Fatma Nihal Yetgin

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CHARACTERIZATION OF DISEASE GENE

M.Sc. 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
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ABSTRACT

CHARACTERIZATION OF DISEASE GENE

Fatma Nihal Yetgin
MSc. in Bioengineering
Advisor: Assoc. Prof. Oktay İsmail Kaplan
January 2025

Being sick is part of human life. Some disease can are common, while others are rare. There are more than 7000 rare diseases in present and is counting. Ciliopathies are one of the rare diseases. Ciliopathies are diseases caused by mutations that affect the function or structure of cilia. Cilia are organelles composed of many compartments that extend outward from the cell and affect some important signalling pathways, such as the Hedgehog signaling pathways.

In 2014, the relationship between EFCAB7 and EVC-EVC2 proteins was discovered. Mutations in the EVC and EVC2 genes cause Ellis van Creveld disease. In 2023, it was found that it causes non-syndromic postaxial polydactyly. However, the relationship between EFCAB7 and cilia has not been sufficiently elucidated. In this study, it was investigated relationship between EFCAB7 and cilia using microscopic methods and functional assays.

Our results indicated that cilia in efcab-7 mutants were significantly shorter than those in the wild type, whereas IFT velocity and particle number did not change noticeably. Moreover, ELMOD-3, which under normal conditions does not enter cilia, remained excluded in efcab-7 mutants and thus reported an intact ciliary gate. Unexpectedly, efcab-7 mutants also displayed attenuated motility and reduced axon number, suggesting an additional function of EFCAB7 in neuronal or muscle function. These findings expand the spectrum of EFCAB7 functions in maintaining ciliary and neuronal integrity and provide new insights into the pathogenesis of its associated rare diseases.

Keywords: Cilia, Ciliopathy, EFCAB7, Rare Disease

ÖZET

HASTALIK GENİ KARAKTERİZASYONU

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Hasta olmak insan yaşamının bir parçasıdır. Bazı hastalıklara sık rastlanırken bazı hastalıklar nadirdir. Günümüzde 7000den fazla nadir hastalık vardır ve sayısı artmaktadır. Silyopatiler de nadir hastalıklardan biridir. Silyopatiler, silyanın fonksiyonunu ya da yapısını etkileyen mutasyonlar sonucu oluşan hastalıklardır. Silya birçok kompartmandan oluşan, hücreden dışarıya doğru uzanan bir organeldir ve Hedgehog sinyal yolağı gibi bazı önemli sinyal yolaklarını etkiler.

2014 yılında EFCAB7'in EVC ve EVC2 proteinleri ile ilişkisi bulunmuştur. EVC ve EVC2 genlerindeki mutasyonlar Ellis van Creveld hastalığına sebep olmaktadır. 2023'de ise sendromik olmayan postaksial polidaktiliye sebep olduğu keşfedilmiştir. Ancak EFCAB7 ile silya arasındaki ilişki yeterince aydınlatılamamıştır. Bu çalışmada, mikroskobik yöntemler ve fonksiyonel deneylerle EFCAB7 ile cilia arasındaki ilişki incelenmiştir.

Sonuçlarımız, efcab-7 mutantlarının silialarının vahşi tipe kıyasla önemli ölçüde daha kısa olduğunu, buna karşın IFT (intraflagellar transport) hızı ve partikül sayısında belirgin bir değişiklik olmadığını göstermiştir. Ayrıca, normal koşullarda silyaya girmeyen ELMOD-3 proteini, efcab-7 mutantlarında da silyaya giriş yapmamış ve bu durum silial geçidin sağlam olduğunu ortaya koymuştur. Beklenmedik bir şekilde, efcab-7 mutantlarının hareket kabiliyetinin azaldığı ve akson sayısının da azaldığı gözlemlenmiştir, bu da EFCAB7'nin nöronal veya kas fonksiyonunda ek bir rolü olabileceğini düşündürmektedir. Bu bulgular, EFCAB7'nin silya ve nöronal bütünlüğün korunmasındaki fonksiyonlarını genişletmekte ve ilişkili nadir hastalıkların patogenezi hakkında yeni bilgiler sağlamaktadır.

Anahtar kelimeler: Silya, Silyopati, EFCAB7, Nadir Hastalık

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LIST OF ABBREVIATIONS

ADPKD	Autosomal Dominant PKD
ARPKD	Autosomal Recessive PKD
BBS	Bardet-Biedl Syndrome
CNS	Central Nervous System
DMD	Duchenne Muscular Dystrophy
EFCAB7	EF-hand calcium binding domain 7
EVC	Ellis van Creveld
GO	Gene Ontology
Hh	Hedgehog
IFT	Intraflagellar Transport
MKS	Meckel-Gruber Syndrome
MTS	Molar Tooth Sign
MRI	Magnetic Resonance Imaging
PKHD1	Polycystic Kidney and Hepatic Disease 1
PKD	Polycystic Kidney Disease
PC1	Polycystin-1
PC2	Polycystin-1
PCD	Primary Ciliary Dyskinesia Syndrome
Shh	Sonic Hedgehog
SCGS	SYSCILIA Gold Standard
TZ	Transition Zone

To my family and members of Kaplan Lab

Chapter 1

Introduction

1.1 Rare Diseases

Diseases are a natural part of life, and they can be categorized in various ways: by the organs they affect, their frequency, or whether they are chronic or acute. Influenza, cancer, diabetes and common cold are among the most well-known common diseases. In contrast, sickle cell disease, cystic fibrosis, Duchenne Muscular Dystrophy (DMD), and hemophilia are known as rare diseases, also known as orphan diseases. Rare diseases often receive less attention, making diagnosis and treatment challenging, yet they significantly impact the lives of those affected.

Countries use the prevalence of a disease to categorize them as a rare disease. A disease is considered rare in the United States if it affects fewer than 200,000 people. In contrast, in Europe, a disease is defined as rare if it affects fewer than 1 in 2,000 people. Also in Japan the number changes to less than 1 in 50000 [1]. China defines rare diseases as those affecting fewer than 1 in 10000 [2]. For India, the threshold adopts less than 1 in 2500 [3]. In our country, the European definition of rare disease has been used since November 2022 with the publication of the Rare Diseases Health Strategy Document and Action Plan by the Ministry of Health [4]. It is predicted that in Türkiye 1 out of every 16 people has a rare disease [5]. According to TÜİK, by 2023 the population of Türkiye is above 85 million. Based on this statistic, there are more than 5 million patients suffering from a rare disease in just Türkiye.

There are over 7000 different rare diseases. 72% of rare diseases are genetic. That means it is caused by genetic mutations which can be inherited from one or both parents or occur spontaneously. Rare diseases can be inherited in an autosomal recessive, autosomal dominant or X-linked manner [6]. The rest of it is non-genetic and includes rare infections, rare cancers and immune deficiencies [7].

Rare diseases' symptoms can vary, not only disease to disease, but there can also be variety in the same disease from patient to patient [8]. The variety and similarity of symptoms can lead to misdiagnosis, as a result in its treatment can be delayed. Because of it, the average time to diagnosis is 5 years. Understanding the mechanism underlying the diseases may help diagnosis and treatment.

Lately improvement in diagnostic techniques such as gene panels and exome sequencing, allows identifying the underlying molecular mechanism of undiagnosed rare diseases [9]. But most of it remains undiagnosed still.

A disease can be rare in one region whereas common in another region. Thalassemia can be give as example. Thalassemia is a kind of genetic anemia that is considered rare in Northern Europe, but in the Mediterranean region and Middle East it is frequent. Periodic fewer syndrome is common in Armenia whereas rare in France [10].

1.2 Ciliopathy

Ciliopathy is a category of rare genetic diseases. Ciliopathy can be explained as functional or structural defects in cilium, its compartment or due to defective cilia biogenesis [11]. More than 35 ciliopathies have been reported, and the number is increasing with advances in genetics [12-14]. In 2018, the total number of human ciliary genes were 747 from Gene Ontology (GO) and SYSCILIA Gold Standard (SCGS). By 2019, the publication of the CiliaCarta article added 209 new genes, bringing the total to 956 [12, 15]. All known ciliopathies to date show either autosomal or X-linked recessive inheritance, mostly caused by single gene mutation [16].

There is no known cure for ciliopathies but there are some approaches such as small molecules and drug therapies, gene therapy and stem cell therapy for managing symptoms aimed at improving life quality of patients.

Ciliopathies can be divided into two categories based on the type of cilia that causes the disease: motile ciliopathies and primary ciliopathies which mean non-motile cilia [17]. The differences between primary cilia and motile cilia result in different phenotypes in motile ciliopathies and primary ciliopathies [17].

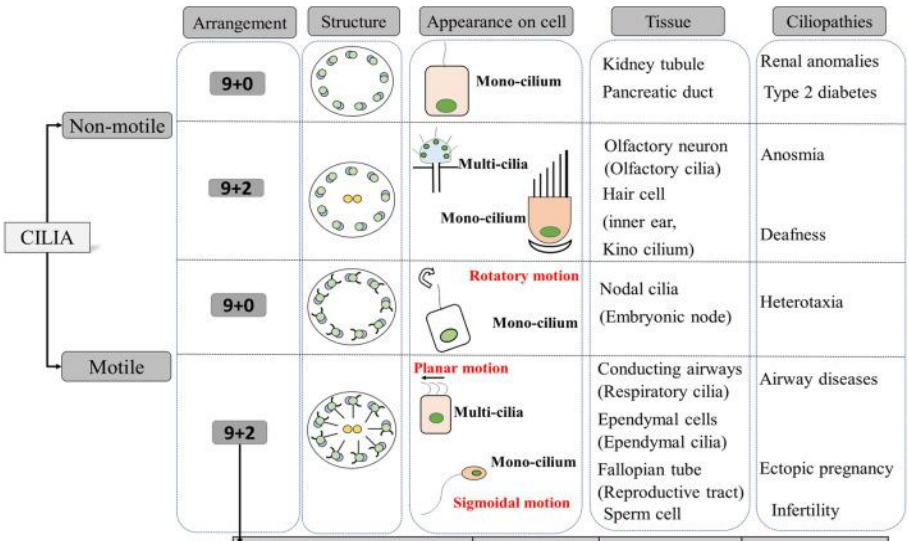
1.2.1 Motile Ciliopathies

Motile cilia are found in the apical epithelial surface of respiratory tract, the brain ependyma and the epithelium of the fallopian tubes [18, 19]. In unicellular organisms, motile cilia play a role in cell movement, while in humans, they are involved in various functions. For example, they capture bacteria and propel mucus in the respiratory tract and they help transport the ovum from the ovary to the fallopian tubes [19, 20]. Dysmotility in brain ependymal cilia may be one of the reasons for hydrocephalus while dysmotility of sperm flagella or fallopian tube cilia can result in reduced fertility [19]. Primary Ciliary Dyskinesia or Kartagener Syndrome patients may exhibit defects in the ultrastructure of the axonemes within their motile cilia [16]. The rhythmic beating of cilia helps carry mucus from the airway, but defective cilia cannot beat like in Primary Ciliary Dyskinesia Syndrome. PCD is generally observed in early childhood and mutations are mostly inherited as autosomal recessive. In rare cases, they can also be inherited X-linked recessive [19]. Symptoms of PCD can vary, but to diagnose the condition, there are some key clinical signs such as airway infections, laterality defects, acute and chronic otitis media, and, in rare cases, hydrocephalus may accompany [18, 19]. Kartagener Syndrome, another motile ciliopathy, is considered a type of PCD. In Kartagener Syndrome, in addition to the PCD's symptoms, situs inversus is also observed [21].

1.2.2 Primary Ciliopathies

For many years, primary cilium was thought to be a non-functional organelle [22]. Recently, it has been understood that it has an important role in signaling pathways to regulate cellular function such as polarity, cell division and metabolism [14]. Primary cilia are non-motile structures that play a role in monitoring and sensing the environment. Due to sensory role, primary cilia are important for development and tissue homeostasis [23].

Primary ciliopathies can affect different organs such as kidneys, eyes, central nervous system, heart and liver [19].



Lower eukaryotes	Cilia/Flagella number	Movement type	Function
Euglena gracilis	one	Corkscrew (euglenoid)	Motility
Chlamydomonas reinhardtii	two	Breaststroke-like	Motility, mating
Paramecium caudatum	multiple	Whiplash	Motility, mating, ingestion

Figure 1.1 Motile and Non-motile cilia with their arrangement, structure, apperence on cell, tissue and ciliopathies [23]

1.2.2.1 Polycystic Kidney Disease (PKD)

There are two types of Polycystic Kidney Disease (PKD) based on inheritance patterns: Autosomal Dominant PKD (ADPKD) and Autosomal Recessive PKD (ARPKD). Both are characterized by kidney cysts, which can grow very large and lead to kidney failure. ADPKD is a common form of PKD, with a prevalence of 1 in 400 to 1 in 1000 in the USA and 1 in 4033 in Japan [24] and its manifests in adulthood [25]. Approximately 12 million people suffer from ADPKD around the world [26]. In contrast, the prevalence of ARPKD is 1:20000 and it is typically seen in early childhood. 30% of newborns with ARPKD die in a couple of weeks [27]. In most cases, ADPKD is caused by mutations in the *PKD1* and *PKD2* genes, which produce polycystin-1 (PC1) and polycystin-2 (PC2), respectively [26, 28]. On the other hand, ARPKD is mostly related to mutations in the polycystic kidney and hepatic disease 1 (*PKHD1*) gene [28]. *PKHD1* gene is found in the primary cilia and basal body and produces fibrocystin [29]. At present, there is only one FDA-approved drug for ADPKD, tolvaptan, and no known treatment or drug for ARPKD.

Tolvaptan helps reduce symptoms of ADPKD but in long-term usage leads to serious side effects [30].

1.2.2.2 Bardet-Biedl Syndrome (BBS)

Bardet-Biedl Syndrome identified in 1920 [31]. BBS exhibits heterogenous, autosomal recessive inheritance [32-34]. 26 genes are found related to the BSS but mutations in the *BBS1* to *BBS18* genes are responsible for approximately 70-80% of Bardet-Biedl Syndrome [14]. The majority of BBS proteins are found at the basal body or within the primary cilium. 8 of the BBS genes, BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9, and BBS18, contribute to the assembly of the BBSome [35].

The prevalence of BBS shows variety in different countries. The prevalence in the UK is 1:125,000 whereas in Switzerland is 1:160,000 [34]. In Tunisia, it is 1:156,000 [36]. However, in some isolated societies, the prevalence can be higher. For example, in the mixed Arab community in Kuwait, it is 1:36,000 and among Bedouins Kuwaitis, it is 1:13,500 [37]. On the other hand, in Faroe Islands, the prevalence is 1:3700 [33].

Clinical features of BBS can be listed as obesity, polydactyly, retinal dystrophy, male hypogenitalism, mental retardation and renal deficiency [33, 34, 38, 39]. Obesity begins to appear in childhood and progresses with age. The occurrence rate of obesity in BBS patients is estimated to be between 72% and 92% [39, 40]. Polydactyly is one of the common symptoms and may be the only symptom seen from birth [39, 40]. Retinal dystrophy, a progressive degeneration of retina, symptoms mostly manifest in the first decade of life and can lead to blindness [14]. Male hypogenitalism is characterized by underdeveloped genitalia, and it is another frequently observed clinical feature. In patients, a wide range of intellectual disability and renal diseases can be seen [14, 41]. There is no known cure for BBS, but current treatments focus on managing symptoms, such as diabetes and hypertension [41].

1.2.2.3 Joubert Syndrome

Joubert Syndrome is autosomal recessive disease characterized by hypotonia, abnormal eye movement, ataxia, episodic breathing dysregulation and developmental delay [42-44]. It is diagnosed by the appearance of the molar tooth sign (MTS) on MRI, caused by midbrain-hindbrain abnormalities [45, 46].

It is associated with more than 40 genes that localize basal body or primary cilia [42, 43]. These mutations disrupt cilia function and lead to different clinical features observed in patients. The identification of these genes enhanced our understanding of the pathophysiology of Joubert Syndrome and related ciliopathies, highlighting the crucial role of ciliary function in human development and disease.

1.2.2.4 Meckel-Gruber Syndrome

Meckel-Gruber Syndrome (MKS) is a lethal ciliopathy inherited in an autosomal recessive manner [47, 48]. MKS was initially identified by Johann Friedrich Meckel in 1822 in two siblings [48]. In 1934, George B. Gruber documented 16 similar comparable cases [49]. This syndrome is characterized by a triad of clinical features: occipital encephalocele, large polycystic kidneys, and polydactyly. Central nervous system (CNS) malformation, hepatic development defects, oral cleft and pulmonary hypoplasia may accompany the symptoms [50, 51]. In this disease, which has a 100% mortality rate, affected individuals die *in utero* or shortly after birth due to mostly pulmonary hypoplasia [48, 50, 51].

Mutations in 13 genes, including CEP290, MKS1, and RPGRIP1L, are responsible for 75% of MKS cases [52]. Many of these genes encode proteins that localize ciliary transition zone (TZ). These proteins are essential for the structure and function of primary cilia, which are critical for many signaling pathways during embryonic development such as sonic hedgehog (Shh) and Wnt [53].

1.2.2.5 Ellis van Creveld

Ellis van Creveld (EVC) is another autosomal recessive rare ciliopathy [47], defined by Richard Ellis and Simon van Creveld in 1940 [54]. Key characteristics of EVC can be listed as chondrodystrophy, polydactyly, congenital heart disease and ectodermal dysplasia [55, 56]. Short ribs and limbs, short stature and dental anomalies such as natal teeth and dysplastic nails may accompany other symptoms [57]. This disease is caused by mutations in one of the two genes, EVC and EVC2, located on the short arm of chromosome 4 [55]. It affects both genders equally [59].

EVC and EVC2 encode transmembrane proteins that form a heterodimeric complex. EVC complex, found primary cilia membrane distal to the transition zone, engage in Hedgehog

(Hh) signaling. Hedgehog signaling has an important role in embryogenesis [60]. Without EVC complex Hh signaling decreases [59].

1.2.2.6 Polydactyly

Polydactyly can be divided into three categories based on where the extra finger is placed; preaxial polydactyly, central polydactyly and most common form, postaxial. Polydactyly term was used by Theodore Kerckring first time in 1670, and it means extra finger in hands and feet. In preaxial polydactyly, patients have two thumbs on their hands or feet, whereas in postaxial polydactyly, an extra finger occurs on the fifth or sixth metacarpal [61]. Poataxial polydactyly frequently seen in ciliopathies, such as Joubert Syndrome, BBS, MKS and NPHP. Addition to these, nodal cilia defects result in situs inversus [32].

Table 1.1 Symptoms of ciliopathies. PCD (Primary Ciliary Dyskinesia), PKD (Polycycstic Kidney Disease), BBS (Bardet-Biedl Syndrome), JBTS (Joubert Syndrome), MKS (Meckel-Gruber Syndrome), EVC (Ellis van Creveld Syndrome), NPHP (Nephronophthisis)

	Motile Ciliopathies		**Primary Ciliopathies**					
	PCD	**Kartgener**	**PKD**	**BBS**	**JBTS**	**MKS**	**EVC**	**NPHP**
Airway Infection	**X**	**X**						
Cognitive Defects				**X**	**X**	**X**	**X**	**X**
Encephalocele				**X**		**X**		
Laterality Defect	**X**	**X**		**X**		**X**		
Liver Diseases			**X**	**X**	**X**	**X**		**X**
Molar Tooth Sign					**X**			
Obesity				**X**				
Polydactyly				**X**	**X**	**X**	**X**	
Renal Cysts			**X**	**X**	**X**	**X**		**X**
Retinopathy				**X**	**X**			**X**
Situs Inversus		**X**			**X**			
Skeletal Defects							**X**	

1.3 Cilia

Cilia are highly conserved, microtubule-based organelles that are composed of subcompartments. These can be listed as the transition zone, basal body, axoneme, ciliary tip, and ciliary membrane [62]. Cilia type can be divided into two depending on whether they have a central pair of microtubules doublets. Motile cilia have 9+2 structure whereas primary cilia have 9+0 structure. Each doublet has 2 strands; A strand with 13 tubulin

protofilaments and B strand with 10 tubulin protofilaments. In motile cilia differ from primary cilia, radial spokes connect inner and outer doublets [47]. There are some specialized cilium types such as nodal cilia or kinocilia. Nodal cilia, which play a crucial role in left-right asymmetry during embryogenesis, are motile cilia but lack central microtubule doublets. On the other hand, kinocilia are one of the primary cilia that have 9+2 axoneme patterns. But they don't have inner dynein arms unlike motile cilia [63]. Kinocilia have passive motion which means they beat after the cell sense sound.

Because of their difference in structure, motile cilia and primary cilia have different roles. Motile cilia have function as fluid movement, transport of particles, cerebrospinal fluid circulation and locomotion in single-cell organism. Brain, lung, kidney, respiratory tract, embryo, sperm and oviduct cells have motile cilia [64]. Function of primary cilia are sensing stimuli such as growth factor, neurotransmitters and light, from the environment, like an antenna.

Basal body initiates ciliogenesis. The main structure of the cilium, axoneme, extends from the cell surface starting at basal body [65].

Transition zone located between the basal body and the axoneme. It acts as a gate, regulating the entry and exit of proteins and other molecules into and out of the cilium. The transition zone is essential for maintaining ciliary integrity and proper function. It serves as a hub for various proteins involved in ciliary assembly and signaling, ensuring that only specific components reach the ciliary compartment.

Cilia have two segments; middle and distal segment. While the middle segment has microtubule doublets, the distal segment has singlet of microtubule. Middle segment connects the basal body to the distal segment. It is important for ciliary stability and function. The distal segment is located at the end of the axoneme. This region is involved in intraflagellar transport (IFT). The distal segment acts as a site where proteins and other molecules are transported to and from cilium. The ciliary tip is also where signal transduction and sensory activities are concentrated, making it essential for the cilium's sensory functions.

1.3.1 Intraflagellar Transport (IFT)

IFT is a transport system, carrying proteins from base of cilium to tip which is called anterograde, tip to base which called retrograde. IFT cargo transport via dyneins and

kinesines. The IFT has two subcomplex: IFT A and IFT B. IFT IFT A consists of 6 proteins whereas IFT B consists of 11 proteins [47]. IFT complexes form at the ciliary base. In anterograde direction kinesin-2 motor proteins and OSM-3 work with IFT B to carry IFT cargo through distal part of cilium, OSM-3 works alone along the distal singlet [66]. In the tip of the cilium IFT complex disassembles and reassembles, and then retrograde direction cargo is transported by IFT A, along with dynein motors. Defective IFT causes some neurodevelopmental disorders [67].

1.3.2 Hedgehog Signaling

Cilia play essential role in several important signaling pathway such as Wnt [68], Hedgehog [69, 70], Notch [71] and mTOR [72, 73] signaling pathways. During development, Hedgehog signaling is essential for intercellular communication homeostasis. In mammals, Hh signaling plays a vital role in almost all organogenesis and maintaining. Three types of HH protein exist in mammalians; Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH). SHH and IHH, both have crucial functions in several tissue. Their function can overlap sometimes [74].

1.4 *C elegans* as a model organism

C elegans is the first multicellular organism whose genome was sequenced, a milestone achieved in 1998 [75, 76]. This well characterized genome serves as a good resource for gene function and their interactions. *C elegans* have 5 autosomes and one sex chromosome. *C elegans* also allows gene manipulations, such as CRISPR-Cas9, to understand gene function. One of the notable characteristics of *C elegans* is transparency, which provides to observe cellular and developmental processes via microscopic techniques and allows real time visualization of cellular events. This transparency provides advantages for studying developmental biology and neurobiology. Short life span and rapid reproductive cycle make *C elegans* an ideal model organism. After hatching 2-3 days later they can produce offspring, allowing researchers to see effects of mutations in multiple generations in a short time.

They have nearly 959 somatic cells, and 302 of them are neurons [77]. There are 60 types of neurons, and these neurons include interneurons, GABAergic neurons and dopaminergic neurons, which are also found in humans. It is manageable yet

comprehensive model for neurobiology studies. Small size and low maintenance expenses make it suitable to work in a laboratory.

1.5 EFCAB7

EF-hand calcium binding domain 7 encodes a protein that is predicted to have calcium binding activity and to positively regulate protein localization to ciliary membrane [78]. EFCAB7 is found in the testis, fallopian tubes, retina, and skeletal muscle, with significant expression in cilia [79]. In primary cilia, EFCAB7 protein localizes in transition zone [80, 81]. In 2014, Pusapati et. al. found that EFCAB7 makes a subcomplex with IQCE. EFCAB7 links this subcomplex to another subcomplex, EVC-EVC2, through EVC2. EFCAB7 is likely important for Hedgehog signaling regulation and the localization of EVC2 within the EvC zone. Lack of EFCAB7 leads to misplacement of EVC-EVC2 complex, and ultimately Hedgehog signaling disruption. Without EFCAB7 [81]. The relationship between EFCAB7 and EVC complex suggested that it may also be associated with EVC Syndrome and Weyers Syndrome. Some clinical features of Weyers Syndrome may overlap with those of EVC Syndrome, but they are generally milder. The other main differences are that Weyers Syndrome has a dominant inheritance pattern and patients with Weyers Syndrome don't have heart defects [82]. Nguyen et. al identified a missense mutation of EFCAB7 gene, along with EVC and EVC2 mutations, in one of 8 Vietnamese patients with EVC syndrome [83]. In 2023, Bilal et. al. reported two different EFCAB7 mutations that cause nonsyndromic postaxial polydactyly in four relative families [79].

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Strains

Table 2.1 The strains work with in this study

N2 - Wild type
N2;jhuEx[CHE-11::GFP+pRF4)
N2[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
N2[unc-25::gfp+lin-15(+)]
N2[unc-25::gfp+lin-15(+)]
efcab-7(T02G5.3)
cdkl-1
bbs-8
dyf-5
kap-1

klp-13

Table 2.2 The strains obtained in this study

efcab-7(T02G5.3)(5x)
efcab-7(5x)*;cdkl-1*
efcab-7(5x)*;bbs-8*
efcab-7(5x)*;dyf-5*
efcab-7(5x)*;kap-1*
efcab-7(5x)*;klp-13*
efcab-7(5x)[CHE-11::GFP+pRF4]
cdkl-1[CHE-11::GFP+pRF4]
bbs-8[CHE-11::GFP+pRF4]
dyf-5[CHE-11::GFP+pRF4]
kap-1[CHE-11::GFP+pRF4]
klp-13[CHE-11::GFP+pRF4]
efcab-7(5x)*;cdkl-1*[CHE-11::GFP+pRF4]
efcab-7(5x)*;bbs-8*[CHE-11::GFP+pRF4]
efcab-7(5x)*;dyf-5*[CHE-11::GFP+pRF4]
efcab-7(5x)*;kap-1*[CHE-11::GFP+pRF4]

efcab-7(5x)*;klp-13*[CHE-11::GFP+pRF4]
efcab-7(5x)[ELMOD-3::GFP;TBG-1::MKATE+rol-6] *cdkl-1*[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
bbs-8[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
dyf-5[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
klp-13[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
efcab-7(5x)*;cdkl-1*[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
efcab-7(5x)*;bbs-8*[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
efcab-7(5x)*;dyf-5*[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
efcab-7(5x)*;klp-13*[ELMOD-3::GFP;TBG-1::MKATE+rol-6]
efcab-7(5x)[unc-25::gfp+lin-15(+)]
efcab-7(5x);*cdkl-1*[unc-25::gfp+lin-15(+)]

2.1.2 Primers

Table 2.3 Primers that used in this study

efcab-7-Forward	GGGAGATACGAAGACTCAGG
efcab-7-Reverse	GACCGGCTACCTTATACTCC
cdkl-1(ok2694)-Forward	gtgatcgaactgtacttcacg
cdkl-1(ok2694)-Reverse	ctgttccctcactgggtctc
bbs-8(nx77)-Forward	catgcaattgcctgaaacactg

bbs-8(nx77)-Reverse	gcaatgcttgtcggattcgac
dyf-5(ok1177)-Forward	gagggaagactgagttgagtg
dyf-5(ok1177)-Reverse	cgaaccttgtttgaactgccg
kap-1(ok676)-Forward	gctcgcttgagtgcttttgtatc
kap-1(ok676)-Reverse	GTGGTACAAGACCTCCATTCACC
klp-13(tm3737)-Forward	gtcggagttgtactagtagaggac
klp-13(tm3737)-Reverse	gtcccgtcacgagtatcactc

2.2 Methods

2.2.1 Dye Filling Assay

Dye filling assay is one of the essential and fast method employed to assess the functionality of cilia. This assay provides crucial insights into whether cilia are defective by evaluating their ability to absorb and transport dye.

The underlying principle of the dye uptake assay is based on the observation that functional cilia can absorb and transport specific dyes effectively, whereas defective cilia exhibit impaired dye uptake. This differential uptake can be visualized and quantified, providing a clear indication of cilia health.

To assess the functionality of cilia using the dye uptake assay, samples were first prepared by cultivating the organisms or cells in optimal conditions. A fluorescent dye solution was then introduced to the samples, allowing sufficient time for cilia absorption. The samples were incubated under controlled conditions to facilitate dye uptake. Following incubation, the samples were rinsed to remove any excess dye not absorbed by the cilia. A fluorescence microscope was used to observe the samples, with functional cilia displaying significant fluorescence due to effective dye absorption and transport, while defective cilia showed reduced or no fluorescence. Images of the samples were captured using the microscope, and appropriate software was utilized to quantify the fluorescence

intensity, enabling a comparative analysis of dye uptake between wild-type and mutant strains.

2.2.2 Cross System

One of the crucial method in this study is making crosses to generate new strains according to the Mendelian principles. To obtain new strain there are several steps and at least 2 crosses. To obtain single mutant with one transgenic; it can be started with wild type males and wild type with transgenic hermaphrodites. Males that have transgenic mate with mutant hermaphrodites. 4-5 hermaphrodites and 10-12 males are taken to the drop plates that contains 5-7 microliter E. coli bacteria. After 2 days mating hermaphrodites are taken to new spread plate separately to see which one is mated. After 3-4 days, 16-20 hermaphrodites at most the L4 stage from the plate with highest number of males are taken and each is placed on separate spread plates. These are F1 progeny, and these progenies contain transgenic and +/- for mutant. One of these F1 plate is chosen and taken F2 progenies. F2 progenies can be +/+, +/- and -/- with transgenic. -/- plate is chosen by PCR and taken F3 progenies. At this step, it is expected that all mothers will be -/-.

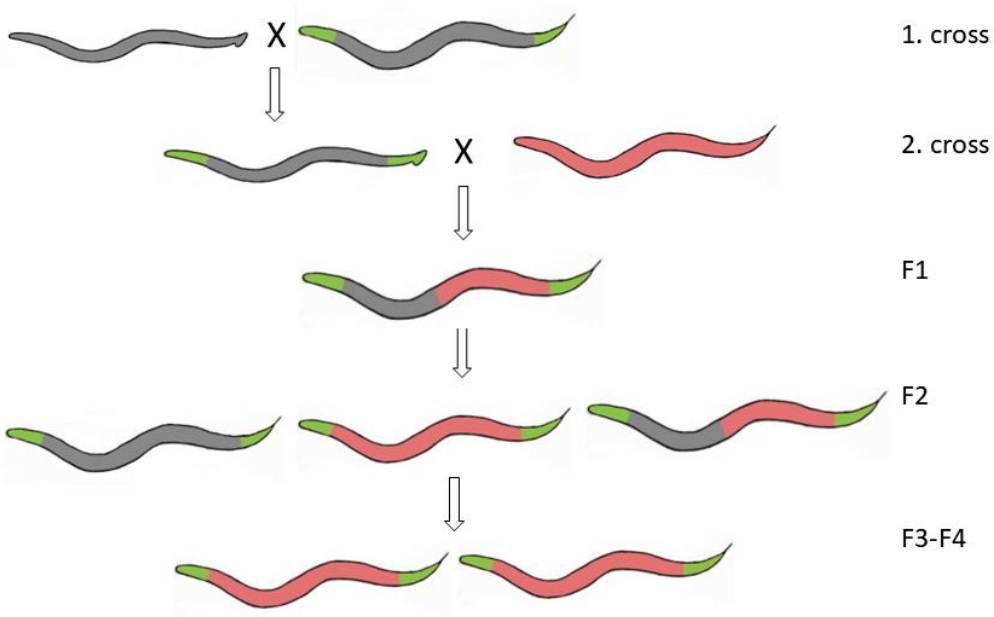


Figure 2.1 *C elegans* crossing system. Greens represent transgenic, red represents mutants.

2.2.3 Microscopic Analysis

In this study, adult worms were used for cilia length, 1 day adult worms for body length and L4 stage worms for axon markers. L4 stage is distinguishable. So L4 stage worms are collected and incubated in 20 degrees centigrade; after 1 day, they become 1 day old adult worms. There are two methods to obtain L4 stage worms. First, worms capable of producing eggs are collected and placed on a spread plate and allowed to lay eggs for 3-4 hours. Then, the adult worms are collected from plates. After give or take 37 hours in 20 centigrade degree, the worms reach L4 stage. The second method is taking a small chunk from a plate with high number of L1-L2 stage worms and transferring a new spread plate. Within 1-2 days, it can be collected L4 stage worms in this plate.

On examination day, worms collected with a pick are transferred to agarose gel on a slide. %2 agarose gel is used. 400 microliter hot agarose is dropped onto the slide. To ensure the agarose is smooth, it is covered with another slide and wait for agarose get cool. Once the agarose has cooled, the slide on top is removed without harming agarose. To fix worms, 2 microliter levamisole is used. Worms are transferred to levamisole on agarose with pick. After worms are collected, a coverslip is placed on it. The image of worms was taken under a compound microscope at 100x magnification for cilia length, 10x magnification for body length and 20x magnification for axons. It was conducted at least 3 independent experiments.

2.2.4 Functional Assay

For functional assay, stereo microscope, Dino eye camera and Dino capture 2.0 program was used. Two types of videos were taken. One of them is tracking assay which examine worms walking. The other one is thrashing assay which examine worms trashing in M9 solution. 1 day adult worms were used for functional assay. 1 day before collected worms were transferred to an empty plate. The camera is placed in one ocular of stereo microscope and record 1 minute video. Add 1 or 2 small drop M9 for thrashing assay. Worms begin thrashing and record video 1 minute again. After videos captured, analyzed in Fiji program. Fiji has a plugins that named wrMTrck. Worms' velocity was measured with wrMTrck.

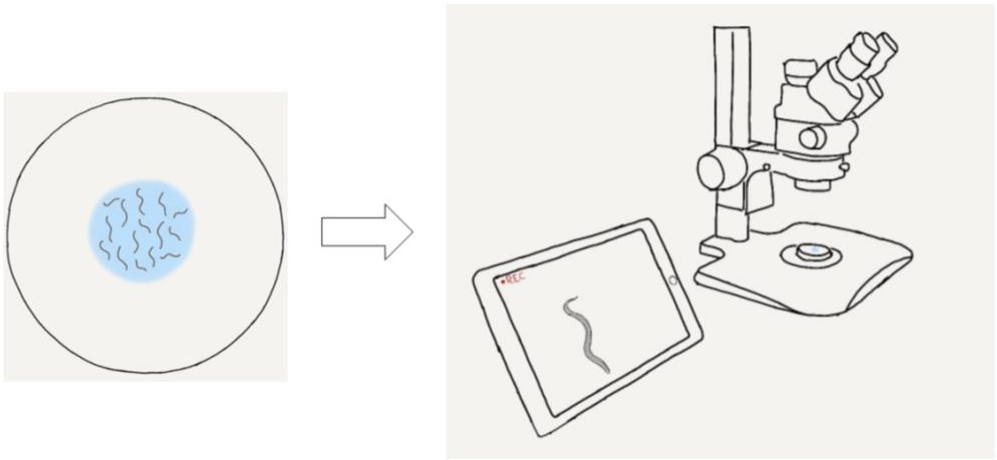


Figure 2.2 Trashing Assay

2.2.5 Statistical Analysis

Cilia length and body length were measured using the Andor program. ImageJ and Kymograph programs were used to measure the number and velocity of IFT particles. To obtain results were collected in Excel file. To determine the significance of results, code was written using the R programming language. A t-test was used to compare the mean values of two data sets and ANOVA test was used to compare the mean values of more than two data sets. The results of P test were visualized using code written in R.

Chapter 3

Results

Orthologs of EFCAB7, IQCE, EVC, and EVC2 were examined, and it was found that only EFCAB7 has an ortholog in *Caenorhabditis elegans*. EFCAB7 has 3 EF-hand domains in human whereas 1 EF-hand domain in *C. elegans.* In this study, RB2480 strain was used which has 1193 base pair deletion. This deletion covers 4 exons out of 10 exons.

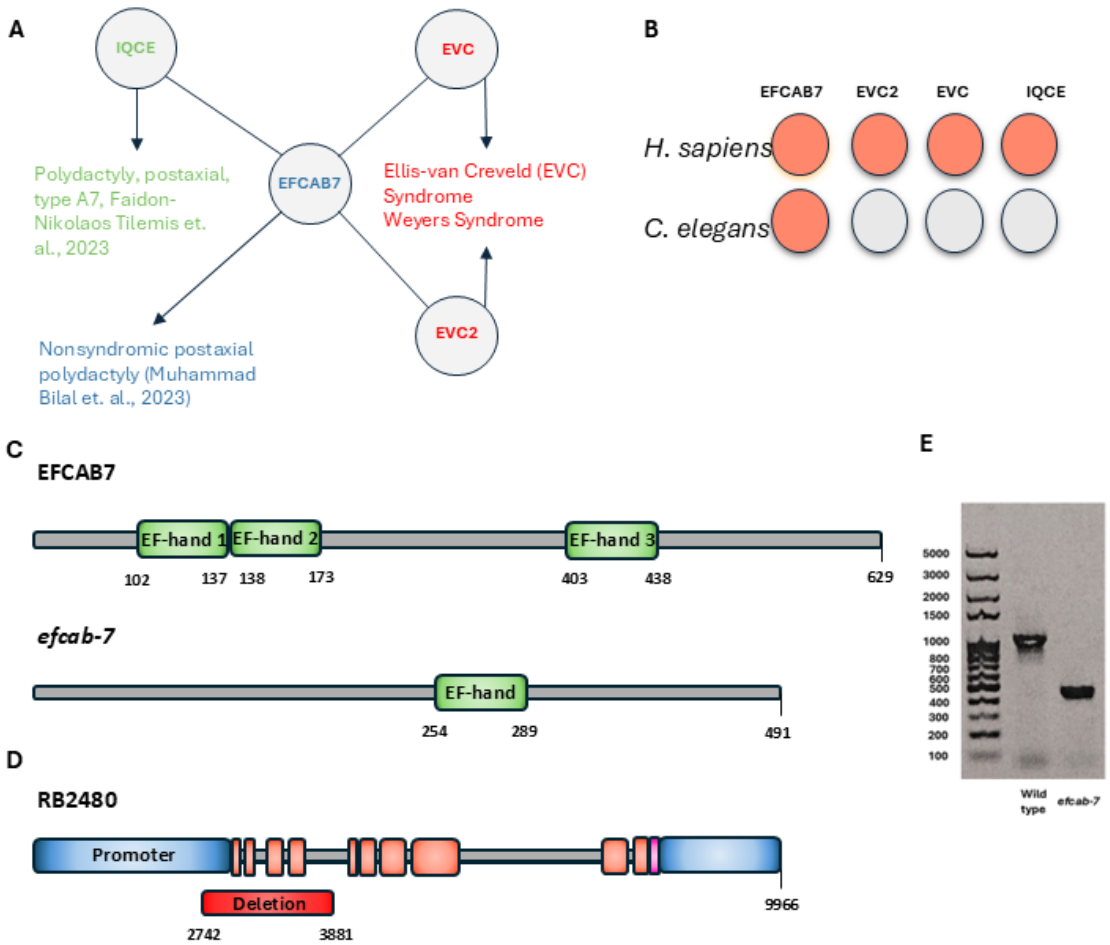


Figure 3.1 Domains of *efcab-7* **A)** Interaction of *efcab-7* and other proteins. **B)** Orthologs of genes in *H. sapiens* and *C. elegans.* **C)** Domains of *efcab-7* in *H. sapiens* (top) and *C. elegans.* **E)** Gel electrophoresis image of wild type and *efcab-7*

3.1 *efcab-7;cdkl-1* double mutant's cilia show difference from wild type

To determine whether cilia functionality was compromised in different genetic backgrounds, a dye uptake assay was performed. Figure 3.2 illustrates the comparative analysis between wild-type cilia and mutant's cilia. It was observed that *efcab-7* and *cdkl-1* mutants exhibited a dye uptake rate of more than 50%, which is considered within the normal range. This indicates that, despite the mutations, these cilia retain a significant level of functionality in terms of dye absorption. But double mutant's uptake rate is less than 25%. This substantial decrease in dye absorption strongly suggests that the double mutant cilia can be defective.

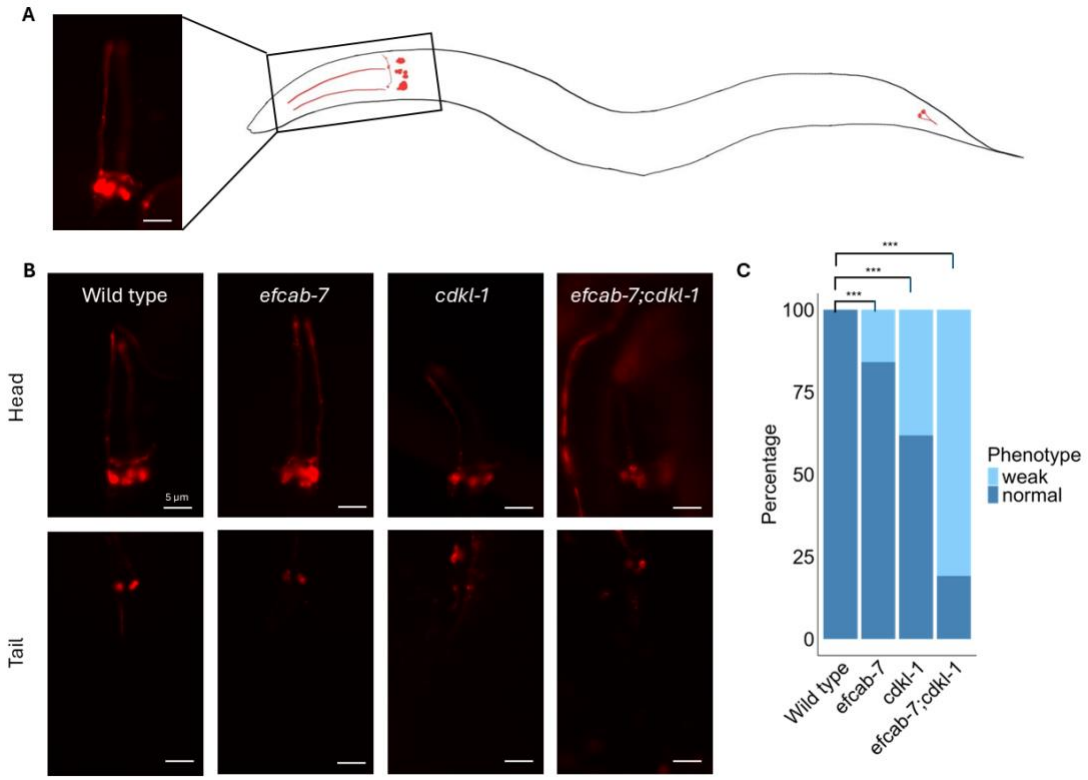


Figure 3.2 Dye uptake assay in wild type and mutant cilia. **A)** Schematic representation of the worm, highlighting the head region. The inset shows a red fluorescent image of the worm's head. **B)** Red fluorescent microscopy images of the head and tail regions of worms with the following genetic backgrounds: Wild type, efcab-7 mutant, cdkl-1 mutant, and efcab-7;cdkl-1 double mutant. Images depict reduced dye uptake in efcab-7 mutant cilia compared to wild type. Scale bar: 5 µm. **C)** Quantitative analysis of dye uptake, presented as a bar graph showing the percentage of worms with "normal" (dark blue) and "weak"

(light blue) phenotypes across four genetic backgrounds: Wild type, *efcab-7*, *cdkl-1*, and *efcab-7;cdkl-1*. Three asterisks (***) indicate statistically significant differences between groups.

3.2 *efcab-7* mutant's cilia shorter than wild type

It was measured cilia length for several mutants and double mutants. CHE-11::GFP transgenic was used for this assay. CHE-11 is transferred along the whole cilia so it allows us to measure cilia length.

Initially, the *efcab-7*(CHE-11::GFP+rol-6) strain was generated. The cilia of the *efcab-7* mutants were found to be shorter than those of the wild-type strain. Subsequently, the *cdkl-1*(CHE-11::GFP+rol-6) and *efcab-7;cdkl-1*(CHE-11::GFP) strains were produced and their cilia lengths measured. Interestingly, the cilia length of the *cdkl-1* mutants was comparable to that of the wild type. However, the double mutant *efcab-7;cdkl-1* exhibited shorter cilia, although the difference was not statistically significant when compared to the *efcab-7* single mutant.

In addition to these strains, we generated CHE-11::GFP transgenic lines with *dyf-5*, *kap-1, efcab-7;dyf-5,* and *efcab-7;kap-1* mutants. The cilia length in the *kap-1* mutants was similar to the wild type, but the *efcab-7;kap-1* double mutants displayed significantly longer cilia compared to both the *kap-1* and *efcab-7* single mutants.

These particular mutants were chosen because of their known or suspected contributions to the structure and function of cilia. The protein CDKL-1 is involved in ciliary functions, and research on its mutations provides light on the processes controlling the development and upkeep of cilia. KAP-1 is a kinesin accessory protein linked to intraflagellar trafficking, while DYF-5 regulates the length of cilia. It was aimed in examining these mutants is to clarify the complex genetic relationships that affect the morphology and function of cilia.

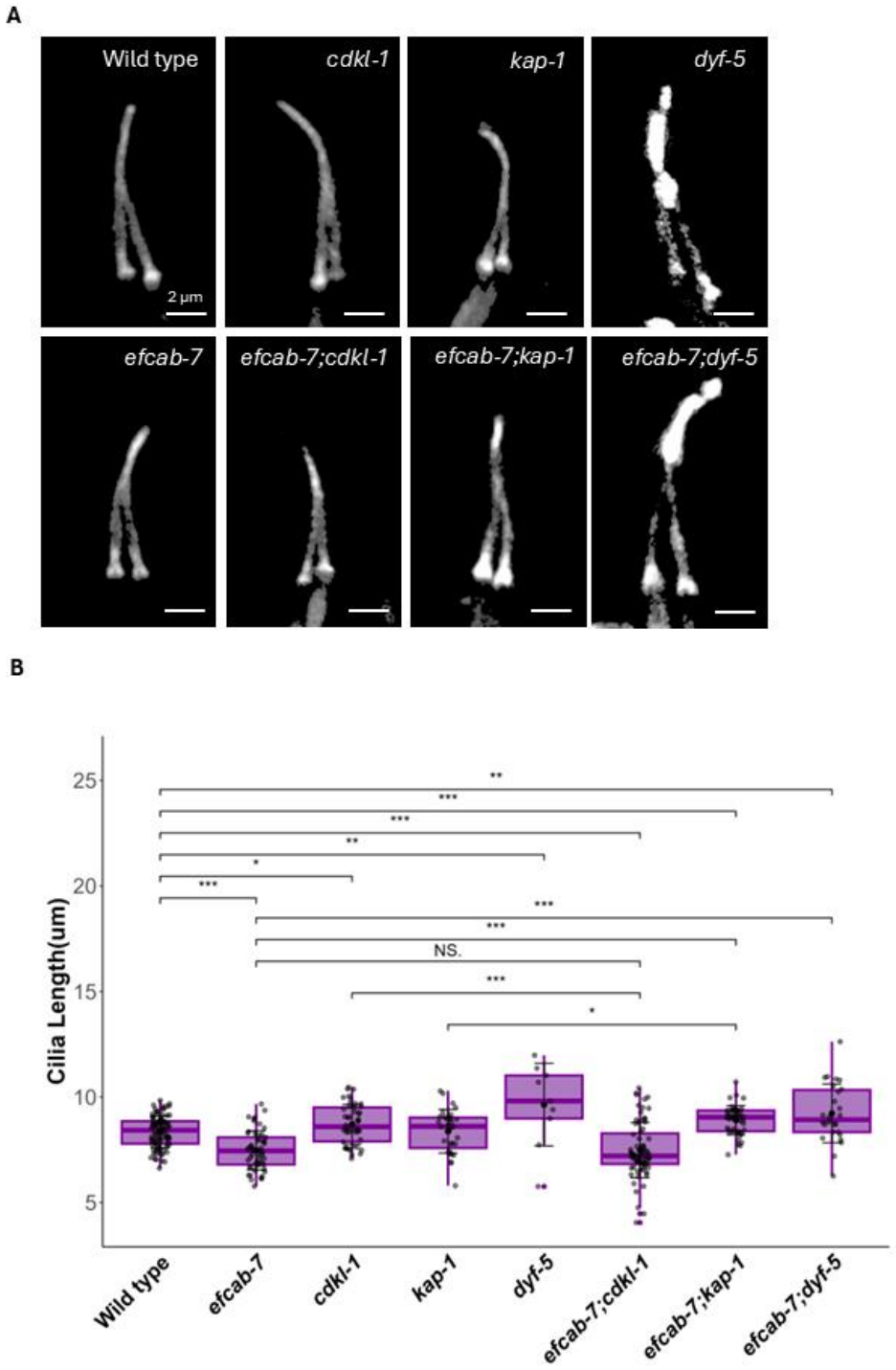


Figure 3.3 Analysis of cilia length. **A)** Representative images of cilia from different genetic backgrounds. The images show cilia from wild type, *efcab-7*, *cdkl-1*, *kap-1*, *dyf-5*, *efcab-7*, *efcab-7;cdkl-1*, *efcab-7;kap-1*, and *efcab-7;dyf-5*. The scale bar represents 2 µm. **B)** Quantification of cilia length in the different mutants. The box plot shows the distribution of cilia lengths for each genotype. Statistical significance is indicated by asterisks: *p < 0.05, **p < 0.01, ***p < 0.001, NS: not significant.

3.3 *efcab-7*'s IFT particles number did not change

To further investigate the functionality of cilia in various strains, we performed an analysis of both retrograde and anterograde intraflagellar transport (IFT) particles. The strains used for this analysis were transgenic with CHE-11::GFP, which allowed for the visualization and quantification of IFT particles in motion.

To measure the IFT particles, 90-frame videos capturing their movement within the cilia were recorded. Using ImageJ software, kymographs were generated from these videos to visualize both retrograde and anterograde transport of IFT particles. The number of IFT particles was then meticulously counted, enabling a detailed comparison across different genetic backgrounds.

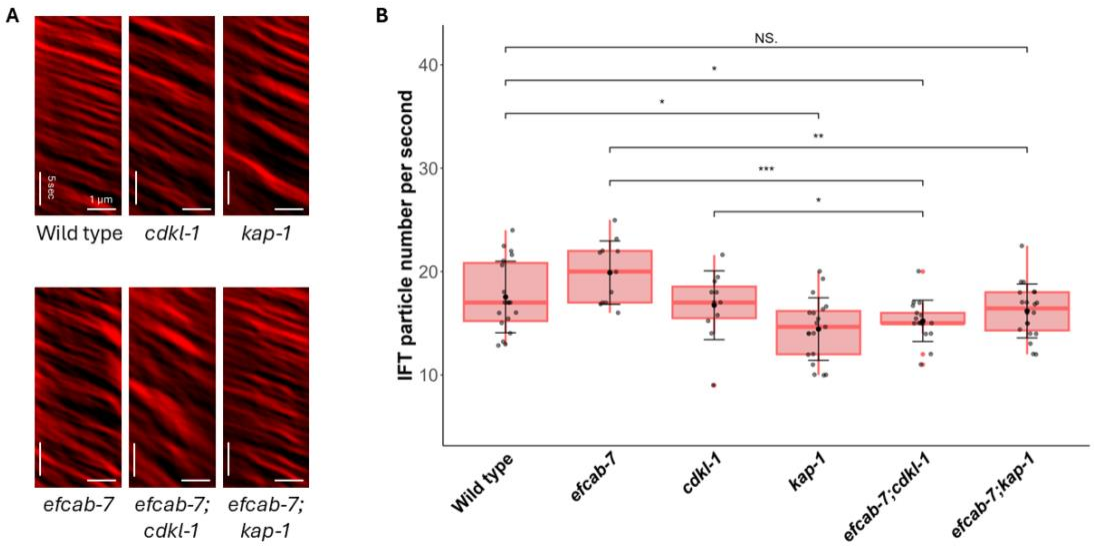


Figure 3.4 Comparative analysis of anterograde IFT particles in different mutants. **A)** Kymograph images of anterograde IFT particles from different mutants. Each image has a scale bar indicating 1 µm and 5 second for size reference**. B)** Box plot showing the number of IFT (intraflagellar transport) particles per second for different genetic backgrounds. The y-axis represents the IFT particle number per second. Statistical significance is indicated with asterisks: "NS" for not significant and "*" for $p < 0.05$.

The analysis of anterograde IFT particles revealed a significant decrease in the number of particles in the *efcab-7;cdkl*-1 double mutants. This reduction indicates that the double mutant cilia are defective in their ability to transport IFT particles efficiently from the base to the tip. In contrast, the single mutants, efcab-7 and cdkl-1, maintained a relatively normal rate of anterograde IFT particle transport. This suggests that while each mutation alone affects cilia function, the combination of both mutations drastically impairs the

anterograde IFT process. These findings further support the hypothesis that both EFCAB-7 and CDKL-1 proteins are crucial for effective anterograde IFT particle transport and that their combined mutations result in a severe functional impairment of cilia.

kap-1 single mutant also showed decrease number of IFT particles whereas the *efcab-7;kap-1* double mutant is non-significant to wild type.

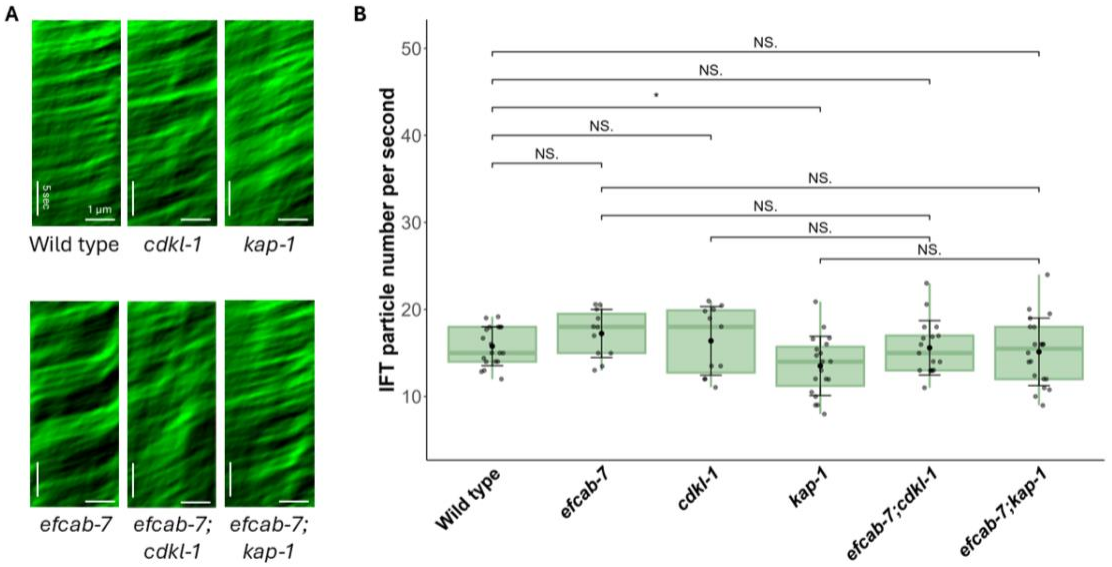


Figure 3.5 Analysis of IFT particles numbers in retrograde. **A)** Kymograph images of cilia from different genetic backgrounds, including wild type, *efcab-7, cdkl-1, kap-1, efcab-7, efcab-7;cdkl-1,* and *efcab-7;kap-1*. The scale bars represent 1 µm and 5 second. **B)** Box plot showing the number of retrograde IFT particles per second for each genetic background. The y-axis represents the number of IFT particles per second, while the x-axis lists the mutants: wild type, *efcab-7, cdkl-1, kap-1, efcab-7;cdkl-1*, and *efcab-7;kap-1*. Statistical significance is indicated by asterisks (*** for $p < 0.001$, ** for $p < 0.01$, * for $p < 0.05$), and "NS" denotes non-significant differences.

The analysis of retrograde IFT particles indicated that there were no significant changes in the particle count for most strains, with the exception of the kap-1 mutants. The kap-1 single mutants displayed a significant decrease in the number of retrograde IFT particles, suggesting a defect in their ciliary transport mechanism. In contrast, the efcab-7, cdkl-1, and efcab-7;cdkl-1 strains did not show significant differences in retrograde IFT particle count compared to the wild type, indicating that these mutations do not notably impact the retrograde transport process.

3.4 Velocity of IFT particles did not show changes in *efcab-7* mutants

Strains obtained with CHE-11::GFP were used to measure velocity of IFT particles. Kymographs that were procured before used. Results were taken from KymographDirect2.1 program. Velocity was measured separately for middle segment, distal segment and retrograde.

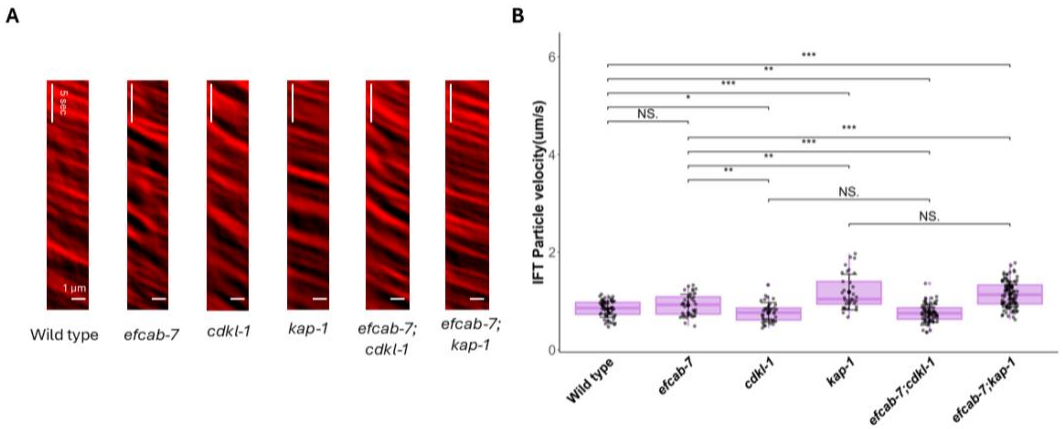


Figure 3.6 IFT particles velocity in middle segment of cilia. Kymographs of IFT particle movement in the middle segment of cilia for various genetic conditions: Wild type, efcab-7, cdkl-1, kap-1, efcab-7;cdkl-1, and efcab-7;kap-1. Each kymograph includes a scale bar indicating 1 µm and a time scale of 5 seconds.

In the middle segment, the velocity of IFT particles in the efcab-7;cdkl-1 double mutants was significantly different from the single mutants, efcab-7 and cdkl-1. The double mutants exhibited a notable decrease in particle velocity, indicating a substantial impairment in the transport process within this segment of the cilia. This significant reduction highlights the combined detrimental effect of both mutations on IFT efficiency in the middle segment.

In retrograde part, IFT particles of efcab-7 mutants did not significantly change. Whereas both double mutants have significantly differ compared to single mutants.

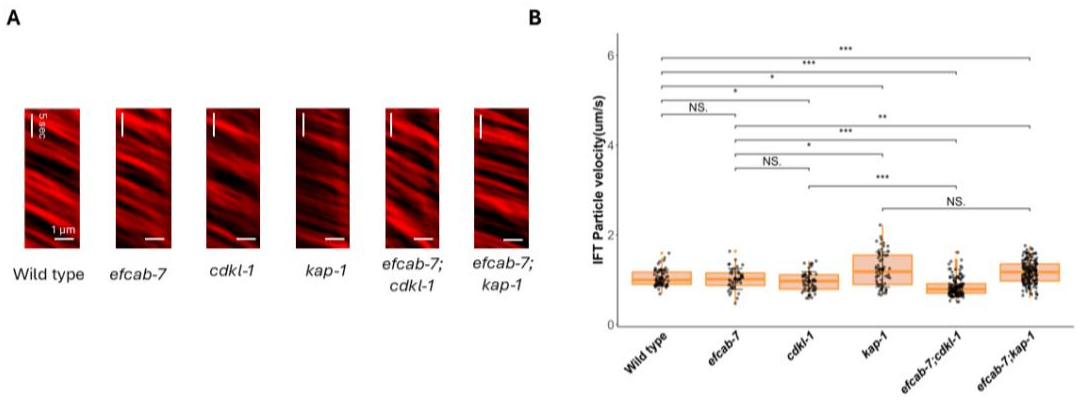


Figure 3.7 IFT particles velocity in distal segment of cilia. Kymographs of IFT particle movement in the middle segment of cilia for various genetic conditions: Wild type, efcab-7, cdkl-1, kap-1, efcab-7;cdkl-1, and efcab-7;kap-1. Each kymograph includes a scale bar indicating 1 µm and a time scale of 5 seconds.

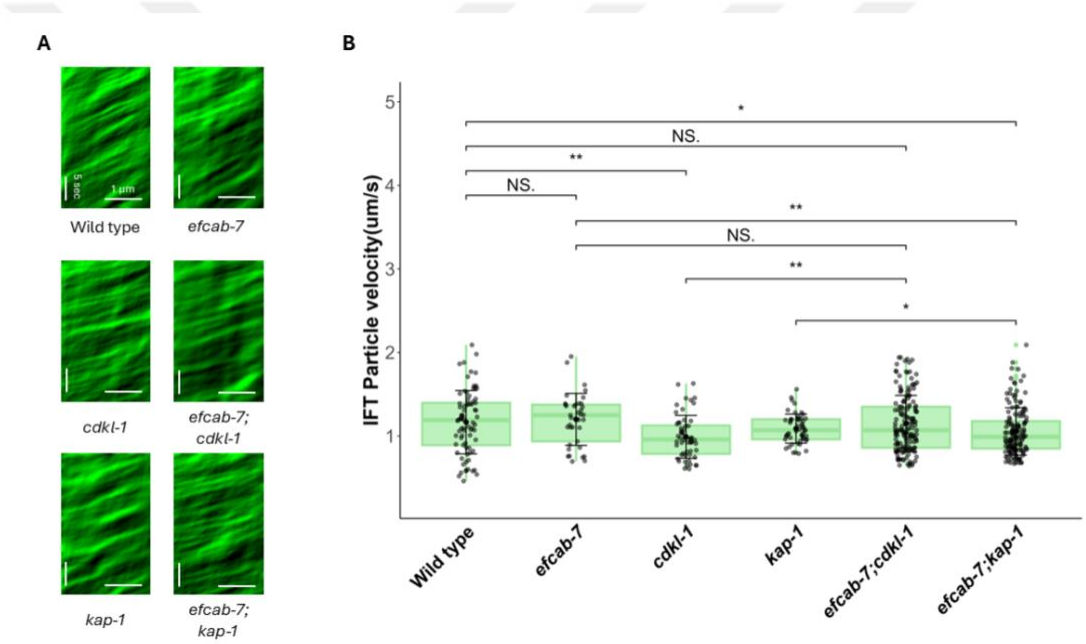


Figure 3.8 IFT particles velocity in retrograde. Kymographs of IFT particle movement in the middle segment of cilia for various genetic conditions: Wild type, efcab-7, cdkl-1, kap-1, efcab-7;cdkl-1, and efcab-7;kap-1. Each kymograph includes a scale bar indicating 1 µm and a time scale of 5 seconds.

3.5 ELMOD-3 does not leak into the cilia in *efcab-7* mutants

ELMOD-3 localizes in periciliary membrane compartment (PCMC) and is not leak to cilia. But if cilia have any defect, ELMOD-3 can leak into the cilia. *efcab-7* localizes in base of the cilia so it made us think that *efcab-7* may have a function in ciliary gate. To

investigate that *efcab-7*(ELMOD-3::GFP; TBG-1::MCHERRY+rol) strain was generated but didn't observe any leak into cilia.

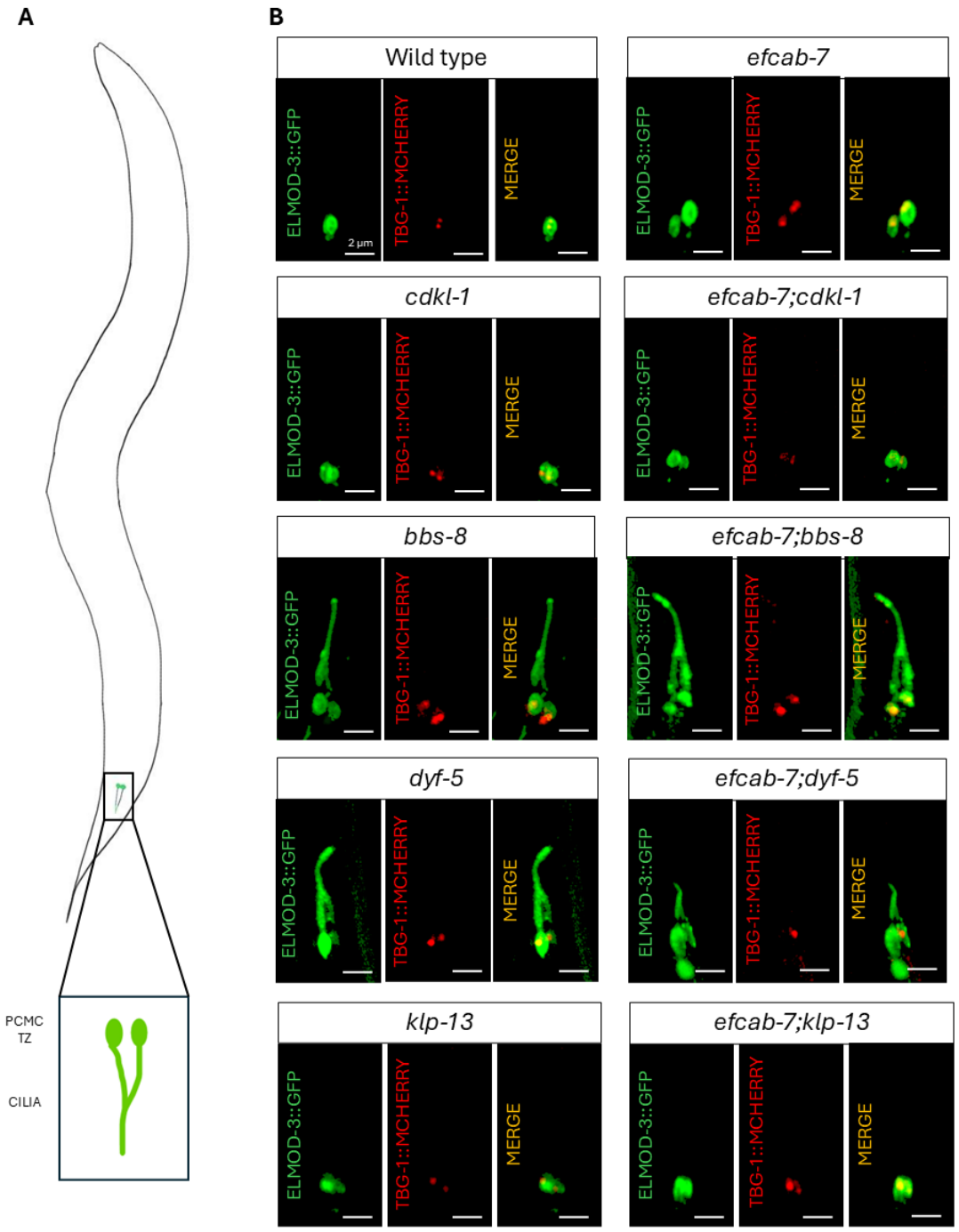


Figure 3.9 ELMOD-3::GFP didn't leak into cilia. A) Representative image of *C. elegans* and PHA/PHB cilia. B) Compound image of ELMOD-3::GFP in wild type and different mutants. scale bar indicating 2 µm

3.6 *efcab-7* mutants are short but *efcab-7;cdkl-1* mutants are shorter

During this experiments, it was observed that *efcab-7;cdkl-1* double mutants are shorter than wild type. Measurement aligned with this observation. *efcab-7* and *cdkl-1* mutants shorter than wild type but *efcab-7;cdkl-1* double mutants even shorter than single mutants. This body shortening indicates that both single mutants independently impact worm body length. However, double mutants have significantly shorter body lengths compared to single mutants.

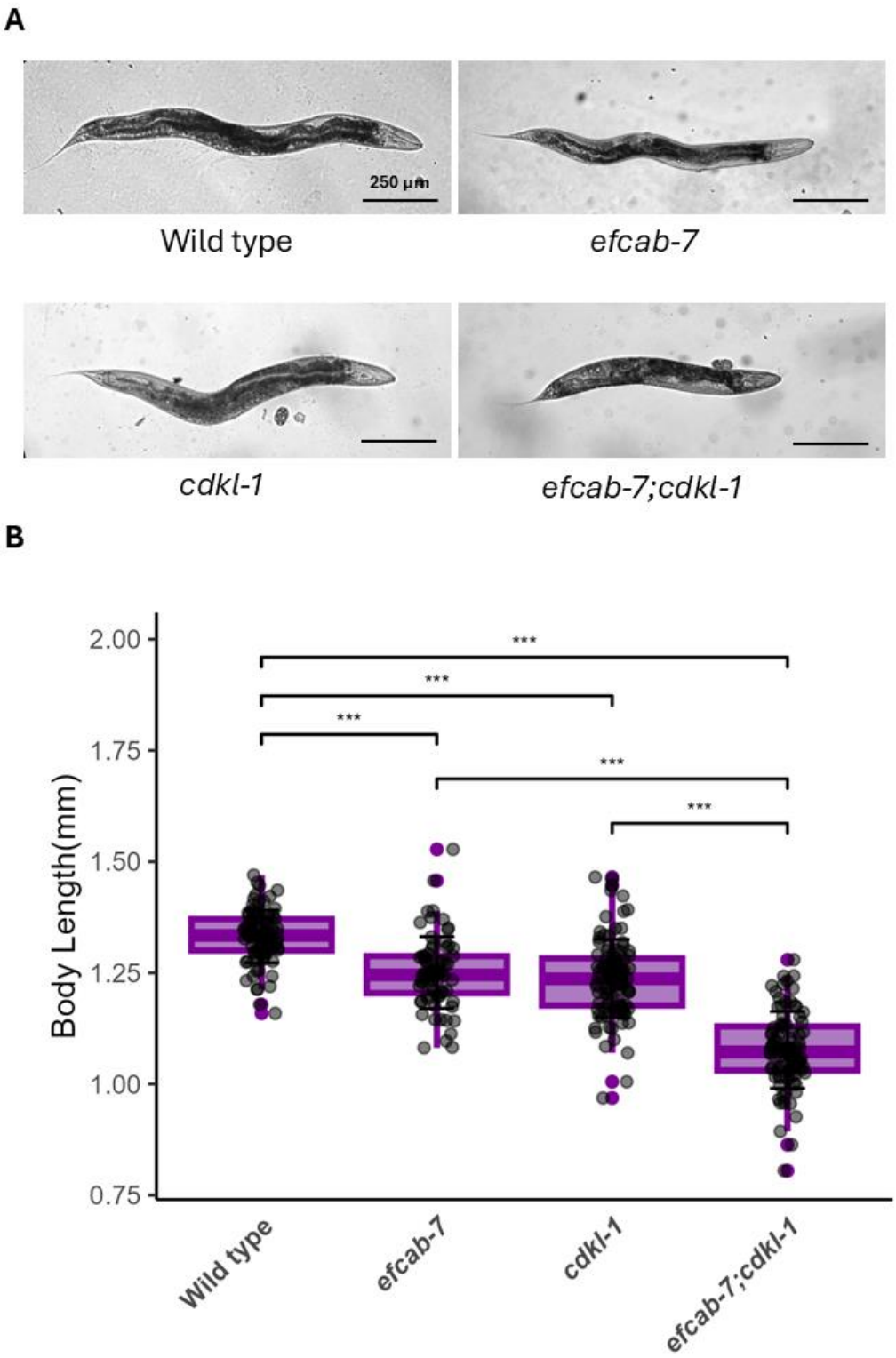


Figure 3.10 Body length. A) Compound microscope images of wild type and mutants. Scale bar indicating 250 µm. B) Body length measurement graphic

3.7 *efcab-7* mutants have immobility defect

During the experiment, it was noticed *efcab-7* mutants walk uncoordinated. This immobility defect surprisingly was not observed in *efcab-7;cdkl-1* double mutants. This unexpected finding suggests a functional interaction between these two genes, highlighting a potential compensatory mechanism in the double mutants. To analyze this walking defect, one-minute videos were recorded using a stereomicroscope, and the walking patterns were extracted using the Fiji program.

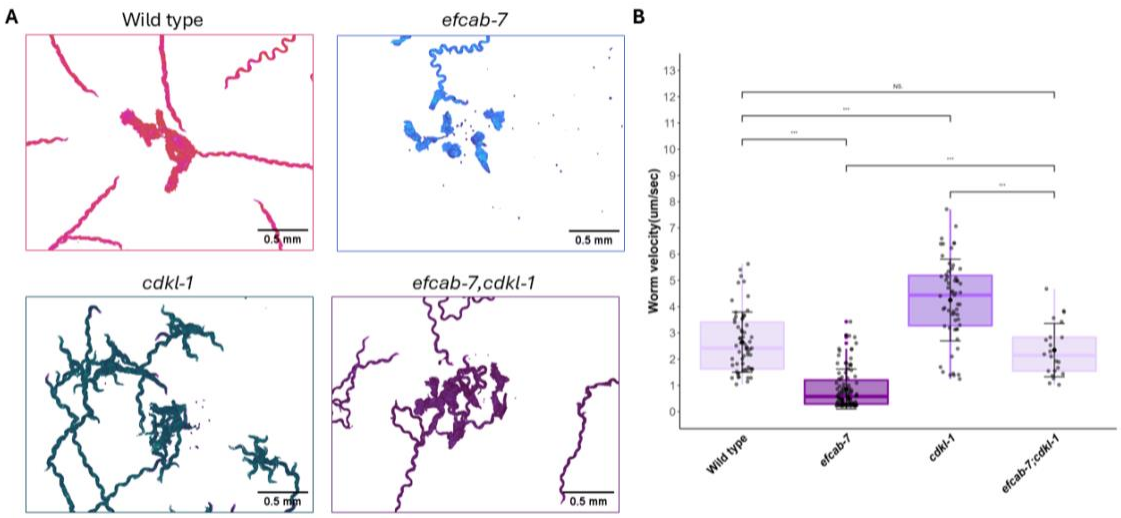


Figure 3.11 *efcab-7* mutants have immobility defects. A) Walking pattern of wild type and mutants. Scale bar is indicating 0.5 μm B) Quantitative results of worms walking speed

3.8 *efcab-7* mutants have fewer axons

A walking defect can arise from various causes, such as muscular or neuronal. To determine whether walking defect was caused by axonal abnormalities, *efcab-7*(unc-25::gfp+lin-15(+)) strain was generated. This marker is used to visualize GABAergic neurons and their axons. Additionally, it was obtained *efcab-7;cdkl-1*(unc-25::gfp+lin-15) to investigate if there is any rescue in double mutant. Results are showed us efcab-7 mutant has less axons compare to wild type and double mutant. Somehow in double

mutant rescued the walking defect and axonal abnormalities.

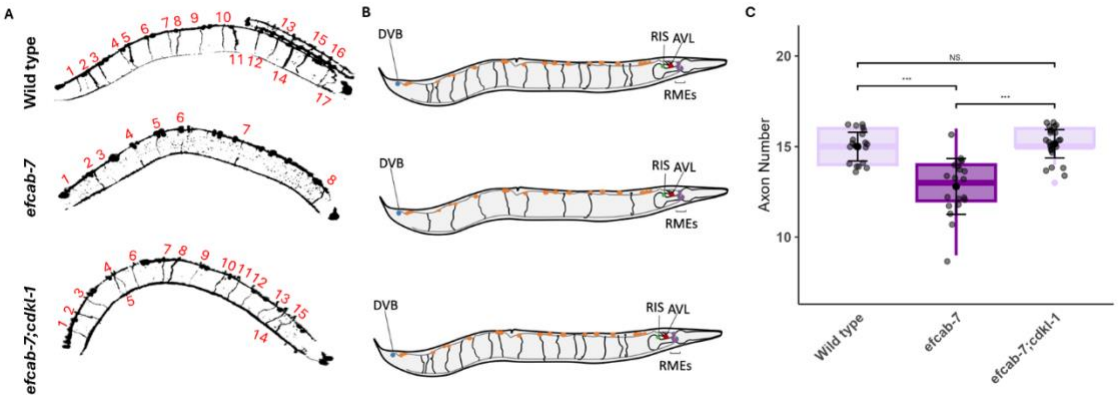


Figure 3.12 Axon numbers A) Confacal image of *unc-25::gfp* marker in wild type, *efcab-7* and *efcab-7;cdkl-1* double mutant. B) Quantitative result of axon number for wild type, *efcab-7* and double mutant

Chapter 4

Discussion

Rare diseases affect more people than expected. Ciliopathy is one of the rare disease and it causes by defective cilia. There is two types cilia so ciliopathies can divided two subcategories; motile ciliopathies and primary ciliopathies. Both of them hae different symptoms depend on functional difference. Ellis van Creveld is a primary ciliopathy and in most cases due to mutations in EVC and EVC2 genes. EVC and EVC2 proteins form a subcomplex and EVC2 protein binds this complex to EFCAB7-IQCE subcomplex. EFCAB7 expresses fallopian tubes, skeletal muscle retina and testis cells' cilia. Because of relationships between EFCAB7 and EVC-EVC2 complex, it may be think EFCAB7 mutation can result in EVC syndrome but there is no evidence. In 2023, it was documented that EFCAB7 mutation cause nonsyndromic postaxial polydactyly yet there is poor knowledge about EFCAB7. EFCAB7 and cilia relationship is also unclear. In this study, we investigated EFCAB7-cilia relationship using *C elegans* as model organism.

The efcab-7 mutants had shorter cilia as compared to wild types. This finding supports the hypothesis that EFCAB7 has a structural role in the maintenance of cilia. IFT particle number and velocity, however, did remain largely unchanged. So, EFCAB7 does not directly affect the transport mechanisms of intraflagellar transport but has an effect on other structural or regulatory elements of height ecilia. Moreover, the lack of ELMOD-3 leakage into cilia in efcab-7 mutants demonstrated that the ciliary gate was intact and so there were no compartmentalization defects at the transition zone.

The study also uncovered a walking defect in *efcab-7* mutants, characterized by uncoordinated movement, which was interestingly rescued in *efcab-7;cdkl-1* double mutants. This extraordinary observation indicates the presence of an important compensatory relationship between EFCAB7 and CDKL-1, possibly through alternative pathways or interactions that restore motor function and axonal structure. Visualization of GABAergic neurons and their axons using *unc-25::gfp* revealed that *efcab-7* mutants

had fewer axons than the wild type, a phenotype also ameliorated in the double mutants. This suggests that the observed walking problem may cause from neuronal defects rather than muscular dysfunction.

Interestingly, axon numbers in efcab-7;cdkl-1 double mutants were normalized, suggesting genetic compensation. Interaction between EFCAB-7 and CDKL-1 could be a new regulatory mechanism for axonal development and maintenance and consequently, provide a better understanding of the concept of genetic resilience in other neurodevelopmental disorders.

These results contribute to a growing understanding of the multifunctional roles of EFCAB7 in ciliary and neuronal biology. They highlight the importance of genetic context in modulating phenotypes, paving the way for future investigations into compensatory mechanisms and therapeutic targets. Further studies should delve into the molecular interactions between EFCAB7 and CDKL-1, exploring whether these findings are conserved in other models and how they translate to human ciliopathies and associated neurodevelopmental disorders.

Chapter 5

Conclusions and Future Prospects

5.1 Conclusions

Cilia of *efcab-7* mutants shorter than wild type, but the mechanism of it still unknown. efcab-7 localize base of cilia. That made us think that efcab-7 mutation may affect ciliary cargo and we examined IFT particles number and velocity. *efcab-7* mutants showed decreased number and velocity of IFT particles. We hypothesized that *efcab-7* may also have role in ciliary gate and used ELMOD-3::GFP transgenic to investigate that. ELMOD-3 localize in TZ and it doesn't get in cilia in wild type. There was not observe any leak into cilia in *efcab-7* mutants.

In meanwhile, we noticed efcab-7 mutants have immobility defect. This defect can caused by neuronal or muscle related. To understand that we use an axon marker; unc-25::gfp. And we observed *efcab-7* mutant have fewer axon. Meanwhile double mutant has the same number of axons as the wilt type. This indicates that the immobility defect may be neuronal.

5.2 Social Impact and Contribution to Global Sustainability

Rare diseases, affecting a small percentage of the population, often go undiagnosed or misdiagnosed due to their complexity and the lack of widespread knowledge. However, advancements in gene characterization have begun to change this landscape, offering profound social impacts and contributing significantly to global sustainability.

The characterization of genes associated with rare diseases has a transformative effect on patients and their families. Early and accurate diagnosis through genetic testing can alleviate the prolonged uncertainty and emotional distress that many families face. For instance, identifying the genetic basis of a rare disease can lead to targeted therapies, improving the quality of life and potentially extending the lifespan of affected individuals1. Moreover, understanding the genetic underpinnings of these diseases can reduce the stigma often associated with undiagnosed conditions, fostering a more inclusive society.

Additionally, gene characterization empowers patient advocacy groups. These groups play a crucial role in raising awareness, funding research, and influencing policy changes. By providing a clear genetic diagnosis, these groups can better advocate for the needs of patients, ensuring they receive appropriate care and support. This advocacy is essential in driving research and development, leading to innovative treatments and therapies that can benefit not only those with rare diseases but also the broader medical community.

From a sustainability perspective, the advancements in gene characterization contribute to the United Nations' Sustainable Development Goals (SDGs), particularly Goal 3: Good Health and Well-being. By improving the diagnosis and treatment of rare diseases, we can reduce healthcare costs associated with misdiagnosis and ineffective treatments3. This efficiency in healthcare resource utilization is crucial for sustainable health systems, especially in low- and middle-income countries where resources are often limited.

Furthermore, the global collaboration required for rare disease research fosters international partnerships and data sharing. Initiatives like the Global Commission to End the Diagnostic Odyssey for Children with a Rare Disease highlight the importance of cross-border cooperation in addressing these challenges1. Such collaborations not only accelerate scientific discoveries but also promote equity in healthcare, ensuring that advancements benefit populations worldwide, regardless of their economic status.

In conclusion, the characterization of genes associated with rare diseases has far-reaching social impacts and contributes significantly to global sustainability. By improving diagnosis, treatment, and patient advocacy, and fostering international collaboration, we can create a more inclusive and equitable healthcare system that benefits all.

5.3 Future Prospects

In efcab-7;cdkl-1 double mutant can walk normally whereas efcab-7 single mutants can not. Understanding the underlying mechanism can help develop a treatment neuronal or muscle related diseases.

Future research should focus on elucidating the specific biochemical and cellular processes that are altered in the absence of EFCAB-7 and how CDKL-1 compensates for these changes. This could involve detailed studies of gene expression, protein interactions, and signaling pathways in both single and double mutants. Additionally, exploring the role of these genes in different types of neurons and muscle cells could reveal tissue-specific mechanisms that contribute to the observed phenotypes.

By uncovering these mechanisms, we can identify potential therapeutic targets for the treatment of neuronal or muscle-related diseases. For instance, if certain signaling pathways are found to be disrupted in efcab-7 single mutants but restored in double mutants, these pathways could be targeted with drugs or gene therapy to mimic the compensatory effects of CDKL-1. Moreover, understanding how these genes interact could also shed light on broader principles of genetic compensation and resilience, which could be applied to other genetic disorders.

Ultimately, this research has the potential to lead to the development of novel treatments that improve motor function and quality of life for individuals with neuronal or muscle-related diseases. It also underscores the importance of studying genetic interactions and compensatory mechanisms as a strategy for discovering new therapeutic approaches.

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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™GP 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	ZRQSVP800
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
MgCl2	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 neddle	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	SCILOGEX	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

2014 – 2018	B.Sc., Primary School Mathmetics Teaching, Akdeniz University, Antalya, TURKEY
2018 – 2022	B.Sc., Molecular Biology and Genetics, Yıldız Technica University, Istanbul, TURKEY
2022 – Present	M.Sc., Bioengineering, Abdullah Gül University, Kayseri, Türkiye