling assay, we included wild type as a control, followed by imaging fluorecence microscope, and wild

type always fills up their ciliated cells with red fluorescence dye via their cilia. We found no dye filling defects in *ceph-41(tur001)* mutants as compared to the wild type, suggesting that there is no major defect in the formation of amphid and phasmid cilia in *ceph-41(tur001)* mutants (Figure 3.4 C).

We next wanted to image individual cilia of *ceph-41(tur001)* mutants in *C. elegans* because maybe there might be some subtle defect in *ceph-41(tur001)* mutants. Fluorescent markers for ASE, AWA, AWB sensory nerve cells in the amphid and PHA/PHB sensory nerve cells in the phasmid provide information about the cilia structure in addition to the fluorescent staining experiment. To investigate the structure of the *ceph-41* mutant, we used the following fluorescence markers: AWB (*str-1*), AWC (*odr-10*), ASE (*gcy-5*), and PHA / PHB (*srb-6*) sensory nerve cells. Here are the transgenic strains: N2; [*odr-10p::odr-10:gfp*], N2; ntIs1[*gcy-5p::gfp+ lin-15(+)*], N2;*gmIs13[srb-6pr::gfp+rfp4]*, [*str-1p::gfp*]. We genetically crossed these markers into *ceph-41(tur001)* mutants and confirmed them with genotyping (PCR base strategy). We obtained *ceph-41*; [*odr-10p::odr-10:gfp*], *ceph-41*;*[str-1p::gfp]*, *ceph-41*; NTIS1 [*gcy-5p::gfp+ lin-15(+)*], *ceph-41*;*gmIs13[srb-6pr::gfp+rfp4]*. As a result of microscopic analysis, we realized that the cilia structure in the *ceph-41* mutant was not different from the wild type. Our data shows that *ceph-41* does not affect the structure of the cilia in *C. elegans* ((Figure 3.5).

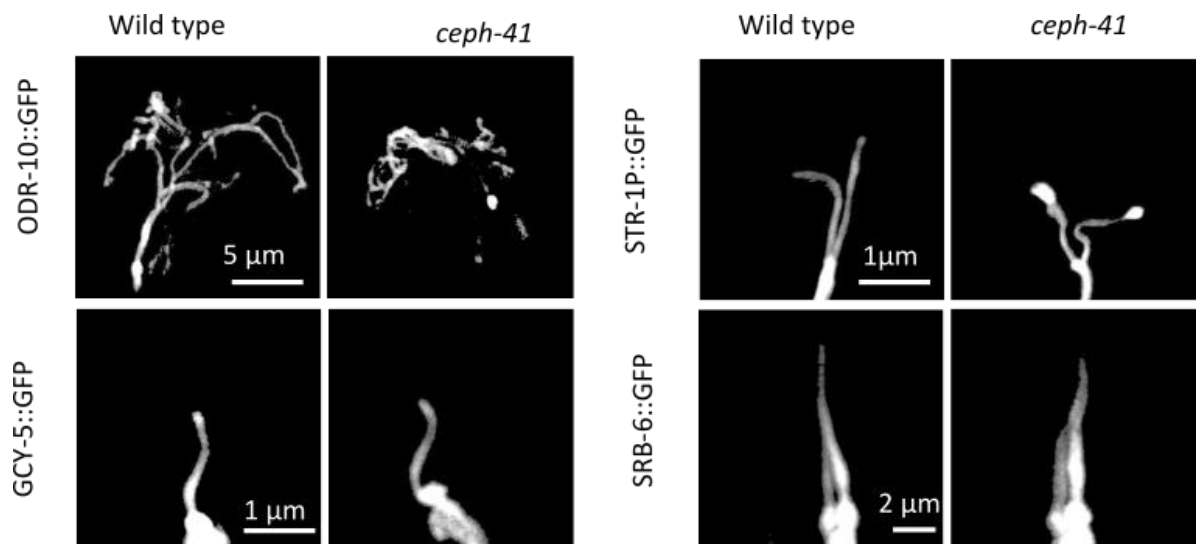


Figure 3.5 *ceph-41* did not affect AWB, AWC, ASE, and PHA/PHB cilia structure. N2;*[odr-10p::odr-10:gfp]*, N2;*ntIs1[gcy-5p::gfp+ lin-15(+)]*, N2;*gmIs13[srb-6pr::gfp+rfp4]*, N2;*[str-1p::gfp]* and *ceph-41*;*[odr-10p::odr-10:gfp]*, *ceph-41*;*[str-*

1p::gfp], *ceph-41*; NTIS1 [*gcy-5p::gfp+ lin-15(+)*], *ceph-41*; *gmIs13[srb-6pr::gfp+rfp4]* are analyzed.

3.4. Investigation of Localization of IFT Proteins in

***CEPH-41* Mutant**

We next examined the IFT protein localization in *ceph-41(tur001)*, and we crossed fluorescence tagged IFT proteins, including N2; Is [OSM6::GFP], N2; *casIs586* [KAP-1::GFP], N2; [OSM-3::GFP+PRF4], N2; *jhuEx* [CHE-11::GFP+pRF4] into *ceph-41(tur001)*. Because maybe *ceph-41(tur001)* may affect the localization of IFT proteins. We used the confocal microscopy to image IFT-A (*che-11*) protein, IFT B (*osm-6*), one heterodimeric kinesin protein (*kap-1*), and one homodimeric kinesin protein (*osm-3*). Confocal microscopic examination of the wild type and *ceph-41* mutant expressing GFP labelled IFT proteins markers revealed that depletion of *ceph-41* did not impair the localization of IFT proteins and kinesin motors ((Figure 3.6).

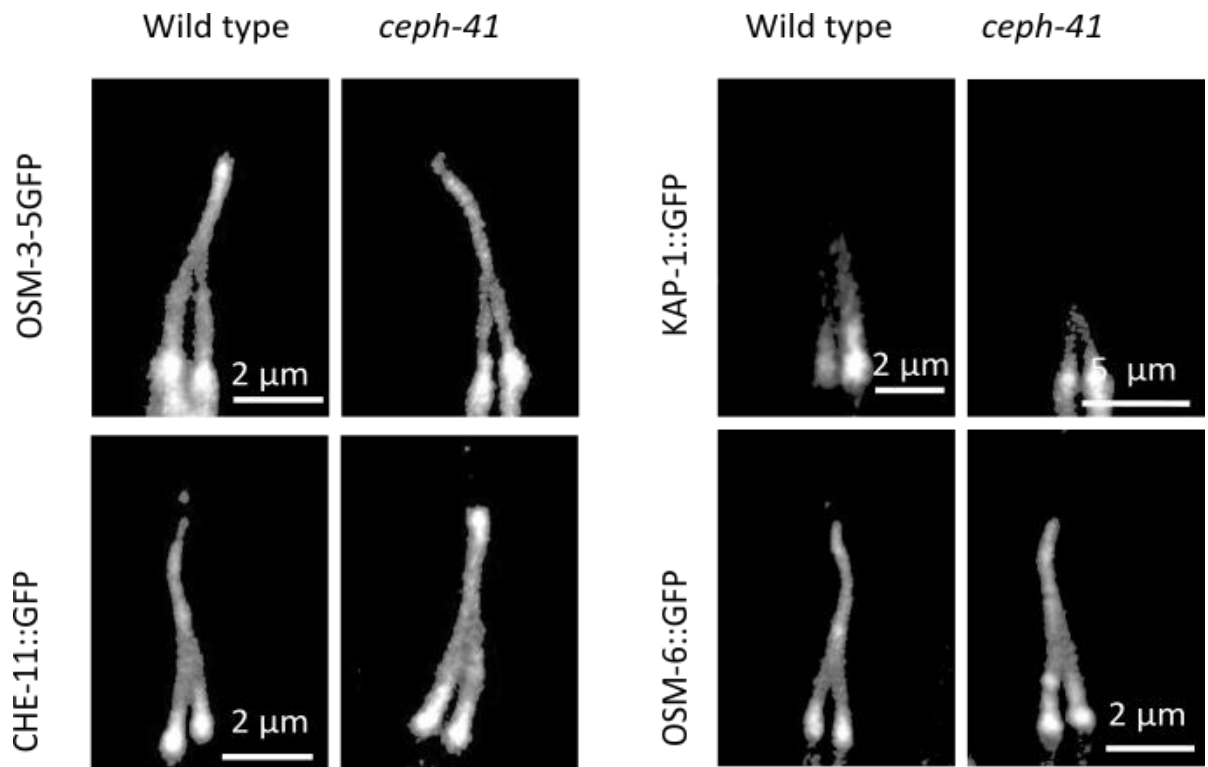


Figure 3.6 *ceph-41* did not affect IFT protein localization.

ceph-41(tur001) mutant crossed with N2; Is [OSM6::GFP], N2;casIs586 [KAP-1::GFP], N2; [OSM-3::GFP+PRF4], N2; jhuEx [CHE-11::GFP+pRF4] to analyzed IFT protein localization.

3.5. *CEP41* mutation does not affect IFT

Given that *ceph-41* mutants do not display defects in cilia structure and IFT protein localization, we proposed that another gene may compensate for the loss of *ceph-41*. We therefore generated a number of double mutants with *ceph-41*, including *ceph-41; bbs-5*. We crossed N2; [*osm-6::gfp*] (IFT protein) into *bbs-5*, and *ceph-41;bbs-5* mutants. We used the confocal microscopy to investigate the cilia structure in *bbs-5*, and *ceph-41; bbs-5*, and found that the length of cilia and IFT protein localization remain unchanged in *ceph-41; bbs-5* double mutants, suggesting that no synthetic genetic interaction between these two genes ((Figure 3.7 A).

When we calculate speed of the protein transport with the *osm6* gene during protein transport, no effect of *ceph-41* was observed in single *ceph-41* and double *ceph-41; bbs-*

5 mutants (Figure 3.7 B). Likewise, when we look at the length of the cilia, we saw that the *ceph-41* does not affect the length of the cilia (Figure 3.7 C).

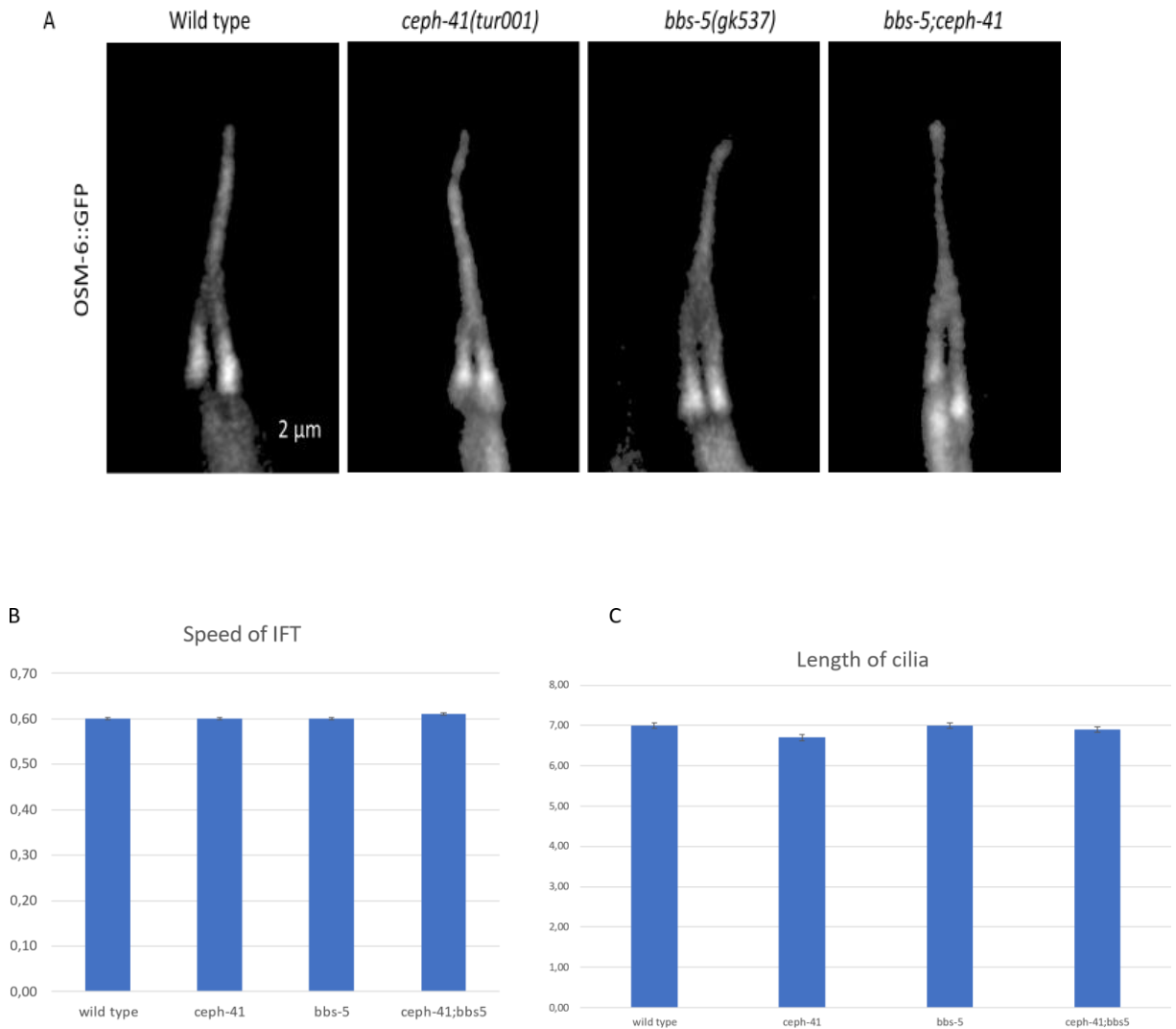


Figure 3.7 *ceph-41* double mutant did not affect IFT protein localization and length of the cilia.

A) For further genetic research of A *ceph-41*, we obtained the double mutant with *bbs-5*. B) *ceph-41* did not affect IFT protein localization and speed of IFT. C) *ceph-41* was found to not affect cilia length

Chapter 4

4. Discussion

Joubert syndrome is a ciliopathy disease that affects about one in every 100,000 people. It is also categorized as a neurodegenerative condition since it causes developmental problems in the cerebellum and brainstem in JS sufferers. Symptoms of abnormal eye movements, fast breathing, delayed intellectual and language development, hypotonia, ataxia, mental retardation, liver cyst, retinal defect/degeneration, genital defect, and cystic kidney might be present in JS patients. [44]. There are now 38 genes known to cause Joubert syndrome, and these genes are given in Table 1 as Human Gene Name, OMIM Gene ID, Ortholog Gene Name, and Wormbase ID. CEP41 is one of the 38 genes that cause JS.

Cells extend an antenna-like organelle called cilia, which is made up of microtubules. The structure consisting of microtubules surrounded by a membrane is called an axoneme. [10] Extending from virtually any cell surface, cilia can act as a sensor and allow the cell to regulate embryo development and move by harboring signaling pathways. Cilia receptors (such as rhodopsin) localized on the ciliary membrane surface allow the environment to be perceived and the cell to respond to them. The transition zone and the basal body of the cilia controlling the entrance and exit of proteins into the cilia are located at the + end of the cilia [26]. The evolutionarily conserved *CEP41* gene encoding the 41 kilo-Dalton protein was found to cause JS and was the first and only study to show the link in 2012 [58]. In that study, *CEP41* was found to be localized in cilia and JS-like abnormalities were shown in the generated mouse and zebrafish JS models. In 2020, loss of CEP41 was reported by Ki et al to have caused vascular impairment in zebrafish and human cell lines and thought that CEP41 could have pro-angiogenic roles [100]. They discovered that CEP41 is necessary for tubulin glutamylation during cilia disassembly and has a function in endothelial cell dynamics like tubulogenesis and migration. However, the molecular mechanism by which CEP41 causes JS is not explained in those studies.

Our lab was interested to understand to the functions of CEP41 in cilia. Our lab uses *C. elegans* as a model system to study cilia biogenesis, and my supervisor identified the *C. elegans* orthologue of CEP41 using BLASP analysis. *C. elegans* F42G8.19 name emerged as the ortholog of human CEP41 and was named CEPH-41. I performed multiple sequence alignment (MSA) of CEP41 protein sequences from a range of organisms, such as *H. sapiens*, *C. lupus*, *D. rerio*, *C. elegans*, *P. troglodytes*, *B. taurus*, *S. pharaonia*, *M. musculus*, *R. norvegicus*, *G. gallus*, *X. tropicalis*, *M. mulatta*, by using multiple sequence alignment (MSA). (Figure 3.1 A). As a result, CEP41 seems to be an evolutionarily conserved gene, and has a rhodanese like domain known to play a role in intracellular transport. A phylogenetic tree was created to calculate its similarity of the *CEP41* gene to *H. sapiens*, *C. lupus*, *D. rerio*, *C. elegans*, *P. troglodytes*, *B. taurus*, *S. pharaonia*, *M. musculus*, *R. norvegicus*, *G. gallus*, *X. tropicalis*, and *M. mulatta*. As a result, *P. troglodytes* most closely resembles the human CEP41 gene, and the most distant ones are *C. elegans* and *S. pharonis*. (Figure 3.1B). My supervisor discovered the presence of potential X-box motif sequence in the promoter of *ceph-41*. The X-box motif present in the many ciliary genes may indicate that this may be expressed in the ciliated cells, and the presence of a possible X-box motif suggests that it is likely expressed in the ciliated sensory neurons (Figure 3.1 C).

To look at the specific localization of the *CEP41*, *ceph-41p:: CEPH-41:: wrmScarlet* plasmid was designed (Figure 3.2 A). As a result of the analysis, it was determined that *ceph-41* was localized specifically in the middle segment of the cilia (Figure 3.2 D). For further investigation, *ceph-41p:: CEPH-41:: wrmScarlet* is co-injected *tmem-145p::TMEM-145 :: GFP* localized in the middle segment, and [*ceph-41p:: CEPH-41:: wrmScarlet* + *tmem-145p::TMEM-145 :: GFP*] was obtained. In the analysis of [*ceph-41p:: CEPH-41:: wrmScarlet*+ *tmem-145p::TMEM-145 :: GFP*], *ceph-41* was conclusively proven to be localized in the middle segment (Figure 3.3).

To examine the *ceph-41* (tur001) mutant how it causes JS, we used the *ceph-41* mutant, which was created in our laboratory and created by the CRISPR / Cas9 technique. PCR results showed that while the wild type was 1790bp, *ceph-41* (tur001) remained 617 bp (Figure 3.4 A). We performed the dye filing assay to examine the cilia structure. As a result, when we compared the wild type (positive control) and the *ceph-41* mutant, we did not find any structural defects in cilia (Figure 3.4 C)

To better examine the effect of *ceph-41* on cilia structure, GFP labeled sensory cilia genes (AWB (*str-1*), AWC (*str-2*), AWA (*str-1*), ASE (*gcy-5*) and PHA / PHB (*srb-6*) We looked at the localization in the *ceph-41* (tur001) mutant. We found that the *ceph-41* (tur001) mutant had no effect on the hair structure (Figure 3.5).

To analyze the effect of *ceph-41* (tur001) mutant on IFT protein localization, we used GFP-tagged IFT-A (*che-11*) protein, IFT B (*osm-6*), one heterodimeric kinesin protein (*kap-1*), and one homodimeric kinesin protein (*osm-3*). When the *ceph-41* mutant and wild type were compared, no defects in IFT protein localizations were observed (Figure 3.6).

We suggested that in the *ceph-41* mutants, another gene may compensate for the loss of *ceph-41*, since the single *ceph-41* mutants have no effect on protein localization and cilia structure. Therefore, by using the [*osm-6::gfp*] (IFT protein) marker, we generated a double mutant with *ceph-41*, including *bbs-5*. We found that IFT protein localization and cilia length remained unchanged in *ceph-41; bbs-5* double mutants (Figure 3.7 B and C). There is no synthetic genetic interaction between these two genes (Figure 3.7 A).

Chapter 5

5. Conclusions and Future Prospects

5.1 Conclusions

In this study, we tried to understand the molecular mechanism of the *CEP41* gene that causes JS. We found that *CEP41* is evolutionarily conserved and has a Rhodanese Like Domain. It was found by the dye filing assay that the *ceph-41* mutant produced by CRISPR / Cas9 did not affect the cilia structure in *C. elegans*. Furthermore, the structure of AWB, AWC, AWA, ASE, and PHA / PHB cilia was examined in the *ceph-41(tur001)* mutant, no structural defect was found. There were no defects in the localization of the IFT proteins examined in the *ceph-41(tur001)* mutant. When we examine the effect of *ceph-41* on the localization of IFT proteins by creating mutants with other genes, it was found that *ceph-41* has no effect.

5.2 Social Impact and Contribution to Global Sustainability

Joubert syndrome is an inherited disease with an autosomal recessive character. Because this syndrome occurs in one in 100,000 births, it is classified as a rare disease. Patients did not develop an area called "vermis" that runs through the middle of the cerebellum and controls balance and coordination. Deformation occurs in the brainstem that controls breathing and swallowing. Since these areas are affected, abnormal breathing is observed in patients from childhood. The group of diseases that cause structural and functional impairments in cilia, including Joubert syndrome, is called "ciliopathy". Motile cilia are involved in the removal of mucus from the respiratory tract and the movement of the cell in the sperm. Nodal cilia in mammalian embryos play a role in correct left-right asymmetry in embryo development. Immobile eyelashes, which are

found on almost all cell surfaces but seen as an unusable structure for a long time, exhibit more mechanical, sensory, and chemical functions. Ciliopathy is a rare and orphan disease, so there is no cure for the patient. We want to contribute to this by investigating the molecular mechanism of the *CEP41* gene, which is one of the 38 genes that cause Joubert syndrome. JS, which shows delayed intellectual and language development, hypotonia, ataxia, mental retardation, liver cyst, retinal defect / degeneration, genital defect, and cystic kidney symptoms in patients, causes many difficulties and reduces the quality of human life.

CEP41, encoding 41 kilodalton protein (kDa), was found to be associated with Joubert syndrome in 2012 [58]. Since Joubert syndrome is a disease associated with cilia, it was predicted that *CEP41* may have a role in cilia, but the study of this gene's function in cilia was limited to the 2012 study. Using the *C elegans* model as an organism, we found that the *ceph-41* gene localized specifically the middle segment of cilia. In our studies, we found that the *ceph-41* mutant in *C elegans* did not cause any structural defects. Also, we tried to understand the role of cilia by doing functional analysis.

5.3 Future Prospects

Since the function of cilia is a special for cell structure, protein entry into cilia is tightly controlled. JS genes *TMEM67*, *RPGRIP1L*, *CC2D2A*, *TCTN1* are involved in this construct that controls protein entry into cilia in the "transition region". In the absence of one of these genes, *TRAM1A* (transmembrane protein) and retinitis pigmentosa 2 (*RP2*), which were not previously localized to the cilia, may enter the cilia [86]. Transgenics expressing N2 [*RPI-2 :: GFP*] and N2 [*TRAM1A :: tdTomato*] will be crossed with *ceph-41(tur001)* to investigate the function of *ceph-41* in the transition zone.

To investigate whether *CEP41* is affected in the transition zone and controls protein entry into cilia, a search can be made with 2 different protein complexes: the NPH module and the MKS module. While the falsoirst protein complex contains the *NPHP1* and *NPHP4* proteins, this complex is called the NPH module. The other protein complex has been found to contain the proteins *TMEM17*, *B9D1 / MKSR1*, *B9D2 / MKSR2*, *CC2D2A / MKS6*, *TCTN1*, and *TMEM231*, and this complex is called the MKS module [86].

Also, it will investigate whether *ceph-41* affects the localization of TTL enzymes and affects CCP5 enzymes and causes tubulin glutamate dysfunction.

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APPENDIX

Detailed List of Materials/Instruments Used in Experiments

Material / Reagent / Instrument / Software	Company	Order number / Model
Compound microscope	LEICA	LEICA DM6 B
Andor iXon Ultra EMCCD	ANDOR	253.3.5/16/14996
Andor iQ 3.6.2 Software	ANDOR	
ZEISS LSM 900 with Airyscan 2	ZEISS	
ZEISS ZEN 3.0 (blue edition)	ZEISS	
Carl Zeiss microscope	CARL ZEISS	Axio Vert.A1
Pressure Supply Port	NARISHIGE	IM-400
Oil Hydraulic Micromanipulator	NARISHIGE	MMO-4
Heater	NARISHIGE	PC-100
Pure nitrogen tank	Gazsan	GA-K2099096
Fluorescence stereo microscope	LEICA	LEICA M205 FA
Stereo microscopes	LEICA	LEICA S9I
Cooled incubator	Panasonic	MIR-554-PA
Ultra-Low Temperature (UTL) freezer	Haier	DW-86L62B
Autoclave (steam sterilizer)	Tuttnauer	3850ELC-D
	Nüve steam Art	OT 90L
Laminar flow hood	Nucleon Laboratory Instruments	Class II Biosafety cabinet
PCR thermal cyclers	Thermo Fisher Scientific	ProFlex PCR System

	Bio-Rad	C1000 Touch Thermal Cycler
Waterbath	Thermo Scientific™	Precision™GMP 02
	Thermo Scientific™	Precision™ GP 10
Electrophoresis	Thermo Scientific™	Owl EasyCast™ B2
	Thermo Scientific™	Owl EasyCast™ B1
	Bio-Rad	Sub-Cell Model 96
Molecular Imager®	Bio-Rad	Gel Doc™ XR+ System
Spectrophotometer	Thermo Scientific™	NanoDrop™ 2000
Centrifuge	Thermo Scientific™	MicroCL 21R Microcentrifuge
	Thermo Scientific™	MicroCL 21 Microcentrifuge
Micro centrifuges	GYROZEN	KGZS23518120872
Electronic balance	Precisa	LS 1200C SCS
	SHIMADZU	ATX 224
Vortex mixer	Stuart Biocote	SA8
Magnetic stirrers	Heidolph	MR HEI-TEC
Microwave oven	Vestel	MD 20 MB
Ice system	Scotsman	AF 80 AS 230/50/1
Water purification system	Merck	ZRQSV800
Agar-Agar, Kobe I	CARL ROTH	5210.2 - 1 kg
Bacto™ Peptone	BD Bioscience™	211677 - 500 gr
Sodium chloride (NaCl)	ISOLAB	969.033.1000 - 1 kg
Cholesterin	CARL ROTH	8866.1 - 100 gr
Nystatin	RPI (Research Products International)	N82020-10.0
MgSO4.7H2O	CARLO ERBA REAGENTS	10034-99-8
CaCl2	CARLO ERBA REAGENTS	10043-52-4
KH2PO4	Merck	104873.1000 - 1 kg
K2HPO4	Merck	105101.1000 - 1 kg
LB Broth, Miller Formulation	VWR Life Science	J106 - 1 kg
Proteinase K	Sigma-ALDRICH	SLBQ1035V
KCl	Merck	7447-40-7
Tris base	Sigma-ALDRICH	T1503 - 1 kg

MgCl ₂	Merck	7786-30-3
10X Easy Taq buffer	TRANSBIO	N21106
High Pure dNTPs	TRANSBIO	AD101-02
Primers	MACROGEN	
Easy Taq DNA polymerase	TRANSBIO	AP111-01
Ultra pure water	Tekkim Kimya	TK.911010.0
Agarose	Prona Agarose Biomax	D00216PR
Ethidium bromide	BioShop	ETB444.1
Tris base	Sigma-ALDRICH	T1503 - 1 kg
Glacial acetic acid	ISOLAB	64-19-7
EDTA	CARLO ERBA REAGENTS	6381-92-6
100bp Plus II DNA ladder	TRANSBIO	BM321-01
6X Loading buffer	TRANSBIO	GH101-01
Bromophenol blue	AMRESCO	115-39-9
Immersion oil	Sigma-ALDRICH	56822 - 50 mL
Sodium azide	SERVA	30175.01
Halocarbon oil	Sigma-ALDRICH	H8898 - 50 mL
HEPES	BioShop	7365-45-9
Ethanol	ALKOKİM	01012018-IR.01
Injection needle	WPI	TW100F-4
Sterile syringe (0.5mL)	AYSET Tıbbi Ürünler	KD8354-00-10/17
Sterile syringe (10mL)	Helmed	20160802
Sterile filter unit with MF-Millipore, 0.22 µm	Millex® Syringe Filters	SLGS033SS
Microscope slide	ISOLAB	I.075.05.003
Cover glass	ISOLAB	075.00.004
Parafilm	Bemis	PM-996
Pipettes 0.2-2 µl 1-10 µl 2-20 µl 10-100 µl 20-200 µl 100-1000 µl"	Thermo Scientific	PH79581 JH97441 PH79581 JH97441 JH95162 JH95573
Pipettes 10-100 µl 100-1000 µl	SCIOLOGEX	YM5D071264 YM5G082883

Pipettes 0.5-10 µl 2-20 µl 10-100 µl	NICHIRYO Nichepet EX II	J15809081 J16317571 J16101431
Multi-channel pipettes 1-10 µl 10-100 µl	Thermo Scientific	OH22524 LJ02605
Pipette tips 10 µl 200 µl 10000 µl	ISOLAB ISOLAB Biosigma	005.01.001 005.01.002 17A0845
S1 pipet filler	Thermo Scientific	187550
Serological pipettes 10 mL 25 mL	Biosigma	N403346 N403347
Individual tubes (0.2 mL)	Thermo Scientific	AB-0620
Cryogen tube (2 mL)	Biosigma	CL2ARBEPSTS
Eppendorf tubes 1.5 ml 2 ml	LAMTEK ISOLAB	LT1003098 MTPPN6020008
Centrifuge tubes 15 mL 50 mL	ISOLAB ISOLAB	CTPPA7015002 CTPPA9050002
Petri dishes 60*15 mm 90*15 mm	FIRATMED FIRATMED	8870000046 8870000011
Weighing boats; 30 mL 100 mL	ISOLAB	WBPSN7030001 WBPSN7100001

CURRICULUM VITAE

2013 – 2017 Molecular Biology and Genetic, Necmettin ERBAKAN Universty,
Konya, TURKEY

2017 – 2021 Present M.Sc., Bioengineering, Abdullah GÜL Universty, Kayseri,
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SELECTED PUBLICATIONS AND PRESENTATIONS

There is no publication and presentation.