TURKISH REPUBLIC OF NORTH CYPRUS
NEAR EAST UNIVERSITY
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GÜLTEN TUNCEL

POSTGRADUATE THESIS IN MOLECULAR MEDICINE

DEPARTMENT OF MEDICAL BIOLOGY

SUPERVISOR
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The Directorate of Institute of Graduate Studies,
This study has been accepted by the Thesis Committee in Molecular Medicine Program as a PhD Thesis.

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STATEMENT (DECLARATION)

Hereby I declare that this thesis study is my own study, I had no unethical behavior in all stages from planning of the thesis until writing thereof, I obtained all the information in this thesis in academic and ethical rules, I provided reference to all of the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behaviour of breeching patent rights and copyright infringement during the study and writing of this thesis.

Gülten Tuncel

ACKNOWLEDGEMENTS

I would first like to express my sincere gratitude to my supervisor Assoc. Prof. Dr. Mahmut Çerkez Ergören for his endless patience, great support and immense knowledge. He always guided and encouraged me to remain on the right path and do the right thing even when the road got tough. Without his persistent help, the goal of this project would not have been realized.

I would like to thank my thesis comittee members; Prof. Dr. Gamze Mocan, Prof. Dr. Munis Dündar, Assoc. Prof. Dr. Şehime Gülsün Temel and Assoc. Prof. Dr. Umut Fahrioğlu. I also wish to thank all the people, especially Dr. Engin Deniz, Irmak Sakın and Assoc. Prof. Dr. Oktay Kaplan, whose assistance was a milestone in the completion of this project.

I am grateful to Assoc. Prof. Dr. Pınar Tulay for her motivational support and for sharing her knowledge whenever I needed. I also want to thank Ayşegül, Nevrez and members of the NEU Hospital Medical Genetics lab for their support and warm friendship. Narden and Seray, you are amazing friends, many thanks for walking beside me all the time. I would like to thank Meryem for making me smile even in most desperate days of our three-year-journey together. This thesis was produced during the Covid-19 pandemic. Nihan, Hazel and all team members- it was and is a great pleasure working with you. Sleepless nights of the Covid-19 lab will always be remembered. I am always particularly thankful to my spiritual sister, Mine Hüryaşar, who helps me stay sane through my life since -I do not remember when- times. It is a great pleasure to grow old with you.

I must express my profound gratitude to my husband Mustafa Dereboylu, who has always been by my side to motivate and support me through all ups and downs of these four years. Finally, I wish to offer my deepest thanks to my parents Şaziye Tuncel and Şeref Tuncel for making me who I am today and for making all these possible with their endless love, trust, support and patience by all means. I also warmly appreciate the support and understanding of my extended family.

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ABBREVIATIONS

5-HTR1A: 5-hydroxytriptamine receptor 1A

AAV: Adeno-associated viruses

AHI1: Abelson helper integration site 1

ALMS: Alström syndrome

ARF: ADP-ribosylation factor

ATP: Adenosine tri-phosphate

AuNP: Gold nanoparticles

BBS: Bardet-Biedl syndrome

C.elegans: Caenorhabditis elegans

C2: protein kinase C conserved region 2

cAMP: Cyclic adenosine monophosphate

Cas: CRISPR associated protein

CC2D1A: Coiled-coil and C2 domain-containing protein 1A

CRISPR: Cluster of regularly interspaced palindromic repeats

dbSNP: Database of single nucleotide variants

DSB: Double strand breaks

EEG: Electroencephalogram

EV: Empty vector

ExAC: Exome Aggregation Consortium database

GFP: Green fluorescent protein

HGMD: Human Gene Mutation Professional Database

HR: Homologous recombination

IDA: Inner dynein arms

IFT: Intraflagellar transport

IKK: IκB kinase complex

Indel: Insertion-deletion

JS: Joubert Syndrome

LB: Luria Bertani medium

LNP: Lipid nanoparticles

MAF: Minor allele frequency

MKS: Meckel-Gruber syndrome

MRI: Magnetic resonance imaging

NF-κB: nuclear factor κ enhancer binding protein

NHEJ: Non-homologous end-joining

NMR: Non-syndromic mental retardation

NTC: No template control

NVD: Normal vaginal delivery

OCD: Obsessive-compulsive disorder

ODA: Outer dynein arms

OMIM: Online Mendelian Inheritance in Man

PAM: Protoscpacer adjacent motif

PCD: Primary ciliary dyskinesia

PKA: protein kinase A

PKD: Polycystic kidney disease

RP: Retinitis pigmentosa

sgRNA: single guide RNA

SNP: single nucleotide polymorphism

TAL: Transcription-activator-like

TALENs: TAL-effector nucleases

TBE: Tris-borate/EDTA

UIC: Uninjected control

UV: Ultraviolet

WES: Whole exome sequencing

WISC: Wechsler Intelligence Scale for Children

X.leavis: Xenopus laevis

X.tropicalis: Xenopus tropicalis

ZFNs: Zinc finger nucleases

SUMMARY

Cilia are protrusions on cell surface, present on almost all cell types in higher eukaryotic organisms and are involved in several processes including neuronal development, cell motility, establishment of left-right asymmetry during embryonal development, clearance of airways and as receptors in internal organs like the kidneys and pancreas. Defects in cilia structure or function result in a group of complex disorders, called ciliopathies. As cilia are present in a variety of cell types and are involved in various cellular processes, ciliopathies can manifest in multiple organ systems with varying severity. Currently there are multiple established ciliopathy genes and developing molecular techniques accelerated the process of identification of new genes. Here we present *CC2D1A* as a novel ciliopathy gene, which was first identified in three patients with ciliopathy-related phenotypes. Gene knock-out with CRISPR-Cas9 system in two model systems, *Caenorhabditis elegans* and *Xenopus tropicalis* revealed the involvement of *CC2D1A* in cilia. *CC2D1A* knock-out was lethal in *C.elegans*, whereas mutant *X.tropicalis* represented disturbed cilia-driven fluid flow over their ciliary membranes, reduced numbers of cilia and disturbed craniofacial structures. Additionally, CC2D1A was shown to be localized in the dendrite and cilia region of *C.elegans* tail. These results suggests that the protein is involved in ciliogenesis and pathogenic variations directly contribute to ciliopathy-related phenotypes.

ÖZET

Silyalar, ökaryotik canlıların neredeyse tüm hücre tiplerinde bulunan, hücre zarının küçük çıkıntıları şeklinde görünen yapılardır. Silyalar nöronal gelişim, hücre hareketliliği, embriyonal gelişim sırasında sol-sağ organ asimetrisinin oluşması, hava yollarının temizlenmesi ve böbrekler ve pankreas gibi iç organlarda da reseptör görevi yaparak çeşitli süreçlerde ve görevlerde rol alırlar. Silyanın yapısal veya işlevsel kusurları, siliyopati adı verilen bir grup kompleks genetik hastalığa neden olur. Silyalar çeşitli hücre tiplerinde bulunduğundan ve çeşitli hücresel süreçlerde yer aldığından, siliyopatiler çoklu organ sistemlerinde değişen şiddette fenotipik özelliklere yol açabilirler. Bugüne kadar literatüre bildirilen çok sayıda siliyopati geni vardır ve gelişen moleküler teknikler, yeni genlerin tanımlanması sürecini hızlandırmıştır. Bu çalışmada, siliyopati ile ilişkili fenotipleri olan üç hastada ilk kez tanımlanan yeni bir siliyopati geni olarak *CC2D1A*'yı sunuyoruz. *Caenorhabditis elegans* ve *Xenopus tropicalis* olmak üzere iki model sistemde CRISPR-Cas9 teknolojisi kullanılarak ile *CC2D1A* geninde oluşturulan fonksiyon kaybı mutasyonları genin silya ile ilişkili olduğunu ortaya koymuştur. Homozigot fonksiyon kaybı *C.elegans* modelinde öldürücü etkiye yol açmıştır. *X.tropicalis* modelinde ise *CC2D1A* fonksiyon kaybı silyalı deri üzerinde yönlü sıvı akışında bozulmaya, silya sayısında azalmaya ve kraniyofasiyal yapı bozukluklarına yol açmıştır. Ayrıca, CC2D1A proteinin *C.elegans* kuyruğunun dendrit ve silya bölgesinde localize olduğu gösterilmiştir. Bu sonuçlar, proteinin silya oluşumunda rol aldığını ve patojenik varyasyonlarının siliyopati ile ilgili fenotiplere doğrudan katkıda bulunduğunu göstermektedir.

CHAPTER ONE: INTRODUCTION

1.1. Introduction

Ciliopathies comprise a group of complex disorders associated with defective cilia. Cilia are protrusions on cell surface, present on almost all cell types in higher eukaryotic organisms and are involved in several processes including neuronal development, cell motility, establishment of left-right asymmetry during embryonal development, clearance of airways and as receptors in internal organs like the kidneys and pancreas. Therefore, ciliopathies can present themselves with a broad range of phenotypes (Mitchison and Valente 2017). Some disorders that are classified as ciliopathies include the Joubert syndrome, Bardet-Biedl syndrome, nephronophthisis and retinal-renal syndromes (Hildebrandt, Benzing, and Katsanis 2011). There are almost 200 established ciliopathy-related genes and studies in the field are revealing more to be involved in cilia structure or function and result in dysfunctional cilia when mutated.

In this study we introduce three patients that have clinical phenotypes associated with ciliopathy diseases and have homozygous variations in Coiled-coil and C2 domain-containing protein 1A *(CC2D1A)* gene. One patient was diagnosed with Joubert syndrome co-occurring with severe obsessive-compulsive disorder. Whole exome sequencing revealed a likely-pathogenic homozygous variation in Abelson helper integration site 1 *(AHI1)* gene, which is an established Joubert syndrome gene, as well as another homozygous variation in *CC2D1A* gene. Other two patients are siblings, with no definitive clinical diagnosis but have ciliopathy phenotypes. Whole exome sequencing of the siblings revealed a homozygous mis-sense variation in *CC2D1A* gene. As all these patients have clinical symptoms related with ciliopathy disorders, we aimed to investigate whether *CC2D1A* gene has a role in ciliogenesis or cilia function. *CC2D2A*, which is a homolog of our gene of interest, is a known Joubert syndrome gene, however any relation of *CC2D1A* with cilia structure or function was not reported to the literature previously.

For this purpose, we analyzed *CC2D1A* gene and protein expression as well as the cilia structure in patient and control samples. Two different animal model systems, *Caenorhabditis elegans (C.elegans)* and *Xenopus tropicalis (X.tropicalis)* were used to study protein localization and effect of *CC2D1A*-knockout on cilia structure and function as well as other phenotypic outcomes.

1.2. General Information

Ciliopathies are complex genetic disorders that result in structural or functional defects in cilia. Cilia are present in almost all cell types in eukaryotic multicellular organisms and are involved in many processes. These include cell motility, neuronal development, establishment of left-right asymmetry, as chemo- and osmo- receptors as well as mucociliary clearance and ocular functions (Mitchison and Valente 2017). Not surprisingly, dysfunctional cilia result in a diverse range of phenotypes affecting several organs of the human body.

There are several genes that have known functions in ciliogenesis and cilia function and more are being established. Depending on the function of the gene, type of variation, presence of additional variations and other factors patients can possess more severe or milder phenotypes. In general, the syndromes that are listed as ciliopathy syndromes are rare disorders, affecting few individuals but collectively there are millions of people being affected (Reiter and Leroux 2017). Therefore, identification of related genes and their functions are important to build a more comprehensive understanding of the disease and for development of potential therapies.

Development of next generation sequencing techniques and genome editing technologies greatly accelerated the identification of novel genes involved in rare and complex diseases and creation of model organisms to reveal their function *in vivo*.

1.2.1. Cilia

Cilia by definition are microscopic hair-like protuberances on the outside of eukaryotic cells. It is structurally preserved in unicellular organisms such as Chlamydomonas reinhardtii and Tetrahymena thermophile and in multicellular organisms such as

Caenorhabditis elegans, Drosophila melanogaster and vertebrates. They are generally referred as the oldest known cell organelles as they were described back in 1675 by Antony Van Leeuwenhoch, who used a form of light microscope to observe a living protozoan in rain water. He described the organism as having a flat belly "provided with diverse incredibly thin feet, or little legs, which moved very nimbly" (Satir 1995). The term "cilia" originates from Latin and means eyelash. It is thought that it was first used by O. F. Muller back in 1786 (Muller 1786).

With the help of technical advances in microscopy, Jan Purkyne and his student Valentin studied amphibian ciliary movement and the cilia of the mammalian oviduct, further proposing formulations for the mechanism of ciliary motility in 1830s (Purkyne and Valentin 1835; Satir 1995). They thought that cilia were hollow structures, water was pushed through them and small muscles at their base were responsible for their movement. Later, in 1900s Dellinger proved that cilia were not hollow, they were formed by a number of filaments (Dellinger 1909). Sir James Gray contributed to the knowledge of motile cilia kinetics by publishing microphotographs of the phases of one ciliary beat and beating of cilia on sea urchin spermatozoan. He also formulated the stroboscopic technique to accurately measure ciliary beat frequency (Gray 1930, 1955). It was not until 1950s, when electron microscopy was invented, the structure and mechanism of motion of motile cilia were correctly described.

As cilia were initially defined under the light microscope by their motility, it was thought that this was their only function for many decades. However, identification of non-motile cilia with the use of advanced microscopy techniques permanently changed the knowledge (Kovalevskij 1867; Zimmermann 1898). Know we know that cilia are present on almost all cell types in the vertebrates and are found ubiquitously across species from ancient protozoa to nematodes possessing diverse critical functions (Mitchison and Valente 2017).

Upon identification of non-motile cilia, motile cilia were referred as 'secondary cilia' and non-motile cilia as 'primary cilia'. Despite sharing some common structural

features, primary and secondary cilia essentially have differences in structure and function.

1.2.1.1. Cilia Function

1.2.1.1.1. Motile Cilia Function

Motile cilia, or secondary cilia, as its name suggests are motile and are responsible for locomotion of cells, creating directional fluid flow and removal of contaminants from the organs either by causing currents in the surrounding fluid or by providing propulsion. Orientation or the polarity of motile cilia is crucial for normal ciliary function (Marshall and Kintner 2008; Mitchell et al. 2007).

Some cells contain hundreds of motile cilia. Ciliates, which are single-celled protozoans, are a diverse group of multiciliated organisms that use the cilia for locomotion and were used as model organisms as their cilia are molecularly and structurally conserved in higher eukaryotes (Bayless, Navarro, and Winey 2019). In vertebrates, epithelial surfaces of the respiratory tract, middle ear, ventricles of the brain and Fallopian tubes in female reproductive tract are lined with motile cilia. In the respiratory tract motile cilia are involved in the removal of contaminants by creating directional outward flow in the surrounding fluid by coordinated beating (Vladar et al. 2015). In the brain ventricles, cerebrospinal fluid flow created by motile cilia is essential for normal spinal curvature and to provide directional cues for brain development (Grimes et al. 2016). Coordinated ciliary beat in the Fallopian tube plays critical role in tubal transport to support fertilization and early embryogenesis (Lyons, Saridogan, and Djahanbakhch 2006).

Conversely, some cells such as the ones in the embryonic node contain only one motile cilia, which generates unidirectional flow critical for the establishment of left-right patterning of the organs together with non-motile cilia (Yoshiba et al. 2012). Sperm cells, on the other hand, possesses a single flagellum that are specialized motile cilia, which enables them to move within the female reproductive tract (Lyons et al. 2006).

1.2.1.1.2. Non-motile Cilia Function

For decades, all attention was on motile cilia as their function was readily observable and non-motile cilia appeared nonfunctional. However, non-motile primary cilia are present ubiquitously on several cell types including kidney epithelial cells, neuronal cells, cone and rod cells in the vertebrate neural retina, cholangiocyte cells of liver and others. Further studies revealed that non-motile cilia act as a sensory organelle and is also involved in various developmental signaling pathways (Anderson et al. 2008; Mitchison and Valente 2017; Pazour and Witman 2003).

The first non-motile cilia to be observed by electron microscopy were the ones on neuronal cells during examinations of brain tissue sections. Almost all neurons display a primary cilium. It took decades to build an understanding of the relevance of this observation. Evidence suggests that primary cilia are involved in developing and adult neural tissue as a key signaling organelle (Del Cerro, Snider, and Lou Oster 1969; Lee and Gleeson 2010). It was revealed that they play great role in cerebellar development and hippocampal neurogenesis through the transduction of Sonic Hedgehog (Shh) signals, which is critical for the proliferation of cerebellar granule neurons and for regulation of progenitor cell maintenance in telencephalic stem cell niches (Corbit et al. 2005; Lee and Gleeson 2010; Pozniak and Pleasure 2006). Model organisms, especially *C.elegans* provided more comprehensive information about neuronal primary cilia. It is suggested that extracellular signals can regulate the architecture of neuronal cilia as it was observed that sensory signaling is required to maintain the size and shape of the specialized neuronal cilia in *C.elegans* olfactory epithelium (Mukhopadhyay et al. 2008).

In kidneys, non-motile cilia on epithelial cells are in direct contact with urine flow as they line the collecting ducts and the nephron tubule. They regulate certain intracellular pathways such as G-protein signaling, mTOR and Wnt signaling in response to changes in urine composition, osmolality and flow (Mitchison and Valente 2017). Similar to renal cilia, primary cilia protruding from the epithelial cells lining the biliary ducts in the liver act as chemo-, mechano- and osmoreceptors by sensing biliary lumen flow, osmolality and composition. Cilia transduce these signals through

modulation of intracellular cyclic adenosine monophosphate (cAMP) and calcium ions (Masyuk, Masyuk, and LaRusso 2008).

In the pancreas, primary cilia are present on different cell types including the ductal cells, α-, β- and δ- cells in the islets of Langerhans. Like in the liver and kidney, non-motile cilia in the pancreatic ductal cells act as sensors and regulate certain signaling pathways. Also, they are involved in key pathways such as Shh, Wnt and Notch during pancreas development (Lodh, O'Hare, and Zaghloul 2014).

Rod and cone photoreceptors of the vertebrate retina, namely the rod and cone cells, depend on the outer segment that is a highly specialized ciliary organelle. It is capable of detecting light through a complex structure of regularly stacked photopigment-filled membranous disks oriented along the axis of the incoming light, which are either fully internalized or in continuity with the plasma membrane. (Mitchison and Valente 2017).

1.2.1.2. Cilia Structure

To fully understand the function of cilia, its structure and involved proteins should be known. However, despite their ubiquity and importance, their protein composition is not yet fully identified.

Cilia protrude outward from the basal body on the cell surface and consists mainly of three different structural regions: axoneme, basal body, and the transition zone **(Figure 1.a)**. During ciliogenesis, a mother centriole migrates to the cell's apical surface and matures to form the basal body, which generates the axoneme composed of a microtubule cytoskeleton and associated proteins. Even though the basal body consists of a mother centriole and a daughter centriole, nucleation of microtubules is initiated from the mother centriole (Vieira et al. 2006). It was reported that the presence of centrioles correlates specifically with the presence of cilia rather than centrosomes, which suggest that the ancestral role of centrioles was directing cilia formation (Breslow and Holland 2019).

The transition zone, which can also be named as the ciliary gate, is located at the most proximal region of cilia and it controls the entry of proteins into and out of cilia.

The ciliary membrane, which surrounds the cilia, is continuous with the apical membrane of the cell but have a different composition hosting different proteins such as the ion channel TRPV4, polycystin 1 and 2 that are implicated in the polycystic kidney disease (Pazour and Witman 2003; Vieira et al. 2006).

Motile and non-motile cilia have structural differences that enable them to adapt their function. Mobile cilia and flagella, consist of nine outer doublet microtubules positioned along the membrane and a single pair located at the center (9+2 pattern) (Blacque, Cevik, and Kaplan 2008; Pazour and Witman 2003). The major components that attach to the secondary cilia microtubules include the outer and inner dynein arms (ODA and IDA), the radial spokes, central-pair projections and other protein complexes required for motility. Dyneins are the molecular motors that hydrolyze adenosine tri-phosphate (ATP) and lead the doublet microtubules to slide over each other asynchronously, yielding a helical beat. The radial spokes are involved in regulation of ciliary beat by interacting with the central pair microtubules and dyneins (Satir and Christensen 2007). So, the coordination mechanism between the central pair- radial spoke- dynein arms are of great importance for controlled cilial beating.

Axoneme of non-motile primary cilia consist of only nine pairs of microtubules surrounded by the membrane, lacking the central pair (9 + 0 pattern) **(Figure 1.b)**. As non-motile cilia do not assemble the central microtubule complex, dyneins and related proteins, they are unable to beat just like the mutants of motile cilia (Satir and Christensen 2007).

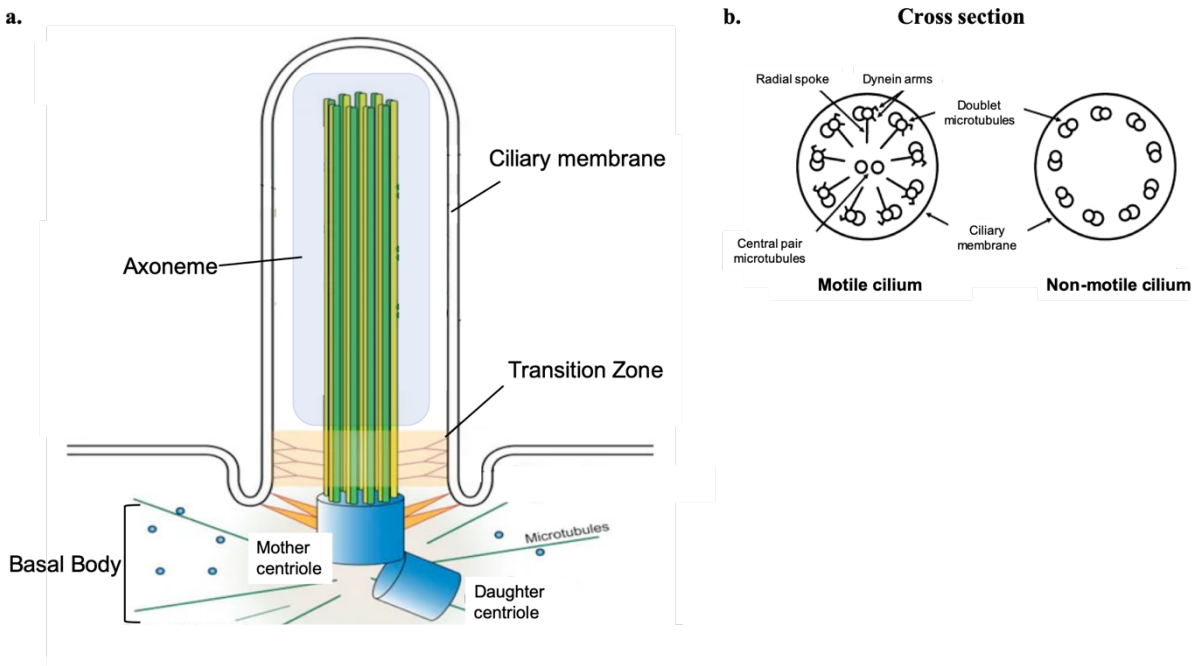

Figure 1.1. General structure of cilia. (ref)

a. Both motile and non-motile cilia are composed of three main parts as the axoneme, the transition zone and the basal body. Axoneme, which is composed of the microtubule cytoskeleton is surrounded by the ciliary membrane. The basal body is a special form of a centriole that consists of a daughter centrile and a mother centriole, which gives rise to the formation of axonemal microtubules.e **b.** Cross section of motile and non-motile cilia are represented. Both are composed of nine pairs of doublet microtubules surrounded by the ciliary membrane. Motile cilia additionally have a central pair, dynein arms and radial spokes that allow them to move. (Figure adapted from (Lai and Jiang 2020))

1.2.1.3. Ciliary Assembly and Intraflagellar Transport (IFT)

As cilia and flagella are not able to synthesize their own proteins, they rely on constant delivery of axonemal precursors from their site of synthesis to the axonemal assembly site at the ciliary tip. This is a process mediated by intraflagellar transport (IFT) (Pedersen and Rosenbaum 2008).

Delivery of ciliary cargo occurs sequentially. It involves sorting and packaging of cargo into carrier vesicles, docking and fusion of these vesicles with ciliary base and assembly of cilia from the base towards the tip. Ciliary targeting and assembly is

mediated by the action of multiple multiprotein complexes including IFT and the BBSome complex that are modulated by ADP-ribosylation factor (ARF) and Rab family proteins (Nachury et al. 2007; Waters and Beales 2011).

Cilia are assembled from the ciliary base by IFT, which utilizes two microtubule associated motor proteins: dynein and kinesins. Membrane receptors and structural components of the axoneme are transported in anterograde manner by the action of kinesin-2 motors along the outer doublet microtubules. The precursors and cargo are unloaded for assembly and use at the ciliary tip. Upon unloading, the empty IFT particles bind to recycled proteins and axonemal turn over products down the axoneme towards the cell body by the action of dynein-2 motor proteins retrogradely **(Figure 2)** (Pedersen and Rosenbaum 2008; Waters and Beales 2011).

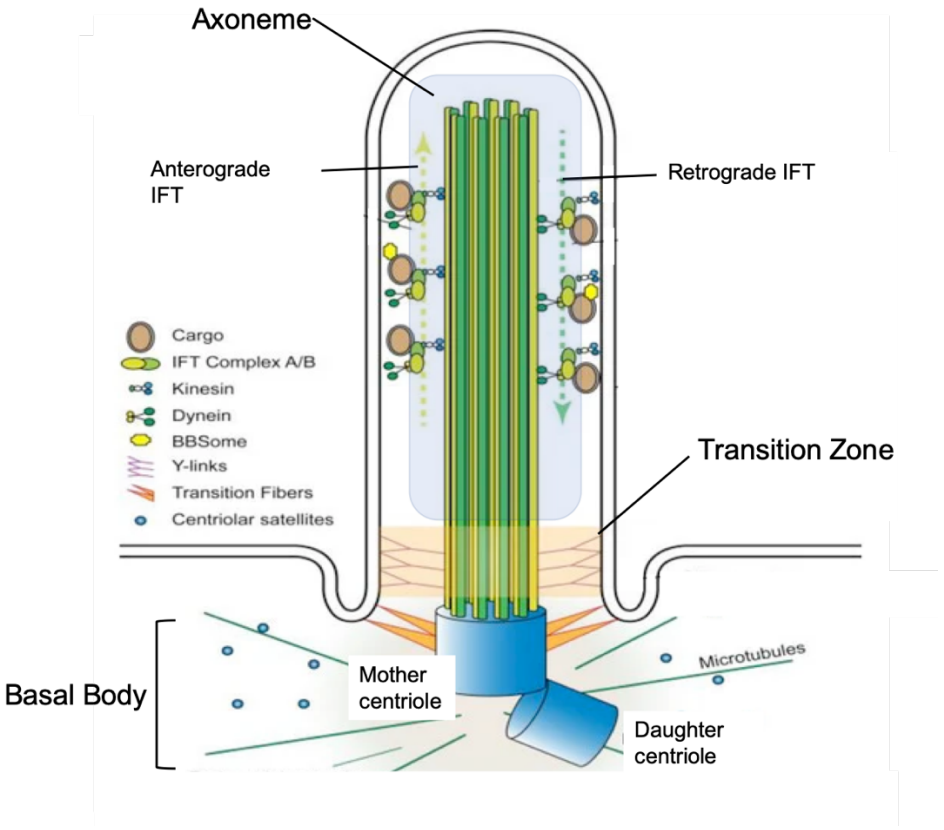

Figure 1.2. Intraflagellar transport (IFT) mechanism in the cilium. (ref)

Figure represents the anterograde and retrograde intraflagellar transport (IFT) mechanisms within the cilium. Cargo molecules are transported from the cytoplasm/ciliary base towards the ciliary tip by anterograde IFT. Used proteins and

cargo molecules are then loaded onto empty IFT complex and transported down towards the cytoplasm by retrograde IFT mechanism. Dynein and kinesins are the molecular motor proteins that are involved in the movement of molecules through the axoneme. (Figure adapted from (Lai and Jiang 2020))

In this context, it is important to note that proteins involved in IFT and related structures are crucial for ciliary assembly as well as cilia-mediated signaling.

1.2.2. Ciliopathies

Ciliopathies comprise a group of rare genetic disorders associated with ciliary dysfunction. Currently, 35 ciliopathy diseases are reported in the literature that include Bardet-Biedl syndrome (BBS), Joubert Syndrome (JS), autosomal recessive and dominant polycystic kidney diseases, Meckel-Gruber syndrome (MKS), Alström syndrome (ALMS). The number is increasing with over 190 established and over 240 candidate ciliopathy-associated genes and related syndromes (Reiter and Leroux 2017). Even though each of these are rare disorders, the number of affected individuals is high.

As cilia are present on almost all cell types, ciliopathies generally show a wide range of phenotypes in multiple organ systems including polydactyly, diabetes, retinal degeneration, mental retardation, hepatobiliary problems, obesity, deafness, laterality defects, skeletal dysplasia, all with different severities, demonstrating the complexity of ciliopathy diseases (Van der Heiden et al. 2011; Reiter and Leroux 2017). So rather than being distinct clinical entities, ciliopathies form a spectrum of disorders with genotypic and phenotypic overlaps.

In the last two decades, discovery of rare variants and molecular mechanisms in disease resulted in an increase in the number of researches in cilia and related diseases as well. Investigating ciliopathies enabled us to further characterize the molecules and mechanisms involved in ciliogenesis, the way transition zone functions in ciliary gating and how intraflagellar transport regulates cargo trafficking and signaling (Reiter and Leroux 2017).

An important characteristic feature of ciliopathy disorders is that the type of the two recessive mutations can determine the severity of the disease phenotype. In this context, protein truncating mutations are classified as 'strong' mutations, whereas missense mutations are 'weak'. In the presence of two strong mutations such as mutations in *CEP290 (NPHP6)* cause a severe, early-onset developmental disorder with a broad-range of organ involvement as in Meckel's syndrome, whereas in the presence of at least one weak mutation causes a milder, late-onset degenerative disorder with less organ involvement, such as a mild form of JS (Hildebrandt et al. 2011). However, other factors such as the involvement of mutations in other modifier genes or the combined effect of two or more recessive genes with heterozygous mutations (true oligogenicity) are also important in determination of genotype-phenotype correlations of ciliopathy phenotype. For example, the presence of heterozygous mutations in *NPHP8* or *NPHP6* in patients with homozygous *NPHP1* deletion results in additional eye or cerebellar involvement in phenotype (Hildebrandt et al. 2011; Leitch et al. 2008).

As mentioned, there are currently over 190 known ciliopathy associated genes and more are being listed. For example the BBSome complex genes *(BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9)* and BBS chaperone complex genes *(BBS6, BBS10, BBS12)*, which are involved in regulation of cilia composition and in intraflagellar trafficking, are common genes that are mutated in BBS (Manara et al. 2019). *PRPF31, PRPF3, PRPF8, RPE65, PDE6A/B* and *RP25* are commonly mutated in autosomal dominant or autosomal recessive retinitis pigmentosa (RP) (Parmeggiani et al. 2011). The two major genes associated with primary ciliary dyskinesia (PCD) are *DNAI1* and *DNAH5*, encoding proteins of outer dynein arm in cilia structure account for over 30% of all PCD cases. Additionally, *HYDIN, RSPH4A* and *RSPH9* in radial spokes, *CCDC151* and *CCDC114* in outer arm docking complex and *CCDC39* and *CCDC40* in dynein-regulatory complex are also involved in PCD (Horani et al. 2016). *MYO7A, CDH23, USH1C, USH2A* and *PCDH15* are common genes associated with Usher syndrome (Jaijo et al. 2007; Tazetdinov, Dzehemileva, and Khusnutdinova 2008).

Some genes, such as *CEP290, KIF7, NPHP1, TMEM67, IFT172* and *WDR19* are involved in more than one ciliopathy disorder phenotypes (Wheway et al. 2019).

1.2.2.1. Joubert Syndrome: a ciliopathy disease

Joubert syndrome (JS) (OMIM #213300) is a rare neurodevelopmental genetic disorder, inherited in an autosomal recessive or X-linked manner. Estimated prevalence of the disease is 1:100,000 live births worldwide (Romani, Micalizzi, and Valente 2013). The disease manifest itself by a malformed brain stem causing the characteristic molar-tooth sign in brain magnetic resonance imaging (MRI). As this region of the brain controls breathing and swallowing reflexes, patients suffer from abnormal breathing patterns, sleep apnea and swallowing difficulties starting from early childhood. An absence or underdevelopment of the cerebellar vermis is also observed in JS. This part of the brain is responsible for balance and coordination so the patients generally suffer from ataxia and oculomotor apraxia. JS is also characterized by other ciliopathy phenotypes such as ocular findings, polydactyly, muscular hypotonia, hepatic fibrosis and renal cysts (Bachmann-Gagescu et al. 2015; Romani et al. 2013).

Currently, genetic cause of around 60% of JS cases are established and research in the area is ongoing to determine other causative genes of this syndrome. The 35 genes to be responsible for JS include *CXORF5, TTC21B, KIF7, TCTN1, TMEM237, CEP41, TMEM138, C5ORF42, TCTN3, AHI1, TMEM216, NPHP1, CEP290, TMEM67, RPGR1P1L, ARL13B, CC2D2A, CEP104, KIAA0556, B9D1, MKS1, TMEM107, ARMC9*, *ZNF423, TMEM231, CSPP1, PDE6D, KIAA0586* and *TCTN2* (Bachmann-Gagescu et al. 2015; Cantagrel et al. 2008; Chaki et al. 2012; Dafinger et al. 2011; Davis et al. 2011; Delous et al. 2007; Edvardson et al. 2010; Garcia-Gonzalo et al. 2011; Lambacher et al. 2016; J. E. Lee et al. 2012; J. H. Lee et al. 2012; Noor et al. 2008; Otto et al. 2009; Parisi et al. 2004; Romani et al. 2014, 2013; Sanders et al. 2015; Sang et al. 2011; Srour et al. 2012, 2015; Thomas et al. 2012; Tuz et al. 2014; Valente, Silhavy, et al. 2006; Van De Weghe et al. 2017). All these genes encode for proteins involved in cilia structure or function. Therefore, pathogenic variations result in

structural and functional abnormalities in the cilia or related structures, classifying JS as a ciliopathy disorder.

As mentioned earlier, the strength of mutations, presence of mutations in modifier genes and heterozygous mutations in different genes can determine the severity of disease phenotype in ciliopathies including the JS (Hildebrandt et al. 2011).

1.2.2.2. Abelson Helper Integration Site 1 (*AHI1*)

AHI1 (Abelson helper integration site 1), also known as Jouberin, ORF1, JBTS3 and dJ71N10.1, is encoded by the *AHI1* gene on chromosome 6 (6q23.3), composed of 35 exons. The protein contains a coiled-coil domain, an SH3 domain and seven WD40 repeats and is described as a putative cytoplasmic adaptor protein (Jiang et al. 2002). *AHI1* is referred as the first gene that was associated with Joubert syndrome as nonsense or frame-shift mutations of the gene was shown to result in Joubert syndrome phenotype, which is a ciliopathy disorder (Dixon-Salazar et al. 2004; Ferland et al. 2004). Currently there are over 15 *AHI1* variations, which are associated with Joubert syndrome, that are reported to the literature (Valente, Brancati, et al. 2006).

Studies revealed that *AHI1* is highly expressed in the brain and the kidneys at the protein level and is primarily localized at the mother centriole and eventually at the basal body of the primary cilium (Eley et al. 2008; Ferland et al. 2004; Hsiao et al. 2009). AHI1 functions in vesicle trafficking and is required for ciliogenesis as it interacts with RAB8A, which is a small GTPase critical for polarized membrane trafficking in the cilia (Hsiao et al. 2009). It is also involved in the canonical Wnt-beta catenin signaling pathway as its loss results in a decrease in endogenous Wnt activity, which was shown to be responsible for cystogenesis in kidneys (Lancaster et al. 2009).

More recent studies suggest that pathogenic variations on the gene may also be associated with increased susceptibility to autism and schizophrenia that are two common disorders showing depression symptoms (Xu et al. 2010).

1.2.3. Coiled-Coil and C2 Domain Containing Protein 1A (*CC2D1A*)

CC2D1A (Coiled-coil and C2 domain-containing protein 1A), which is also named as Freud-1, Aki1 or MRT3, is encoded by the *CC2D1A* gene on chromosome 19 (19p13.12) and contains 31 exons (Basel-Vanagaite et al. 2006). The protein is composed of 951 amino acids and is 104.1kDa. CC2D1A has four Drosophila melanogaster 14 (DM14) motifs at the N-terminus a protein kinase C conserved region 2 (C2) domain at the C-terminus, and a predicted helix-loop-helix DNA binding domain (Ou et al. 2003).

CC2D1A was reported to be localized in the nucleus and has DNA binding properties. It regulates the expression of 5-hydroxytriptamine (serotonin) receptor 1A (*5-HTR1A*) in neuronal cells by binding to a conserved 14 base pair repressor element in those cells and acts as a transcriptional repressor (Ou et al. 2003). It can also act as a transcriptional activator for nuclear factor κ enhancer binding protein (*NF-κB*) through the IκB kinase complex (IKK) pathway (Zhao, Li, and Chen 2010). These DNA binding and transcriptional regulator activities of CC2D1A can be inhibited by the action of calcium within the cells (Basel-Vanagaite et al. 2006). Additionally, CC2D1A can also be found in the cytoplasm where it acts as a scaffold protein in PI3K/PDK1/AKT pathway and in the centrosomes where it regulates spindle pole localization of the cohesin subunit SCC1/RAD21, thereby mediating centriole cohesion during mitosis (Nakamura et al. 2008; Nakamura, Arai, and Fujita 2009).

Studies in *Droshophila Melanogaster* indicated that the ortholog of *CC2D1A* (Drosophila *Lpd*) is involved in the endosomal trafficking of Notch signaling pathway and mutant models have abnormal neuronal development and function (Gallagher and Knoblich 2006). Notch signaling was shown to be a key pathway for cilia length control in deltaD zebrafish mutants (Lopes et al. 2010). However, there is no information about the role of CC2D1A in the Notch signal pathway and cilia length control that may explain the possible involvement of CC2D1A in cilia.

In the last decade, use of mouse models revealed more information about the function of the CC2D1A protein. There were no major morphological changes in respiratory

organs of the KO mouse, but *CC2D1A-KO* neurons had alterations in evoked neurotransmitter release events during the maturation of synapses. Also, it is thought that *CC2D1A* is involved in the regulation of endocytic rate of synaptic vesicles (Zhao et al. 2011). Another mouse model that produces a truncated form of CC2D1A lacking the C2 domain and three of the four DM14 domains, showed that the protein is important in differentiation of neurons by transducing signals to the cyclic adenosine 3',5' – monophosphate (cAMP)-protein kinase A (PKA) pathway (Al-Tawashi et al. 2012). A recent study indicated that conditional deletion of *CC2D1A* from excitatory neurons of male mouse forebrain reduces hippocampal synaptic plasticity and impair cognitive function through hyperactivation of Rac1, which may implicate Rac1 inhibitors in the treatment of intellectual disability caused by *CC2D1A* mutations (Yang et al. 2019).

In humans, a mutation resulting in a genomic deletion of 3589 nucleotides in the *CC2D1A* gene was found to create a truncated protein, which probably present a loss of function effect and cause autosomal recessive non-syndromic mental retardation [30]. In another study, haplotypes of *CC2D1A* and *CC2D2A* were found to be associated with mental retardation in a Han Chinese population (Shi et al. 2012). However, cellular and molecular mechanisms by which the *CC2D1A* is involved in these neuropsyhiatric-related behaviors are not yet clear.

1.2.4. Use of Model Organisms in Cilia Research

People including physicians, scientists and even philosophers that lived before the common era have always been interested in revealing the mechanisms of development, especially embryology, as well as the disorders they observed in humans and other animals. Members of the Hippocratic school, the Greek scientists Aristotle, investor and artist Leonardo da Vinci, German zoologist and evolutionist Ernst Haeckel are among the first people who contributed to the understanding of these subjects by their drawings of animal and human dissections and observations (Eisen 2019).

Since then, the use of a variety of animal model organisms to reveal principles underlying development and certain disorders has been an important concept in

science and medicine. Model organisms are chosen based on their experimental tractability for addressing a particular research question.

1.2.4.1. *Caenorhabditis Elegans*

Caenorhabditis elegans (C.elegans) are small, free-living soil nematodes (roundworms) that are widely used as a powerful model for biomedical research for over 50 years. Even though they are recognized as soil nematodes, they can be isolated easily from rotting vegetable matter and recovered by placing onto a standard nematode culture plate that are agar plates containing a lawn of E.coli (Barrière and Félix 2014). *C.elegans* are easily observable under dissecting microscopes for handling and confocal or compound microscopes allow observation at much higher resolution for various experiments (Corsi, Wightman, and Chalfie 2015). *C.elegans* has two sexual forms. First is the self-fertilizing hermaphrodites (XX) and the second is males (XO). Males frequently arise through mating of hermaphrodites with males (50%) or quite infrequently by spontaneous non-disjunction in the hermaphrodite germ line (0.1%) (Corsi et al. 2015). Self-fertilization of the homozygous hermaphrodite worms allow generation of genetically identical progeny, whereas mating with males facilitate passing mutations between strains as well as the isolation and maintenance of mutant strains.

Around 60-80% of all human genes and 40% of disease-associated human genes have orthologs in the *C.elegans* genome. These include most of the genes involved in signaling pathways in various cellular processes (Culetto and Sattelle 2000; Kaletta and Hengartner 2006). Also at least 38% of the *C.elegans* protein-coding genes have predicted orthologs in the human genome (Shaye and Greenwald 2011). Thus, novel discoveries and designed studies in *C.elegans* have relevance to the study of human health and disease.

They are useful models to study several cellular processes including cell signaling, cell polarity, development and neuroscience. There are several key features that make the *C.elegans* an outstanding experimental system. They are very easy and cheap to culture in the laboratory as they are fed by *E.coli* on agar plates. Although they are

multicellular eukaryotic organisms, their size is small - where larvae are around 0.25 mm and adults are 1mm long, they are easily grown on small agar plates and assays can be performed even in 96-well plates very easily. They have very short generation time (3 days at 25^0C) as the self-fertilizing hermaphrodites enable the large-scale production of several million worms per day. Their life-cycle is composed of embryonic stage, four larval stages (L1-4) and adulthood **(Figure 3)**. When not in use, the worms can be frozen and kept for several years. Also, at L1 larval stage, *C.elegans* can be starved, which triggers a developmental arrest and can be kept on those agar plates for weeks. When needed, a piece of agar from the starvation plate is cut and transferred to a new plate with fresh *E.coli* allowing them to develop into further stages.

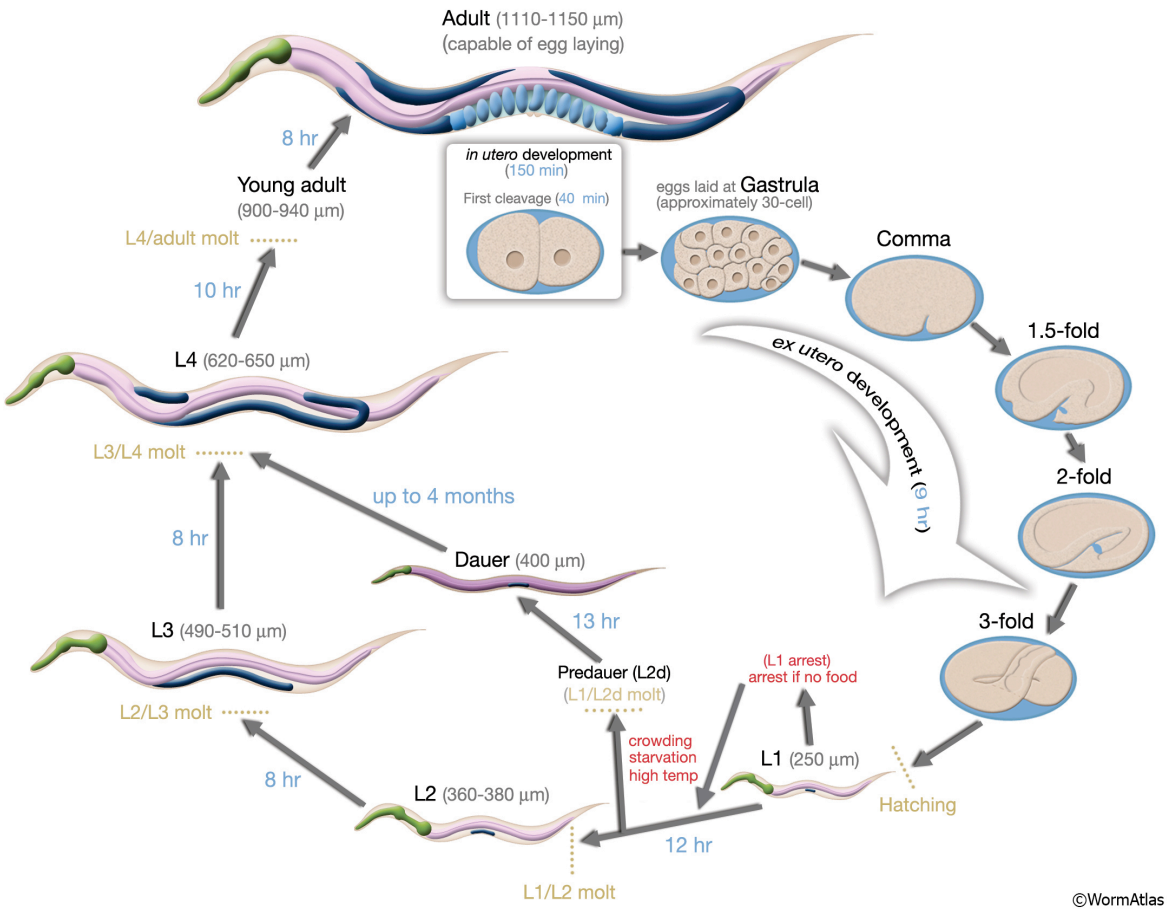

Figure 1.3. C.elegans life-cycle.

C.elegans life-cycle at 22^0C is represented in this figure. The life-cycle is comprised of the embryonic stage, four larval stages and the adulthood. Where fertilization is 0

min, first cleavage after fertilization happens in 40 mins. The eggs are laid outside of the body at 150 mins post-fertilization until the end of gastrula stage. Right after hatching, at L1 stage, the worms can enter developmental arrest if no food is available. Between L1 and L2 stages, environmental conditions such as crowding, starvation and high temperatures can initiate the formation of a morphologically distinct, arrested state called dauer larva. Dauer larva can survive 4-8 times the normal life-span in these conditions. When the environment becomes favorable again, they molt to the L4 stage. At the end of each larval stage, which is marked with a molt, a stage-specific cuticle is formed and the old one is shed. (Figure is adapted from the WormAtlas (Altun and Hall 2021))

They are transparent, which enable us to study several cellular processes to be studied in the living animal by fluorescent labeling. It is one of the first organisms that have complete genomic sequence studied and is easy to genetically manipulate. As they have genetically determined number of cells and developmental fate of every single somatic cell has been mapped, so changes due to manipulation can easily be tracked. Also, a comprehensive connectivity map of their neuronal structure is available (Hall and Altun 2007; Kaletta and Hengartner 2006; Towlson et al. 2013)

C.elegans are one of the most widely used model organisms to investigate cilia structure, function and related proteins as the nematode cilia are evolutionarily well conserved through mammals. Both hermaphrodites and males present 60 ciliated neuronal cells in common, whereas males have additional ciliated cells, most of them implicated in the regulation of mating behaviors. All ciliated cells in both sexes are non-motile, postmitotic sensory neurons, located at the dendritic endings of these neurons at the head (amphid) or tail (phasmid) parts of the worm (Nechipurenko and Sengupta 2017).

For example, the functions of many genes that are evolutionarily conserved in higher organisms such as *DYF-1, DYF-2, DYF-13* and *IFTA-1* which are involved in intraflaggelar transport, were first described in *C.elegans* models. In subsequent studies, it has been shown that these genes are involved in the formation of cilia in

humans and other organisms (Blacque et al. 2005, 2006; Efimenko et al. 2006; Ou et al. 2005). Likewise, *C.elegans* is widely used to investigate the cilia functions of genes that are thought to be related to cilia and are first seen in humans or another organism.

1.2.4.2. Danio Rerio

Danio rerio, or with its more common name the zebrafish, is a small (2.5-4 cm long), freshwater fish originating from South Asia. Zebrafish were introduced as a model organism back in 1960s by George Streisinger and Franklin Stahl, two molecular biology pioneers, to study the genetic basis of vertebrate neural development (Eisen 2019). Since then, zebrafish are being used as a powerful model system based on several advantages of the organism.

Zebrafish are not seasonal breeders, unlike many fish, therefore embryos can be obtained anytime of the year. Besides their generation time is short. A single fish produces hundreds of eggs at once, so it is cheap to obtain. Fertilization and embryo development occurs in the water column and they are transparent, enabling scientists to manipulate and observe the cells throughout their developmental period by appropriate microscopic methods- because of this most zebrafish research is performed in these stages (Eisen 2019; Malicki et al. 2011).

The zebrafish genome-sequencing project was initiated in 2001 and a comprehensive sequencing and annotation of its genome was submitted in 2013. Around 70% of human genes have at least one orthologue in the zebrafish genome and 69% of zebrafish genes have at least one human orthologue. When potential disease-related human genes in Online Mendelian Inheritance in Man (OMIM) database was compared, 82% was shown to have a zebrafish orthologue (Howe et al. 2013). So, it is a good vertebrate model to study human genetic disorders.

Cilia are abundant and can easily be visualized in different organs of zebrafish embryos and larvae, such as the neurons of sensory systems, the pronephric duct and the neural tube. The most common technique to visualize zebrafish cilia is labeling of the ciliary axoneme by microtubule antibodies, mostly acetylated alpha tubulin.

Alternatively, gamma tubulins can be labelled to detect basal bodies and locate cilia. Also high-speed videomicroscopy allow analysis of cilia motility by measuring cilia beat frequency, amplitude etc (Malicki et al. 2011).

Studies in zebrafish has revealed that *ARL13B*, which is mutated in Joubert syndrome, is localized in the cilium and is involved in cilia formation in multiple organs. Knockdown of the gene result in multiple cilia-associated phenotypes (Duldulao, Lee, and Sun 2009). Genetic screens to identify genes possibly associated with specific human genetic diseases are being performed in zebrafish genome. An example is polycystic kidney disease (PKD). 12 genes were identified in an insertional mutagenesis screen in zebrafish of which can be candidates for novel human PKD genes (Sun et al. 2004).

1.2.4.3. *Xenopus Tropicalis*

Xenopus tropicalis are small amphibians originating from West Africa and are closely related to *Xenopus laevis (X.leavis)*, which is a South African species and another model organism that has been in use before *Xenopus tropicalis*.

Both *Xenopus laevis* and *tropicalis* were being used as powerful models for vertebrate developmental mechanisms since 1990s. However, they were not considered as bona fide models to study genetics and human diseases until their genome were fully sequenced in 2010 (Hellsten et al. 2010). It was confirmed that *Xenopus tropicalis* are diploid organisms and have genome organization much like mammals, compared to *Xenopus laevis* (Grainger 2012). With the advent of genome editing technologies, *Xenopus tropicalis* are being used to test candidate genes and alleles for human genetic diseases.

In addition to being the only amphibian with a diploid genome, *Xenopus tropicalis* have other advantages as a model system as well. They have shorter generation time than *Xenopus laevis* and are easy to maintain as they are raised at 24-26^{0}C, whereas *Xenopus laevis* require 18^{0}C water temperature **(Figure 4)**. Almost 90% of human disease genes have homologs in *Xenopus* genome and sequence conservation is high.

This is an important prerequisite for any predictive animal model. Another important feature of *Xenopus* is the ease of gaining access to eggs and embryos. Females lay thousands of eggs each time, which are fertilized either by natural mating or artificially by sperm taken from male testes. Egg laying is inducible by hormone priming. The eggs are also large in size, allowing intracellular microinjections into zygote or into blastomeres of early developmental stages. A unique and very important feature of Xenopus embryos is the possibility of one-sided injections. By this method only one side of the embryo is manipulated and the contralateral side serve as control. This eliminates the risk of variable wild-type gene expression and presence of variable background mutations in different embryos (Blum and Ott 2019).

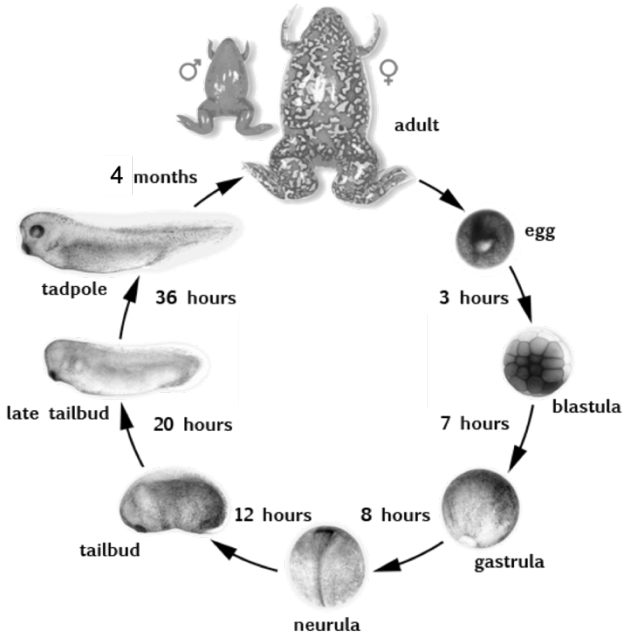

Figure 1.4. Life-cycle of Xenopus tropicalis.

Figure represents the full life-cycle of *X.tropicalis*. Frog embryos develop externally. Within 36 hours post-fertilization, a tadpole with fully functional set of organs is formed. *Xenopus tropicalis* reach adult stage in 4 months, which is much shorter compared to *Xenopus leavis* that require 12 months. (Figure adapted from the Xenbase (Karimi et al. 2018))

Xenopus are used to monitor the effect of human gene orthologs and model human diseases in several organs. For example, knock-down of the *Xenopus* ortholog of

homeodomain transcription factor *IRX5* lead to disruption of migration of progenitor cells in gonads and branchial arches, reproducing the human phenotype where mutations in this gene impair germ cell migration and craniofacial development (Blum and Ott 2019; Bonnard et al. 2012). Another example is the mutations in *PYCR1*, which is involved in proline metabolism, associated with autosomal recessive cutis laxa syndrome. It is characterized by lax and wrinkled skin in humans. Knockdown of *Xenopus* ortholog of the gene, resulted in wrinkles and hypoplasia of the tadpole skin (Blum and Ott 2019; Reversade et al. 2009)

Besides, ciliopathies are particularly well qualified group of diseases to be studied in *Xenopus* model system. The larval skin of *Xenopus* is covered with the epithelium that harbors multiciliated cells (Dubaissi and Papalopulu 2011). These can easily be visualized either by staining ciliary axonemes by histochemical stains or by high-speed videography.

The larvae move in a hovering motion across the agar plate when their cilia are fully functional and beat normally. However, any defect in their cilia at that stage can be observed by measuring the speed of their hovering movement. Knock-down of the *dnah9*, which is an axonemal dynein critically required for ciliary movement is an example for this type of defect. As it is possible to create one-sided mutations in *Xenopus*, these observations and comparisons can be done in one larvae at the same time (Vick et al. 2009). This enables us to obtain preliminary observations at a reduced time and cost. Left-right asymmetry defects and hydrocephalus are among other defects that are readily studied in the *Xenopus* model system (Blum et al. 2009; Hagenlocher et al. 2013).

1.2.5. Genome Editing Technologies

Genome editing, by definition, is the technologies that allow scientists to make specific changes or manipulations in the genomic sequence of eukaryotic cells, basically by creating double strand breaks (DSB). After the formation of a DSB, the cell repairs the break to maintain genomic integrity either by non-homologous end-joining (NHEJ) or by homologous recombination (HR). NHEJ mostly results in disruption of the

sequence and gene knockouts at the target sequences, whereas HR can be used to introduce new sequences into the target site. However, the efficiency of HR in higher eukaryotic cells is very low (Capecchi 2005; Kim 2016).

The earliest form of gene targeting is the use of restriction enzymes. In 1988, Rudin and Haber reported that use of HO endonuclease for site-specific DSB boosted HR efficiency in *Saccharomyces cerevisiae* genome (Rudin and Haber 1988). A similar effect by the use of I-*SceI* restriction enzyme to induce DSB in a targeted manner in the mouse genome was demonstrated in 1994 by the Jasin group. They integrated I-*SceI* cleavage sites into the mouse genome in a two tandem reporter gene region. Both HR by the use of a donor DNA as a template and non-homologous mechanisms were used by transfected cells to repair the breaks (Rouet, Smih, and Jasin 1994). These two studies initiated the progress in the field of genome editing technologies.

Even though the meganucleases were very useful to cleave chromosomal DNA in a site-specific manner due to their long DNA recognition sequences, the main drawback was that they were not reprogrammable (Epinat et al. 2003). Therefore, further studies were focused on developing programmable reagents to target any region on the genome.

1.2.5.1. Zinc-Finger Nucleases (ZFNs)

In 1996, the Chandrasegaran team reported that they successfully induced site-specific cleavage of target DNA *in vitro* by fusing *FokI* restriction enzyme's nuclease domain to zinc finger proteins (Kim, Cha, and Chandrasegaran 1996). Zinc finger nucleases (ZFNs) consists of at least three zinc finger domains, where a single domain interacts with a 3 base pair sequence. This enables recognition of target DNA in a modular fashion and create custom-designed ZFNs to make sequence-specific DNA binding-proteins (Kim 2016).

Genome editing in animals was first achieved by the injection of ZFNs into *Drosophila* embryos in 2002 and others followed in various other animals including *C.elegans* and zebrafish, plants as well as human cells (Bibikova et al. 2002; Doyon et al. 2008; Hye

et al. 2009; Kim 2016; Lloyd et al. 2005; Morton et al. 2006). Even though ZFNs were more efficient in creating targeted and programmable genome editing compared to meganucleases, target specificities still remained as a challenge. Additionally, many ZFN applications were cytotoxic, likely as a result of non-specific (off-target) site cleavages (Hye et al. 2009).

Another breakthrough in the field came in 2009 with the identification of transcription-activator-like (TAL) effector nucleases (TALENs) (Boch et al. 2009; Moscou and Bogdanove 2009).

1.2.5.2. Transcription-activator-like (TAL) Effector Nucleases (TALENs)

TALENs were identified in a bacterial plant pathogen, the *Xanthomonas*, in 2009. Like ZFNs, TALENs also use the *FokI* nuclease to create DSBs. The difference is that each repeat domain in TALENs recognize a single base. Therefore, four different repeat domains can be mixed/matched and used to create new DNA-binding domains that can be fused to the *FokI*.

TALEN-encoding plasmids are being developed by several groups, bringing TALENs forward in genome editing technology.In 2011, TALENs was used for genome editing in human cells. Compared to ZFNs, TALENs showed less cytotoxicity in human cells (Miller et al. 2011).

However, the dominance of TALENs technology in the field ended shortly after, in 2012, with the introduction of cluster of regularly interspaced palindromic repeats (CRISPR)- CRISPR associated protein 9 (Cas9) as a new tool for genome editing.

1.2.5.3. Cluster of Regularly Interspaced Palindromic Repeats- CRISPR Associated Protein 9 (CRISPR-Cas9) System

CRISPR-Cas system is based on the natural adaptive defense system of bacteria and archaea that protect them against invading viruses and plasmids (Jinek et al. 2012).

In general terms; when a virus infects a host, its genome is cleaved into short fragments, which are then integrated into the CRISPR locus of the host genome as spacer sequences between identical repeats by the action of Cas1 and Cas2 (adaptation stage). In case of a repeated infection with the same virus or plasmid, the repeat-spacer element is transcribed to form pre-crRNA and then processed to form a mature guide crRNA. Maturation is performed either by endoribonuclease complexes in type I and III or by an alternative mechanism that involves bacterial RNase III directed by a trans-encoded small RNA with around 24 nucleotide complementarity to the repeat regions of crRNA, named as the tracrRNA, in type II (Deltcheva et al. 2011). Upon formation of the mature crRNA, either one (Cas9 in type I) or several (types I, III) of Cas proteins bind crRNA and are directed to pair with complementary protospacer sequences of invading plasmid or viral genome (expression stage). The CRISPR-Cas complex in types I and II can target genomic sequences that contain the tri-nucleotide protoscpacer adjacent motif (PAM). Target recognition is followed by creation of DSBs by Cas enzymes and silencing of the foreign sequences (interference stage) **(Figure 5)** (Bhaya, Davison, and Barrangou 2011; Makarova and Koonin 2015; Terns and Terns 2011; Wiedenheft, Sternberg, and Doudna 2012).

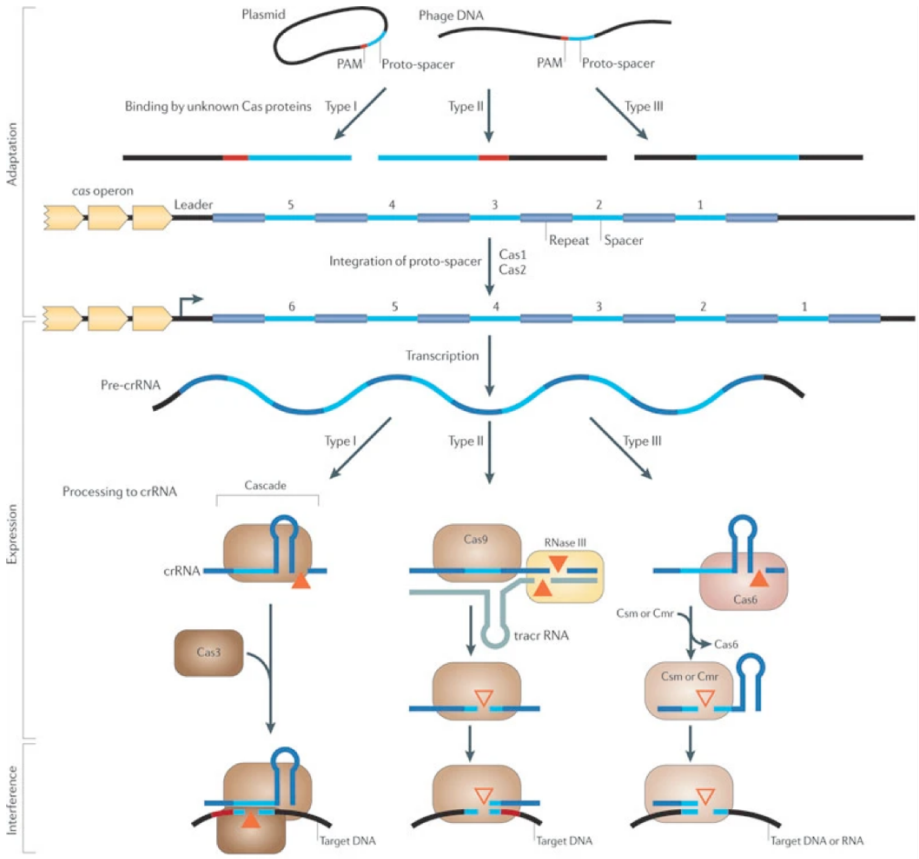

Figure 1.5. Schematic representation of the CRISPR-Ca((ref)

CRISPR-Cas9 system consists of three stages as the adaptation, expression and interference stages. Foreign DNA fragments are integrated into the host genome during an infection inside the CRISPR locus as spacer sequences. The locus is transcribed during the repeated infections and the spacers are used by the Cas enzymes to recognize and interfere with the invading agents' DNA and create DSBs. There are three types (type I, II and III) of CRISPR-Cas systems that use different Cas enzymes and have slightly different action mechanisms as represented in the figure. (Figure taken from (Makarova et al. 2011))

There are three types of CRISPR-Cas systems (Type I, II and III) and diverse Cas proteins that are involved in different steps of the processes **(Figure 5)** (Makarova et al. 2011). Jennifer Doudna and Emmanuelle Charpentier re-engineered the Cas9 nuclease, which functions in type II, to a two-component system by merging the tracrRNA and crRNA into a 'single guide RNA' or 'sgRNA'. Custom designed sgRNAs guide the Cas9 endonuclease to the target sequence and initiate DSB

formation. The system was then used to induce site-specific genome modifications in human cells by different groups simultaneously (Cho et al. 2013; Cong et al. 2013; Jinek et al. 2013; Mali et al. 2013). As mentioned, when a DSB is introduced to the sequence by the nuclease, the cell mostly repairs the break by NHEJ. NHEJ generally result in insertion-deletion (indel) mutations that results in a frameshift and loss of function in the targeted gene. Alternatively, the cell can also use HR to repair the break, which in this case can be a sequence introduced with the system, can be used introduce the desired genome alteration (He et al. 2016; Zhang 2021).

When compared to ZFNs and TALENs, an important advantage of CRISPR-Cas9 system is that it does not require protein engineering. Customized CRISPR-Cas9 systems are readily constructed by changing guide RNA sequences (Kim 2016). This greatly reduced the cost and time required for genome editing, increasing its feasibility when compared to previous technologies, creating a giant leap in the field of genome editing. This way, the use of model organisms to unravel novel gene functions, creating genetically engineered plants and studies in personalized medicine and gene therapy fields became both cost and labor effective.

On the other hand, as *FokI* is a dimer and cleave the DNA only when dimerized, but Cas9 functions as a monomer. Therefore, site specificity of *FokI* can be higher. Also, the fact that Cas9 is derived from prokaryotic cells and has no known function in higher eukaryotes raised the concerns about reduced site specificity and possible off-target cleavages. Larger genome size also increases the probability of off-targets. As anticipated, several groups reported off-target effects of Cas9 in human genome (Cradick et al. 2013; Fu et al. 2013; Kim 2016; Pattanayak et al. 2013). Both the gRNAs and Cas9 has been modified to overcome this problem. For example, Cas9 enzyme was fused with the *FokI* domain of TALE or zinc finger proteins to take advantage of their dimerization property to increase the specificity of genome editing (Bolukbasi et al. 2015; Guilinger, Thompson, and Liu 2014). Later in 2015, another Cas enzyme called Cas12a or Cpf1 was identified in prokaryotic cells. Cpf1 creates staggered cuts on DNA as opposed to the blunt cuts created by Cas9, it is guided by a

single sgRNA- lacking tracrRNA and utilizes a T-rich PAM sequence (Zetsche et al. 2015).

Delivery of Cas enzyme and sgRNA oligos into cells is an important consideration and can be achieved by different methods. Cas9 enzyme can be delivered into the cell either in plasmid DNA, mRNA or protein forms. As transcription and translation steps from plasmid DNA will take time, mRNA and protein options enable faster editing. However, it is cost effective and also enables sustained expression of the protein. mRNA delivery allows faster application but the fact that RNAs are unstable and can easily be degraded before being translated, reduces efficiency of editing. Delivery of Cas9 in protein format, on the other hand, enables immediate gene editing but is the most transient and least cost effective format (Yip 2020). Cas9, regardless of the form, and the sgRNA expression vectors are delivered into the cell by different methods **(Figure 6)**. These include microinjection, electroporation, transfection by chemicals or by using extracellular vesicles such as viral vectors (Glass et al. 2018; Yip 2020).

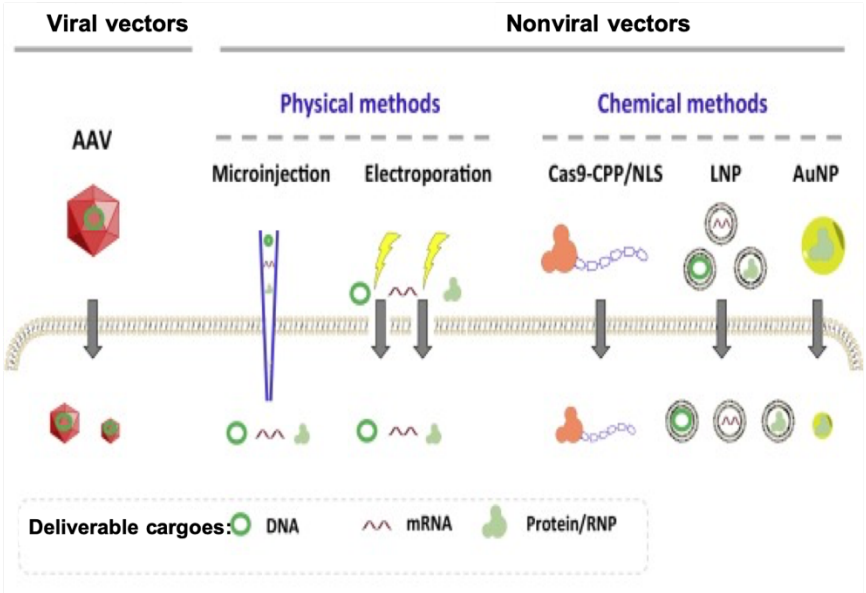

Figure 1.6. Delivery mechanisms of CRISPR-Cas9 components into cells. (ref)
Different methods by which the CRISPR-Cas9 components can be delivered into the cells are represented in this figure. The Cas9 enzyme can either be delivered in the form of plasmid DNA, mRNA or protein, whereas the sgRNAs are inserted into sgRNA expression vectors. These can be inserted into the cells either by physical

methods such as microinjection and electroporation or by transfection with extracellular particles such as gold nanoparticles (AuNP) and lipid nanoparticles (LNP) using chemical reagents. Use of viral vectors, mostly adeno-associated viruses (AAV) is another option. (Figure taken from (Glass et al. 2018))

Currently, gene editing with CRISPR-Cas system is being used effectively in research, generation of animal models to study and treat human genetic diseases, agriculture, food and farming industry effectively, as well as in therapy mostly as clinical trials (Chemello, Bassel-Duby, and Olson 2020; Cruz and Freedman 2018; Yan et al. 2020; Zhang 2021).

1.2.6. Work in this thesis

In this thesis we aimed to investigate the involvement of *CC2D1A* gene in cilia and cilia-related phenotypes. Two different model systems were used to create gene-specific knock-out animals and analyse the effects on cilia function and structure. Also, protein localization was determined using a transgenic model.

In the first chapter, general information about the concepts discussed in the thesis were explained. Brief information about the techniques used is also given in the first chapter. In the second chapter, materials used throughout the project and all techniques are explained in detail. In the third chapter, detection of the *CC2D1A* gene as a candidate ciliopathy gene was explained, providing detailed results obtained from clinical and genetic analysis of three patients. In chapter four, experimental results obtained from *CC2D1A* knock-out and transgenic expression of fluorescently labelled protein in *Caenorhabditis elegans (C.elegans)*is explained. Chapter five explains functional and structural data obtained from *CC2D1A* knock-out in Xenopus tropicalis *(X.tropicalis)* model. In chapter six, all results are discussed, data is explained and compared to previous studies in the field.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Materials

2.1.1. Suppliers

Thermo Scientific (Pittsburg, USA), Qiagen (Hilden, Germany), Sigma-Aldrich (Poole, UK), Zeiss (Oberkochen, Germany) Bio-Rad (California, USA), Leica (Wetzlar, Germany), Addgene (Massachusetts, USA), TransGen Biotech (Beijing, China), Macrogen (Seoul, South Korea), Intron (Seoul, South Korea), Biological Industries (Beit HaEmek, Israel), Narishige Scientific Instrument Lab. (Tokyo, Japan), Illumina (San Diego, USA), Beckman Coulter (California, USA), Invitrogen (California, USA).

2.1.2. Chemical Reagents

2.1.2.1. Enzymes

T4 ligase enzyme and *BsaI* restriction enzyme were supplied by Thermo Scientific and Protease K was obtained from Intron.

2.1.2.2. Molecular Weight Markers and Loading Dyes

100bp Plus II DNA Ladder obtained from TransGen Biotech and DNA Gel Loading Dye (6X) from Thermo Scientific were used in agarose gel electrophoresis experiments.

2.1.2.3. Oligonucleotides

All sgRNAs and control primers were supplied by Macrogen, Inc.

2.1.2.4. Vectors

pRB1017 sgRNA backbone vector, pDD162 Cas9 expressing vector and pRF4 plasmid containing the rol-6 (sulO06) gene ‘roller’ marker was supplied by Addgene.

2.1.2.5. Isolation Kits

Plasmid isolation was performed with EasyPure Plasmid MiniPrep Kit supplied by TransGen Biotech and genomic DNA isolation from whole blood was done with QIAamp DNA Blood Mini Kit from Qiagen.

2.1.3. Devices

Stemi 508 by Zeiss and DM6 B by Leica were the microscopes that were used. Bio-Rad ChemiDoc^{TM} XRS+ gel imaging system and Image lab software were used for agarose gel visualization. IM-400 Electric Microinjector from Narishige was used for microinjections. Illumina MiSeq sequencer and Beckman Coulter CEQ8800 sequencer were used for DNA sequencing.

2.1.4. Standard solutions

Phosphate buffered saline (PBS) for *X.tropicalis* embryo storage and 10X Tris-borate/EDTA (TBE) buffer and ethidium bromide solution for agarose gel electrophoresis were prepared as described in (Anon 2000).

2.1.5. Preparation of *C.elegans* Lysis Buffer

Lysis mix contains 0.7 µl 500mM KCl, 0.7 µl 100mM Tris Base, 0.7 µl 25mM $MgCl_2$, 0.07 µl 1% gelatin, 0.7 µl 4.5% tween, 4.13 µl ddH_2O and 1.4 µl protease K.

2.1.6. Preparation of *C.elegans* Recovery Buffer

Recovery buffer was prepared with 5mm HEPES pH 7.2, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 66 mM NaCl, 2.4 mM KCl, 4% Glucose (w/v).

2.1.7. Human subjects

Three human subjects from two families participated in this study. Patient 1 was a 17-year-old male, who was admitted to the Near East University Medical Faculty Hospital Neurology outpatient clinic. His major complaints were imbalance and eye movement abnormalities, ongoing since birth.

Patient 2 was a 10-year-old female that was admitted to Uludag University Hospital Department of Pediatric Psychiatry as she had speech delay at the age of 4. Patient 3 is her sibling, 6-year-old male, who also present autistic features.

Informed consent forms were signed by both parents.

2.2. Methods

2.2.1. Human Genomic DNA Extraction

Genomic DNA was isolated from peripheral blood samples drawn into EDTA-containing tubes. Isolation was performed with QIAamp DNA Blood Mini Kit (Qiagen, 51104).

2.2.2. Next Generation Sequencing

Whole exome sequencing (WES) was performed with DNA isolated from peripheral blood sample taken from the patients. In-solution target enrichment was performed according to the manufacturer's protocols before Illumina TruSightOne Sequencing Panel and MiSeq sequencer (Illumina, San Diego, CA) were used for the targeted re-sequencing of the DNA sample.

Sequence alignment to the reference genome, variant calling, annotation and filtering to remove benign single nucleotide polymorphisms (SNPs) with allele frequencies ≤ 0.03 was performed using an in-house pipeline. Rare homozygous variants with minor allele frequency (MAF) values lower than <1% in the 1000 genomes phase III, single nucleotide variants (dbSNP) and Exome Aggregation Consortium (ExAC) databases were screened. Human Gene Mutation Professional Database (HGMD) was then used to screen previously reported clinical significances of the detected variants.

2.2.3. Sanger Sequencing

Upon selection of candidate variations, they were confirmed by PCR coupled with direct sequencing of target regions using a CEQ8800 Sequencer (Beckman Coulter) according to the manufacturer's protocols.

2.2.4. Generation of *CC2D1A* Knockout *Caenorhabditis elegans* (*C.elegans*) Model

2.2.4.1. *C.elegans* Primer and sgRNA Design

For the generation of the knockout model, sgRNAs were designed to target the *C.elegans* homolog of *CC2D1A*, the *Y37H9A.3.1. Y37H9A.3.1* is composed of 17,531 base pairs and have a total of 11 exons. Primers to check deletions and determine genotypes of the organisms after CRISPR-Cas9 experiment were also designed. Locations of the sgRNAs and control primers are represented in **Figure 9**. Expected band sizes for wild type and all possible mutants with the designed primer pair were: 4219bp wild type, 2812bp deletion with sgRNAI and II, 1751bp deletion with sgRNAII and III, 344bp deletion with sgRNAI and III.

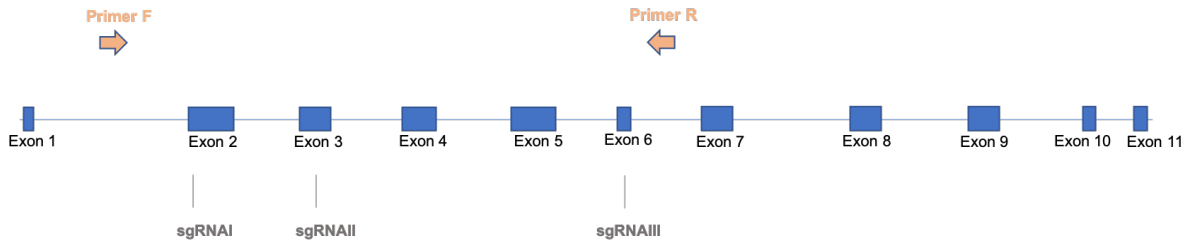

Figure 2.1. Target regions of control primers and sgRNAs designed for Y37H9A.3.1 gene.

Represents the structure of exons (rectangle boxes) and introns (straight lines) of *Y37H9A.3.1* gene. sgRNAI was designed to target exon 2, sgRNAII was designed to target exon 3 and sgRNAIII targets exon 6. Primer F represents the forward control primer and primer R represents control reverse primer.

2.2.4.2. Primer Optimization

Primers were optimized by gradient PCR using PCR Master Mix (2X) by Thermo Scientific™ (Thermo Scientific™, K0171) according to the manufacturer's instructions. Gradient PCR is set up using the following parameters: 95^0C for 3 minutes, 30 cycles of 95^0C for 30 seconds, 66-69^0C for 45 seconds and 72^0C for 4:40 minutes followed by 72^0C for 5 minutes. Wild-type *C.elegans* DNA, which was isolated before, is used as template. Expected band size was 4219bp.

2.2.4.3. Gel Electrophoresis

Agarose gels with 1% concentration were prepared by standard 1X TBE buffer and 1% UltraPure™ agarose by Thermo Scientific (Thermo Scientific, 16500100). Ethidium bromide was added to the buffer in 1/100 volume ratio. PCR products were loaded with 1/10 volume of DNA gel loading dye (Thermo Scientific, R0611) and were run on a horizontal submarine format with 1X TBE buffer. 100bp Plus II DNA ladder (TransGen Biotech, BM311-01) was used to compare and detect the PCR product sizes.

PCR products from gradient PCR were run on 1% agarose gel at 120V for 40 minutes and visualized under ultraviolet (UV) light by Bio-Rad ChemiDoc™ XRS+ gel imaging system.

2.2.4.4. sgRNA Annealing

sgRNAs were obtained as forward and reverse oligos. Annealing of the oligos was performed by preparing annealing mixture for all three sgRNAs separately (1µl forward oligo + 1µl reverse oligo + 2µl annealing buffer + 6µl ddH_2O) and using the following parameters in the PCR machine: 95^0C for 5 minutes then decrease to 4^0C slowly (0.01^0C/sec).

2.2.4.5. Cloning sgRNAs into Expression Vector

Annealed sgRNAs were cloned into pRB1017 sgRNA backbone vector (Addgene plasmid #59936, (Arribere et al. 2014)) by preparing the cloning mixture for each sgRNA separately (1µg PBR1017 vector + 1:1 annealed oligos + 2µl T4 ligase buffer + 0.5µl T4 ligase enzyme (Thermo Scientific, EL0011) + 0.5µl *BsaI* restriction enzyme (Thermo Scientific, ER0291) + ddH_2O up to 20µl) and using the following parameters in the PCR machine: 37^0C for 60 minutes followed by 50^0C for 5 minutes followed by 65^0C for 20 minutes.

PCR was set up to confirm the insertion of sgRNAs. For each sgRNA, a forward primer designed previously from inside the pBR1017 vector and the sgRNA's own

reverse oligo were used. PCR was set up using the following parameters: 95^0C for 3 minutes, 30 cycles of 95^0C for 30 seconds, 60^0C for 45 seconds and 72^0C for 0:40 seconds followed by 72^0C for 5 minutes. PCR products were run at 120V for 30 minutes and visualized under ultraviolet (UV) light by Bio-Rad ChemiDoc™ XRS+ gel imaging system as described in section 2.2.7.3. Bands approximately 580bp long were expected to confirm the presence of sgRNA inside the vector/plasmid and no bands in control (empty vector, EV). After confirmation of cloning, transformation was performed.

2.2.4.6. Transformation of Competent *E.coli* with sgRNA Vectors

Previously prepared competent *E.coli* cells were used for transformation. For each sgRNA, all cloning product and 100µl of competent *E.coli* from glycerol stock was mixed in a microtube. A control tube with competent cells but no cloning product was also included as a control. They were incubated on ice for 30 minutes and heat-shocked for 2 minutes at 42^0C. After the heat shock, mixture was put back on ice immediately. 900µl Luria Bertani medium (LB broth) was added into the tube and incubated at 37^0C for 30 minutes. Then the transformation product was centrifuged for 1 minute at 8000rpm, 900µl of the supernatant was discarded and the cells were resuspended in the remaining 100µl supernatant. Then all of the ligation product was plated on LB agar plates containing ampicillin for selection. Plates were incubated at 37^0C overnight.

Ten colonies from each plate (sgRNAI, sgRNAII, sgRNAIII) was taken by the help of a pipette tip and diluted in 20µl ddH_2O in a microtube. PCR was set up with forward primer designed previously from inside the pBR1017 vector and the sgRNA's own reverse oligo using the following parameters in the PCR machine: 95^0C for 3 minutes, 30 cycles of 95^0C for 30 seconds, 60^0C for 45 seconds and 72^0C for 30 seconds followed by 72^0C for 5 minutes.

PCR products were run on 1% agarose gel at 120V for 30 minutes and visualized under ultraviolet (UV) light by Bio-Rad ChemiDoc™ XRS+ gel imaging system as described in section 2.2.7.3.. An 'only vector' tube was included as a control. Expected

band size was approximately 580bp for colonies containing the cloned vector. Selected colonies were put in 10ml LB broth in a falcon tube and incubated at 37^0C, shaking at 250rpm in an incubator overnight.

2.2.4.7. Plasmid Isolation

750µl of overnight grown bacteria is mixed with 750µl 50% glycerol and stored in -80^0C for further use. Rest of overnight grown bacteria were centrifuged at 8000rpm for 10 minutes. Supernatant was removed. EasyPure® Plasmid MiniPrep Kit (TransGen Biotech Co., EM101) is used for plasmid isolation according to the manufacturer's instructions. Plasmid concentrations were measured using NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific™, ND-2000). Results were; 910ng/ µl for sgRNAI, 662.7ng/ µl for sgRNAII and 910ng/ µl for sgRNAIII.

2.2.4.8. Microinjection of *C.elegans*

Microinjection is performed in the gonad area of hermaphrodite *C.elegans* at L4 developmental stage (1-day adult) as this is the stage that their germ cells start to develop and is prone to genetic manipulation. The worms must be stable during injection. 'Agar pads' were prepared and used for this purpose.: 2% agarose gel prepared with ddH_2O was dropped between two cover slides and separated when dried. Then halocarbon oil (Sigma Aldrich, H8898) was dropped all over the agarose drop. After the agar pads were prepared, 2-3 *C.elegans* were placed onto the slide and waited until they sink into the halocarbon oil and become stable.

A mix containing 15ng pDD162 (Peft-3::Cas9+Empty sgRNA) vector (Addgene plasmid #47549, (Dickinson et al. 2013)), 50ng of each pRB1017 Y37H9A.3.1-targeted sgRNA vectors and 25ng of pRF4 plasmid (Mello et al. 1991) containing the rol-6 (sulO06) gene 'roller' marker was prepared and injected. The roller marker creates a rolling phenotype and is used as a selection marker to identify worms in F1 generation that were most likely have been genetically modified by the sgRNAs.

After injection, recovery buffer was dropped onto the halocarbon oil, enabling the worms to start moving. Recovery buffer was prepared with 5mm HEPES pH 7.2, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 66 mM NaCl, 2.4 mM KCl, 4% Glucose (w/v). Then each worm was placed into a separate plate (nematode growth medium spread with OP50 strain of *E.coli*). They were then incubated at 25^0C for 3-4 days. After incubation, 6 F1 worms that showed the roller phenotype were selected and each one was placed into a separate plate. They were then incubated at 25^0C for 3-4 days, again, until they produce eggs.

2.2.4.9. Confirmation Genome Editing in *C.elegans*

Then the F1 *C.elegans* are lysed and PCR is performed with designed control primers to confirm knockout. For lysis each worm is placed in a PCR tube and 7µl of lysis mix is added. Lysis mix contains 0.7 µl 500mM KCl, 0.7 µl 100mM Tris Base, 0.7 µl 25mM $MgCl_2$, 0.07 µl 1% gelatin, 0.7 µl 4.5% tween, 4.13 µl ddH_2O and 1.4 µl protease K. Tubes are placed in -80^0C for at least 30 minutes and then placed in the PCR machine for the lysis programme: 65^0C for 60 minutes, and 95^0C for 15 minutes. After lysis, these samples were used as template for PCR. PCR was set up with control primers using the following parameters: 95^0C for 3 minutes, 30 cycles of 95^0C for 30 seconds, 67^0C for 45 seconds and 72^0C for 4:40 minutes followed by 72^0C for 5 minutes. PCR products are run on 1% agarose gel at 120V for 30 minutes and visualized under ultraviolet (UV) light by Bio-Rad ChemiDoc^{TM} XRS+ gel imaging system as described in section 2.2.7.3.

F1 that have heterozygous *CC2D1A* knockout genotype were detected and 12 clones from each plate were taken into separate plates and after 3-4 days lysis and control PCR were performed again, to detect homozygote mutants.

2.2.5. Generation of Transgenic *Caenorhabditis elegans*

C.elegans were used to study protein localization of CC2D1A as they are transparent and are easy to visualize. For this purpose, transgenic *C.elegans* were generated that express green florescent protein (GFP)-tagged CC2D1A protein with a cilia-specific promoter region.

Cilia-specific *Arl-13* gene's promoter region, *Y37H9A.3* coding region and GFP with unc-54 3'UTR region (arl-13p::Y37H9A.3::GFP_UNC-543UTR) was cloned into the empty *C.elegans* expression vector pPD49_79 (Addgene plasmid #1447, deposited by Andrew Fire).

Upon delivery, plasmids were transfected into *E.coli*, multiplied and isolated as described in sections 2.4.3. and 2.4.4. above. 50ng vector was microinjected to the gonad area of hermaphrodite *C.elegans* as described in section 2.2.7.8. Another vector tagged with wrmScarlet (red), *CEP-41* gene (CEP-41::wrmScarlet) was also injected to the worms as a control. *CEP-41* is a known ciliopathy gene, expressed in the cilia.

F1 progeny were then visualized under fluorescent microscope to monitor CC2D1A::GFP and CEP-41::wrmScarlet expression.

2.2.6. Generation of *CC2D1A* Knockout *Xenopus tropicalis* (*X.tropicalis*) Model

X.tropicalis studies are being performed in collaboration with Dr. Engin Deniz from Yale Medical School. *CC2D1A* gene in *X.tropicalis* is composed of 2922 nucleotides. To generate *CC2D1A* knockout model, sgRNAs targeting exon 1 of *CC2D1A* were designed and purchased ready as cloned in expression vectors. 1.6 ng Cas9 protein (PNA Bio, CP03) with 200pg or 400pg sgRNA expression vector and a fluorescent tracer, Alexa Flour^{TM} 488 (Invitrogen) were injected post fertilization at one cell stage.

CC2D2A knockout was used as a positive control, as a known ciliopathy gene. An uninjected control group and an only Cas9 injected control group were included to demonstrate the results of any damage created by microinjections. Embryos were cultured in 3% Ficoll in 1/9 x MR with gentamicin until stage 30, post fertilization day 1 at 25 ℃.

Genetically manipulated larvae were used for further analysis.

2.2.7. Optical Coherence Tomography (OCT)

Xenopus larvae possess cilia on the skin epidermal cells, which act as a mucociliary clearance agent as a defense against pathogens (Blum and Ott 2019). Similar to the cilia in human upper respiratory tract, they beat in a coordinated manner and move mucus from anterior to posterior (head to tail) (Brooks and Wallingford, 2014).

Post fertilization day 1 stage 28 *Xenopus* larvae raised at 25°C were used for optical coherence tomography to analyze the functional consequences of the flow created by the cilia, as described in (Date et al. 2019). *CC2D1A* knockout, *CC2D2A* knockout and control animals were used. Images were obtained with particle speed colorization and were processed in Fiji, Image J (Schindelin et al. 2012). *Xenopus* larvae were classified and quantified as normal, slow or absent due to flow created over epidermal cilia.

2.2.8. Staining of Epidermal Cilia of *Xenopus tropicalis*

CC2D1A knockout and control *Xenopus* embryos were fixed at stage 30 and were stored in phosphate buffered saline (PBS). Upon permeabilization of epithelial tissue in PBS with 0.1% Tween-20, they were fixed in 4% paraformaldehyde for staining. Actin filaments (F-actin) were stained with highly selective phalloidin (Alexa Fluor™ 647 Phalloidin, Invitrogen A22287) and ciliary axonemes were stained with anti-acetylated α-tubulin antibody (Sigma-Aldrich, T6793).

Differences in cilia morphology were visualized by confocal fluorescent microscopy. Ciliated cell per area was counted to quantify the difference between control and F0 *CC2D1A* knockout organisms.

2.2.9. Visualization of Craniofacial Features of *Xenopus tropicalis*

Craniofacial features of the *CC2D1A* knockout and control *Xenopus* tadpoles were analyzed at stage 41-42 of embryo development under light microscope after immobilization by anesthesia.

CHAPTER THREE: *CC2D1A* IS A CANDIDATE GENE FOR CILIOPATHY PHENOTYPE IN HUMANS

3.1. Introduction

Investigation and molecular characterization of novel genetic variations that are detected in patients is important to reveal genotype-phenotype relations between those genes and diseases. Adding the results to the growing literature and publicly available universal databases further facilitates the diagnosis and development of personalized therapies, especially for individuals with rare genetic disorders.

Here we report three patients that have ciliopathy-related phenotypes and have homozygous variations in the *CC2D1A* gene. Importantly, two of these patients are siblings, with no definitive diagnosis and carry a novel nonsense variation in *CC2D1A*.

3.2. General, Neurological and Neuropsychiatric Examination of Patient 1

17-years-old patient was the first child born to non-consanguineous parents from Turkish Cypriot heritage and has a healthy sister. Family pedigree is presented in **Figure 7a.** Parents had no known neurological disorders. He was born by normal vaginal delivery (NVD) as 3250 grams.

Neurological examination revealed oculomotor apraxia and truncal ataxia as he was not able to initiate voluntary saccades in a head-fixed position but he could manage it by the vestibo-ocular reflex. Brain magnetic resonance imaging (MRI) revealed cerebellar vermis hypoplasia, mesencephalon and superior cerebellar peduncles constituting the typical molar tooth sign of Joubert syndrome (JS) **(Figure 7b)**. There was no Joubert-related retinal dystrophy, ocular coloboma, cystic renal disease and hepatic fibrosis in the patient.

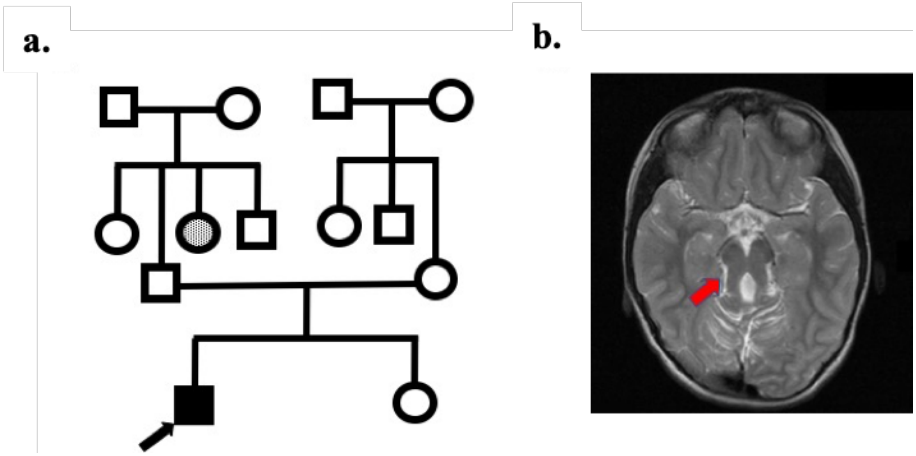

Figure 3. 1. Familial and clinical information of patient 1.

a. Family pedigree of Patient 1 is shown. Patient 1 is the first child of non-consanguineous parents. Parents and younger sister are not affected. **b.** Typical molar tooth sign of Joubert syndrome observed in the brain magnetic resonance imaging (MRI) of the patient is represented by the arrow.

Further evaluation by child and adolescent psychiatry department was performed using the Wechsler Intelligence Scale for Children (WISC) and he was diagnosed with moderate intellectual disability and obsessive-compulsive disorder (OCD). His psychiatric symptoms were characterized by repetitive questions, worrying about getting sick and extreme anxiety and anger when non-answered by his mother.

The patient was then directed to Near East University Medical Faculty Hospital Medical Genetics department for molecular genetic analysis.

3.3. Molecular Genetic Analysis of Patient 1

Whole exome sequencing results revealed a homozygous c.2106G>A (rs1276908141) variation in Abelson's Helper Integration 1 (*AHI1*) gene and another homozygous c.1739C>T (rs202057391) variation in Coiled-Coil and C2 Domain-Containing Protein 1A (*CC2D1A*) gene, both inherited from heterozygous parents **(Figure 10)**.

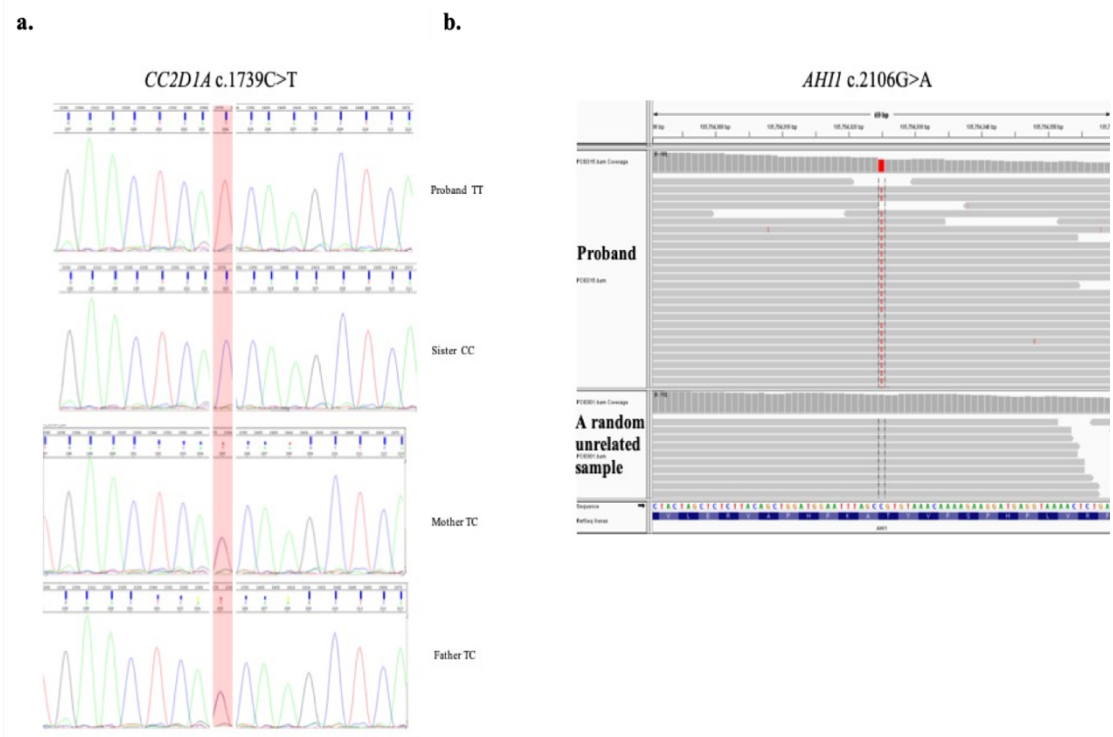

Figure 3. 2. Molecular genetic analysis results of patient 1.

a. Sanger sequencing results of the *CC2D1A* c.1739C>T variation in the proband (homozygous T), sister (homozygous C) and parents (heterozygous). **b.** Whole genome sequencing results of the *AHI1* c.2106G>A variant of the proband compared to random unrelated samples.

AHI1 c.2106G>A rs1276908141 is a silent change at codon 702. It was reported in heterozygous state in one JS patient, *in trans* with another known pathogenic JS variant in the HGMD and ClinVar databases.

CC2D1A c.1739C>T rs202057391 is a missense variant causing substitution of threonine with isoleucine at position 580 of the amino acid sequence. This variant was previously reported to be associated with Smith-Magenis Syndrome-like and Mental retardation autosomal recessive 3 disorder in the ClinVar and HGMD databases.

3.4. Neurological Examination of Patients 2 and 3

Neurological and neuropsychiatric evaluation of patient 2 revealed symptoms of autism spectrum disorder and non-syndromic mental retardation (NMR). She also has

obese phenotype, suggestive of a ciliopathy phenotype. Her sibling, 6-year-old male, patient 3, also present autistic features and NMR and he had left renal cysts. Electroencephalogram (EEG) and MRI brain tests of both siblings were normal.

They were born to healthy consanguineous parents from Turkish heritage by NVD as 3750 and 3500 grams, respectively. Family pedigree is shown in **Figure 8**. After neurological examination and screening tests, patients were directed to Uludag University Hospital Department of Medical Genetics for molecular analysis.

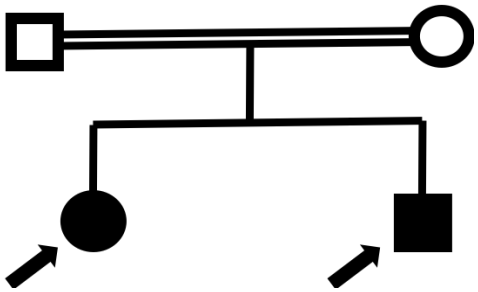

Figure 3. 3. Familial information of patients 2 and 3.

Represents the family pedigree of the two siblings. Patient 2 is the daughter and patient 3 is the younger son, who were born to healthy consanguineous parents.

3.5. Molecular Genetic Analysis of Patients 2 and 3

A homozygous novel *CC2D1A* c.1186C>T (p.Arg396*) variant was identified in both siblings, inherited from heterozygote parents **(Figure 11)**. No other candidate variations potentially related to their phenotype was detected in whole exome sequencing analysis. *CC2D1A* c.1186C>T (p.Arg396*) variation creates a truncated CC2D1A protein, potentially causing loss of function effect.

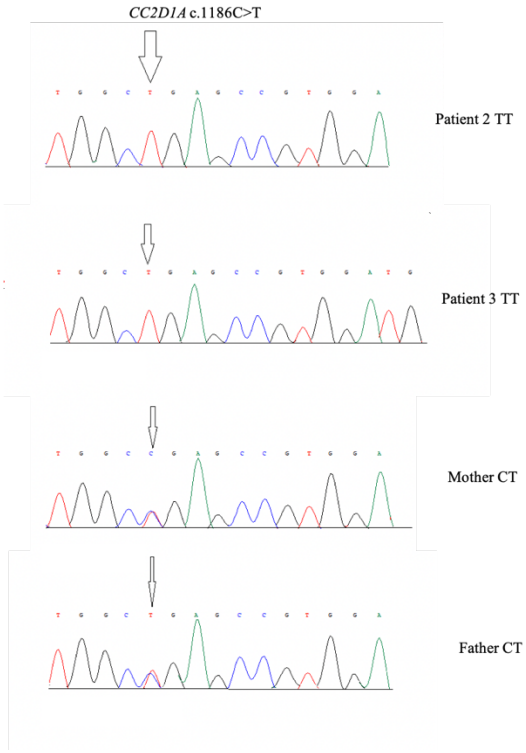

Figure 3. 4. Molecular genetic analysis results of patients 2 and 3.

a. Sanger sequencing results of the *CC2D1A* c.1186C>T variation detected in patients 2 and 3 are represented in this figure. Patients inherited the variation from heterozygote parents.

3.6. Discussion

Ciliopathies are a wide range of disorders with multiple organ involvements and affect thousands of patients worldwide. Ciliopathies are caused by pathogenic gene variations in cilia-related proteins. Advances in molecular genetic analysis and genome editing technologies enable detection and identification of new genes and novel variants in patients, especially with rare genetic disorders. Identification of homozygous variations in the *CC2D1A* gene in three patients representing ciliopathy phenotypes led us to investigate the role of this gene in cilia using model organisms.

Patient 1, has two homozygous mutations that may result in a combined effect in the phenotype. He was diagnosed with pure JS co-occurring with severe OCD and has a synonymous homozygous mutation in the *AHI1* gene, which is an established JS gene. However, this is the first study that report this synonymous variation in homozygous

form in a JS patient. As it is unlikely that the presence of this variation alone to result in the strong phenotype observed in our patient alone, we thought that *CC2D1A* mutation is enhancing the phenotype. Detection of another novel *CC2D1A* variation in two siblings with ciliopathy-related phenotypes strengthened our hypothesis.

CHAPTER FOUR: USING *CAENORHABDITIS ELEGANS* AS A MODEL TO STUDY *CC2D1A*

4.1. Introduction

C.elegans are an excellent model to study neurodevelopmental and cilia-related disorders and genes as a comprehensive connectivity map of their neuronal structure is readily available and a substantial amount of adult hermaphrodite neurons possess cilia at the ends of their dendritic processes. Easy genetic manipulation allows creation of knockout and transgenic animals. Their transparency make *C.elegans* an ideal model organism to investigate protein expression and localization by fluorescent labelling. We used *C.elegans* to investigate the effect of *CC2D1A* in cilia structure or function by creating *CC2D1A* knockout organisms by CRISPR-Cas9 technology. Additionally, localization of CC2D1A protein was determined by creating models that express fluorescently-labelled CC2D1A.

4.2. Optimization of Control Primers

Designed primers were optimized by gradient PCR, using wild-type *C.elegans* DNA as template **(Figure 13)**. Expected band size was 4219bp. Even though there were non-specific bands at approximately 2600bp, 67^0C was determined as the optimal annealing temperature for the control primers.

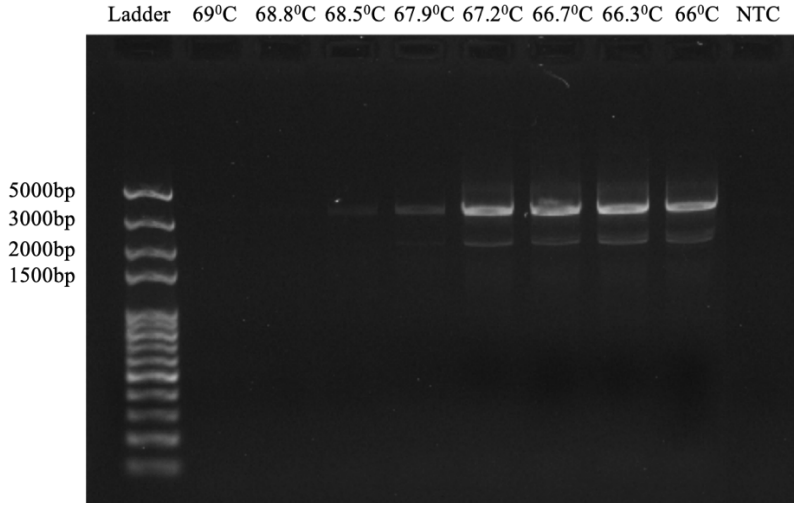

Figure 4. 1. Optimization of control primers targeting the Y37H9A3.1 gene in *C.elegans*.

Agarose gel image of gradient PCR products set with control primers and wild-type *C.elegans* DNA as template. Expected band size was 4219bp. Thicker bands at 4219 base pairs indicate *Y37H9A3.1* in wild type *C. elegans*, other bands at 2600bp are non-specific. There were no products above 67.2^{0}C. 67^{0}C was determined as the optimal annealing temperature for the control primers. (NTC: no template control)

4.3. Confirmation of sgRNA Ligation Into the pRB1017 sgRNA Backbone Vector

Three sgRNAs (sgRNAI, sgRNAIIa and sgRNAIII) targeting *Y37H9A.3.1, C.elegans* homolog of *CC2D1A*, were cloned into the pRB1017 vector to be used in CRISPR-Cas knockout of the gene. For each sgRNA, a ready forward primer targeting the vector and the sgRNA's own reverse oligo was used as the reverse primer **(Figure 14a)**. Bands around 580bp were expected to confirm cloning of sgRNAs into the backbone vector. Cloning procedure was repeated for failed sgRNAs until correct bands are observed (**Figure 14b**).

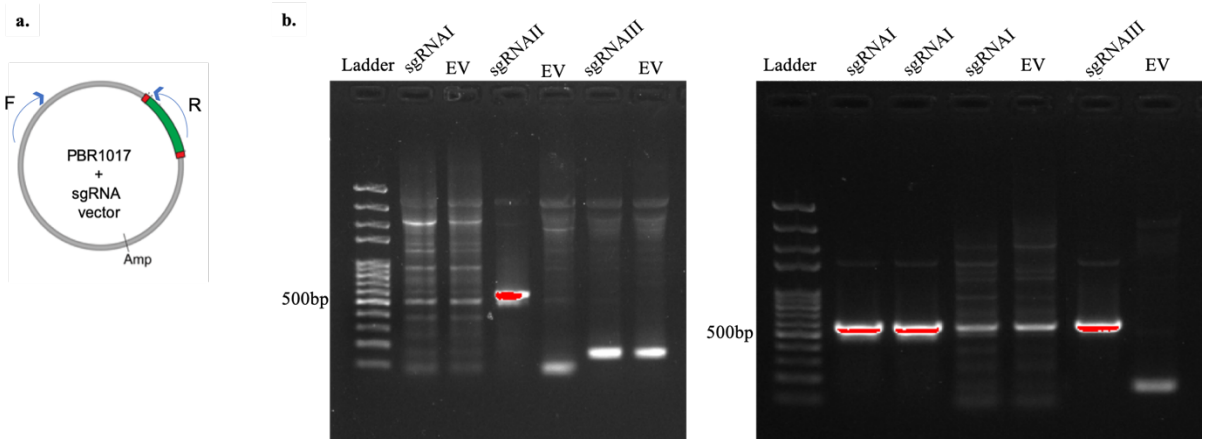

Figure 4. 2. Confirmation of sgRNA insertion into the backbone vector.

a. Represents the locations of forward and reverse primers used to confirm the insertion of sgRNAs into the backbone vector pBR1017. **b.** Agarose gel images of pBR1017 vector after cloning and transfection with sgRNAI, II and III. Strong bands at 580bp indicates the presence of sgRNA in the vector. No or faint bands indicate no insert. (EV: empty vector)

4.4. Confirmation of Transformation of E.coli with Ligated Vectors

Upon transformation of competent *E.coli* cells with previously prepared sgRNA expressing pRB1017 vectors, colony PCR was performed to select transformed colonies. For each sgRNA, forward primer targeting the vector and the sgRNA's own reverse oligo was used as the reverse primer **(Figure 14a)**. Bands around 580bp were expected to confirm transformation of *E.coli* colony with the vector. Agarose gel images are shown in **Figure 15**. Colonies labelled as 1.8 (for sgRNAI), 2.6 (for sgRNAII) and 3.6 (for sgRNAIII) were selected. Vectors isolated from these cells were used for injection, together with the Cas9 expression vector for CRISPR-Cas9 experiments in *C.elegans*.

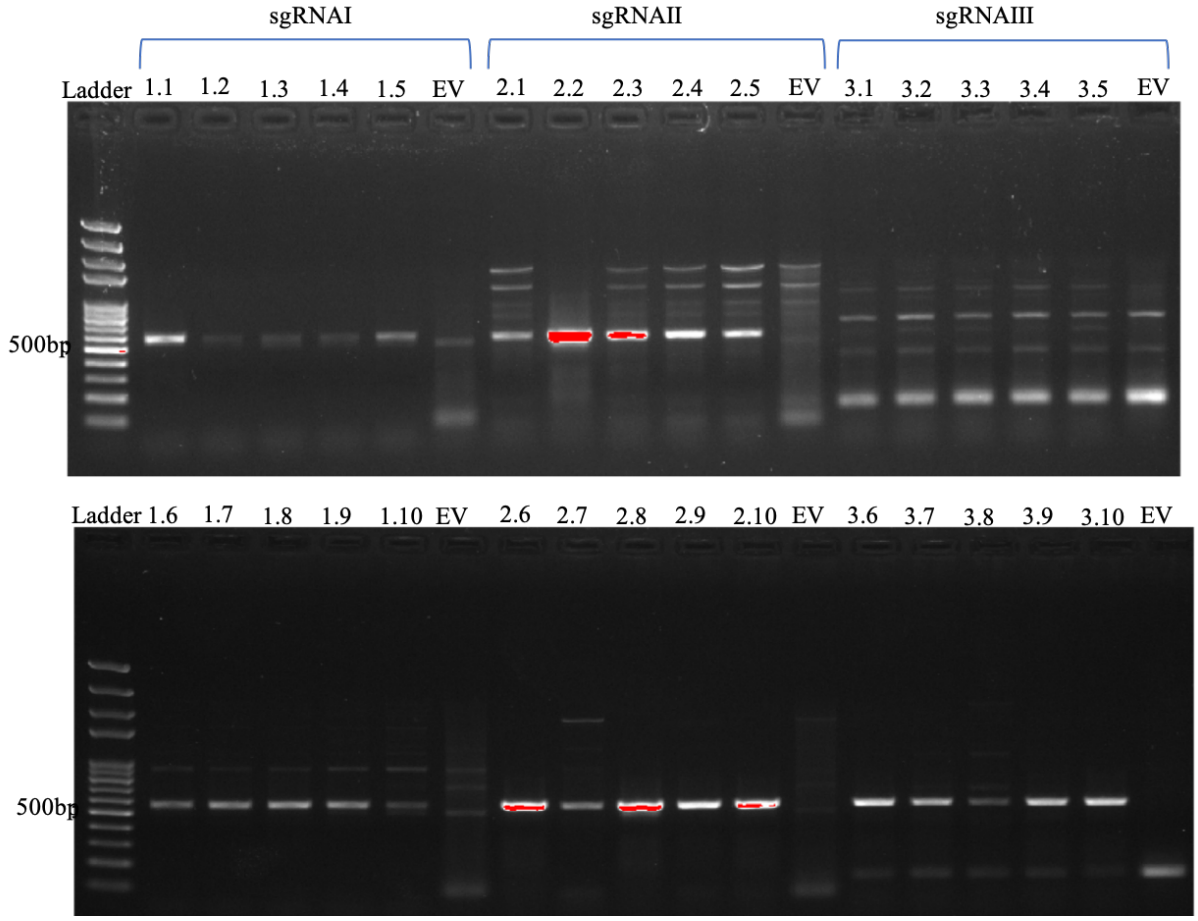

Figure 4. 3. Confirmation of *E.coli* transformation with sgRNA-ligated vectors.
Gel images of colony PCR performed with bacteria transformed with sgRNA-containing plasmids. Wells labelled 1.1-1.10 are the ten colonies selected for sgRNAI, wells labelled 2.1-2.10 are the ten colonies selected for sgRNAII and wells labelled 3.1-3.10 are the ten colonies selected for sgRNAIII. (EV: empty vector)

4.5. Confirmation of *CC2D1A* Knockout in *C.elegans*

After the injection of over 25 *C.elegans* with CRISPR-Cas9 components, they were grown and 6 F1 worms with a roller phenotype were selected. After production of eggs, control PCR was set up to detect the F1 organisms that has heterozygous or homozygous deletion in *CC2D1A* **(Figure 16)**.

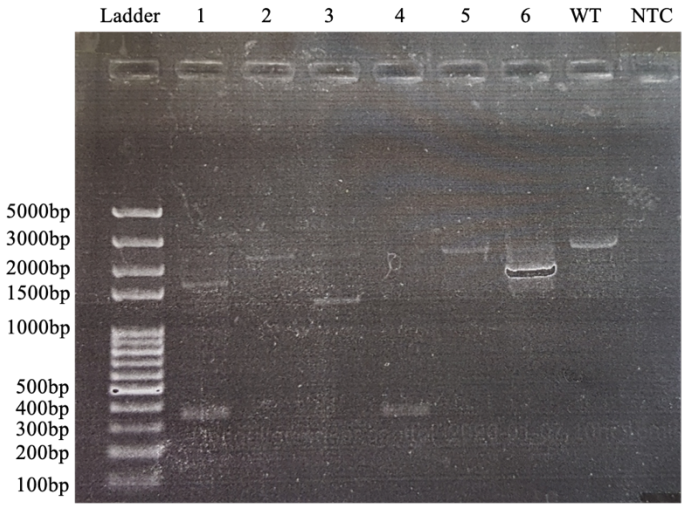

Figure 4. 4. Confirmation of *CC2D1A* knock-out and selection of mutant *C.elegans*.

Agarose gel image of 6 F1 *C.elegans* that show the 'roller' phenotype. Worms 2 and 5 had the wild-type genotype. Worms 1,3,4 and 6 were heterozygotes. (WT: wild type, NTC: no template control)

Worms labelled as 1,3,4, and 6 had bands suggestive of heterozygous deletion in *CC2D1A*. Their offspring (F2 generation) were planned to be analyzed to detect homozygous *CC2D1A* knockout mutants. Unfortunately, even though the experiments were repeated at least 3 times and with different sgRNA oligos as well, no worms with this genotype could be obtained. Complete knockout of *CC2D1A* is likely lethal in lower eukaryotes like *C.elegans*.

4.6. Localization of CC2D1A in *C.elegans*

Transgenic *C.elegans* expressing CC2D1A::GFP and CEP-41::wrmScarlet were visualized under fluorescent microscope **(Figure 17)**. CC2D1A::GFP expression was observed in cilia and dendritic cells of *C.elegans* tail. The protein largely co-localises with the CEP41, suggesting a possible role in tubulin structure / function. Further functional analyses will be needed to understand the precise function of the protein.

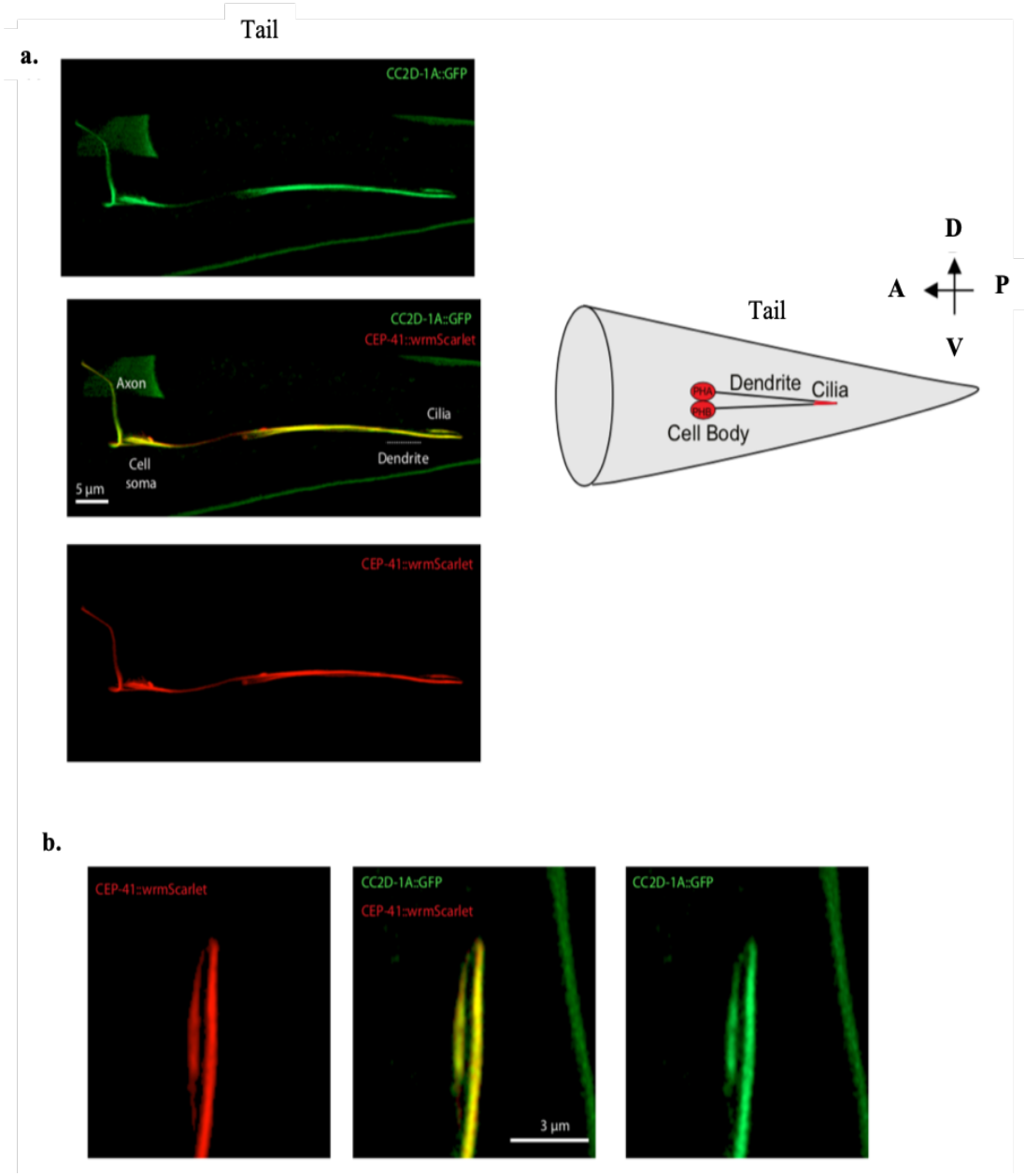

Figure 4. 5. CC2D1A protein expression in *C.elegans* cilia.

a. Fluorescent imaging of CC2D1A::GFP (green), CC2D1A::GFP and CEP41::wrmScarlet merged (yellow), CEP41::wrmScarlet (red). Tail structure of the *C.elegans*, including the cilia, dendrites and cell body are also represented in the figure. **b.** Close-up image of the cilia expressing CC2D1A::GFP (green), CC2D1A::GFP and CEP41::wrmScarlet merged (yellow), CEP41::wrmScarlet (red).

4.7. Discussion

Even though heterozygous *CC2D1A* knockout *C.elegans* were obtained and mated, no homozygous knockouts were detected in the following generations. These results suggest that complete loss of this protein is lethal in early stages of life in lower eukaryotic organisms like the *C.elegans*. Further molecular characterization and functional analyses will enable us understand the precise role of CC2D1A protein. Determining protein localization is an important starting point to reveal its function.

Expression of fluorescently-tagged CC2D1A protein revealed that it is localized in the cilia and in dendrites of *C.elegans* tail. This may suggest a possible role in cargo transfer, which is a crucial aspect of ciliogenesis and normal cilia function.

CHAPTER FIVE: USING *XENOPUS TROPICALIS* AS A MODEL ORGANISM

5.1. Introduction

Xenopus tadpoles have long been used as model organisms to study ciliary structure and function as well as neurodevelopmental diseases. Larval skin of *Xenopus* possesses multiciliated cells on epithelial surface that can easily be visualized either by staining ciliary axonemes or by high-speed videography to assess structure and function. Compared to *X.leavis, X.tropicalis* has much shorter generation time and has a diploid genome, which makes it more advantageous (Dubaissi and Papalopulu 2011).

We created *CC2D1A* knockout *X.tropicalis* animals to study the possible role of this protein on the cilia present on their epidermal surface. Effect of gene knockout on structure was visualized by immunohistochemical staining and effect on ciliary function was analyzed using optical coherence tomography to assess cilia flow in control and knockout organisms.

5.2. Epidermal Cilia Flow is Disturbed in *CC2D1A* Knockout *X.tropicalis*

Cilia function in control and *CC2D1A* knockout *X.tropicalis* groups was evaluated by visualizing the flow created over ciliated skin at stage 28 by optical coherence tomography (OCT). OCT is described as the optical analogue of ultrasound imaging. It is non-invasive and non-distructive, therefore can be used to capture micrometer-resolution 2 or 3 dimensional images or videos of tissues *in vivo*.

Coordinated beating of healthy cilia creates a directed fluid flow. Disruption in normal fluid flow is caused by defects during ciliogenesis or cilia polarity. OCT images of flow over the ciliated epidermal surface of an embryo from uninjected control group and a *CC2D1A* knockout embryo are shown in **Figure 18a**. Normal, directed flow over the ciliated skin is observed in the uninjected control *X.tropicalis*, whereas the directionality of the fluid flow is clearly disturbed in *CC2D1A* knockout model.

Total OCT results obtained in multiple measurements demonstrated the difference in cilia function between uninjected control group, only Cas-9 injected controls, *CC2D1A* knockout group and *CC2D2A* knockout group quantitatively (**Figure 18b).** The number of organisms with normal cilia flow decreased significantly in *CC2D1A* knockout group compared to UIC and Cas-9 control groups. The decrease in the amount of normal cilia flow in *CC2D1A* knockout group is even greater than the *CC2D2A* knockout group, which is an established ciliopathy gene. These results clearly demonstrate the decrease in cilia function due to the absence of CC2D1A protein, suggesting a possible role in ciliogenesis.

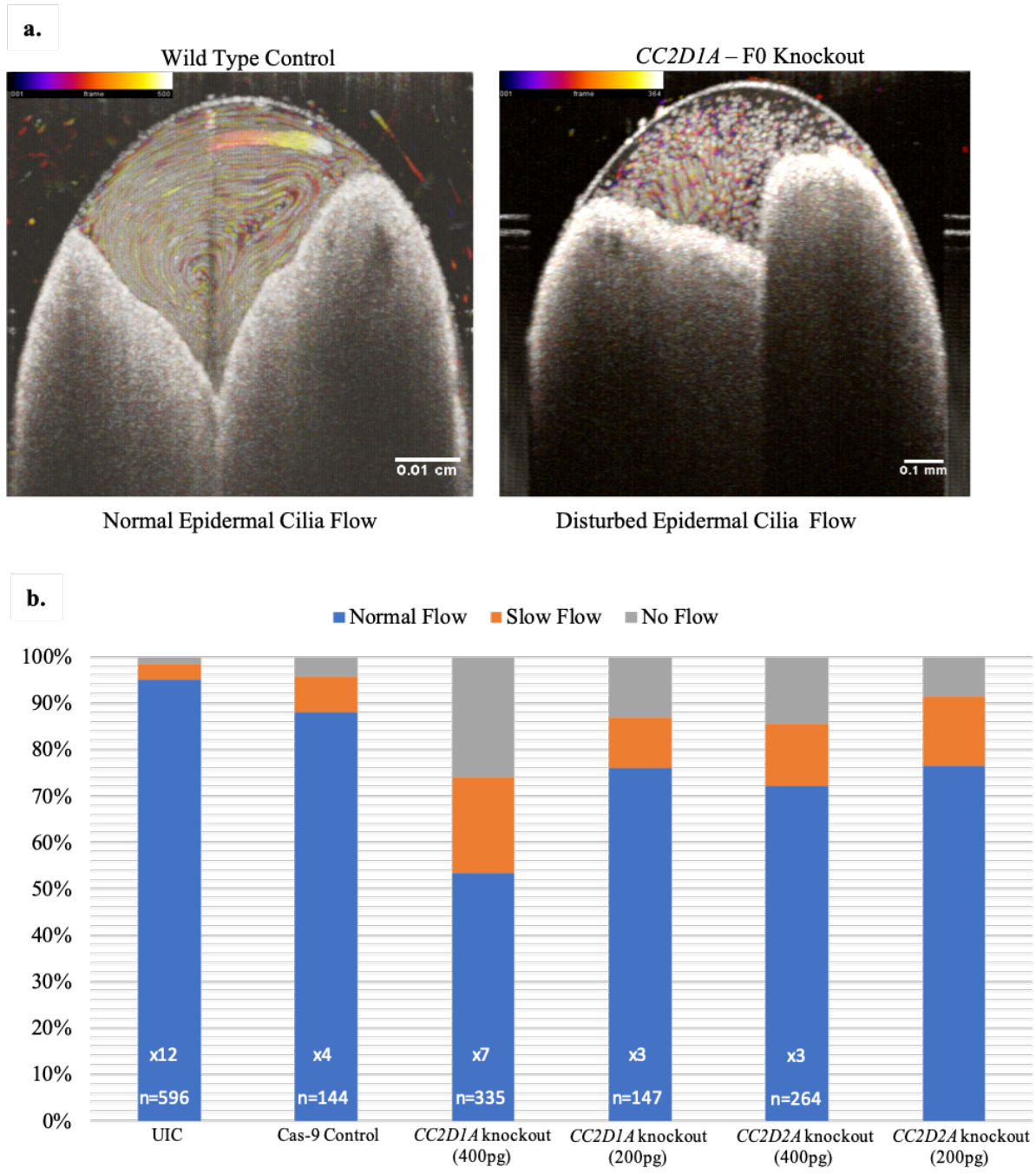

Figure 5. 1. Optical coherence tomography images and results representing epidermal cilia flow in control and *CC2D1A* knockout *X.tropicalis*.

a. Fluid flow over epidermal cilia was captured by OCT and images were represented in this figure. Image on the left panel shows normal directional flow created over ciliated skin of wild type *X.tropicalis* epidermis. On the right panel, disturbed epidermal cilia flow is visible in *CC2D1A* knockout animal. **b.** Total OCT results are summarized in the chart. In each column, the times that the experiment was repeated

(x) and the total number of *X.tropicalis* evaluated (n) are shown. Cas-9 control group demonstrate the results of the damage created by microinjections. There is a significant decrease in function of embryonic epidermal cilia in *CC2D1A* knockout embryos compared to UIC and Cas-9 control groups. (UIC: uninjected control)

5.3. Loss of Epidermal Cilia is Observed in *CC2D1A* Knockout *X.tropicalis*

After the establishment of the changes in cilia function, immunostaining on UIC tadpoles with normal epidermal flow and *CC2D1A* and *CC2D2A* knockout tadpoles with disturbed epidermal flow was performed to observe changes in amount, distribution and morphology of epidermal cilia. A significant decrease in the number of multiciliated cells on *CC2D1A* knockout epidermis was observed compared to UIC and Cas-9 injected controls as well as a clear disruption in morphology and distribution of cilia over the skin **(Figure 19.a.)**. Cilia count per area was calculated to understand the difference in the consistency. **Figure 19.b.** shows the significant decrease in the number of epidermal surface cilia in *CC2D1A* knockout samples with abnormal epidermal cilia flow compared to UIC group. This further confirms a possible role for CC2D1A protein in ciliogenesis. Consistent with the results obtained from OCT, the amount of decrease in cilia number per area in *CC2D1A* knockout samples was greater than *CC2D2A* knockout samples.

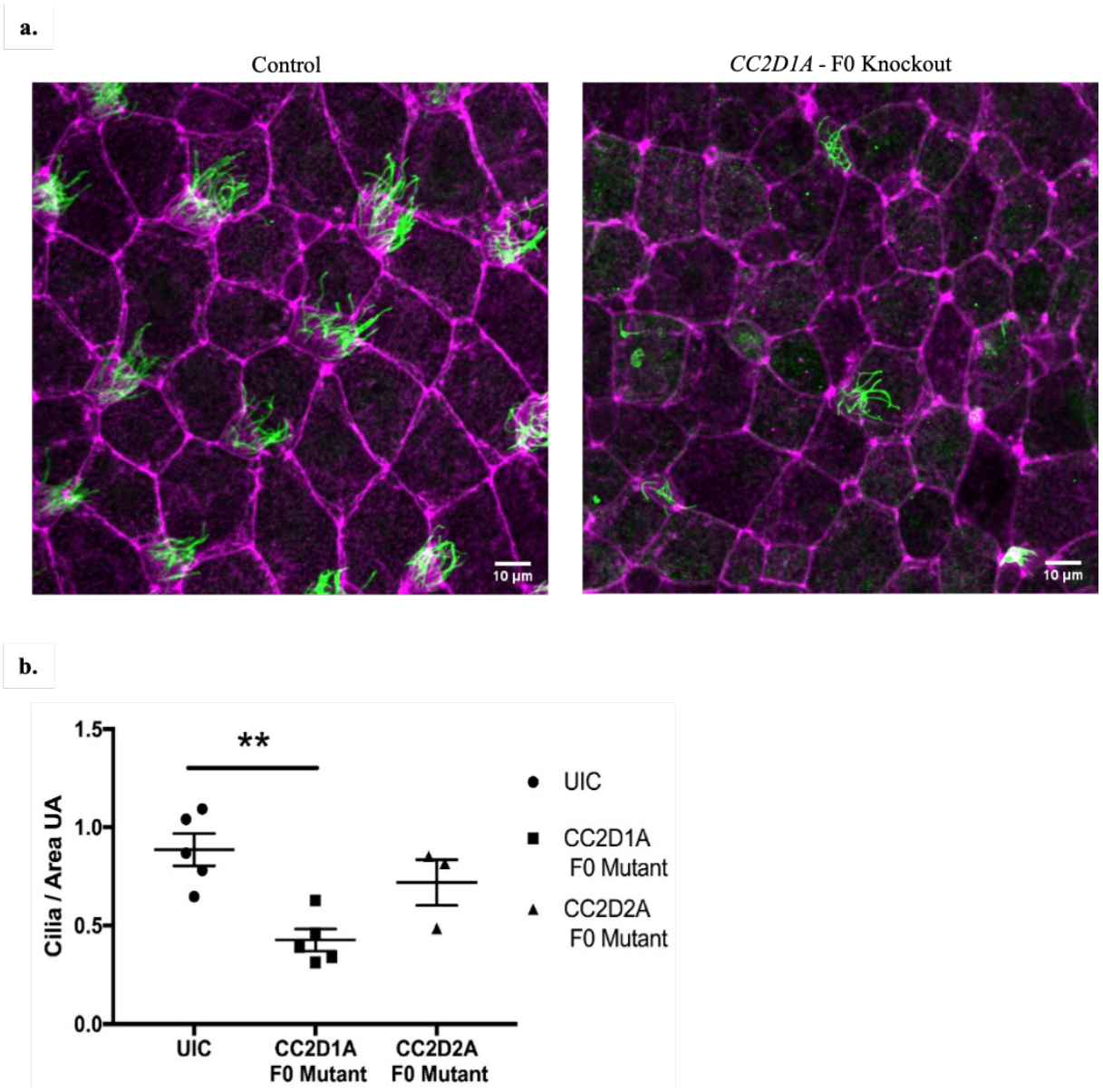

Figure 5. 2. Immunohistochemical staining results of epidermal cilia in control and *CC2D1A* knockout *X.tropicalis*.

a. Immunohistochemical staining images of control and *CC2D1A* knockout *X.tropicalis* multiciliated epidermal skin obtained by confocal fluorescent microscopy is shown. Phalloidin dye (violet) selectively stains the actin filaments of epidermal cells. Ciliary axonemes were stained with acetylated α-tubulin antibody (green). Disruption in amount, morphology and distribution of cilia in *CC2D1A* knockout model is clearly visible in fluorescent images. **b.** Cilia count per area in UIC, *CC2D1A* and *CC2D2A* knockout animals is demonstrated in the chart. The difference between the UIC and *CC2D1A* knockout group is statistically significant ($p<0.05$).

5.4. Knockout of *CC2D1A* Results in Craniofacial Malformations in *X.tropicalis*

Control and *CC2D1A* knockout larvae were visualized under light microscope at stage 41-42 to investigate any change in gross morphology of craniofacial features. Malformations caused by *CC2D1A* knockout were greater than malformations resulted from *CC2D2A* knockout **(Figure 20)**.

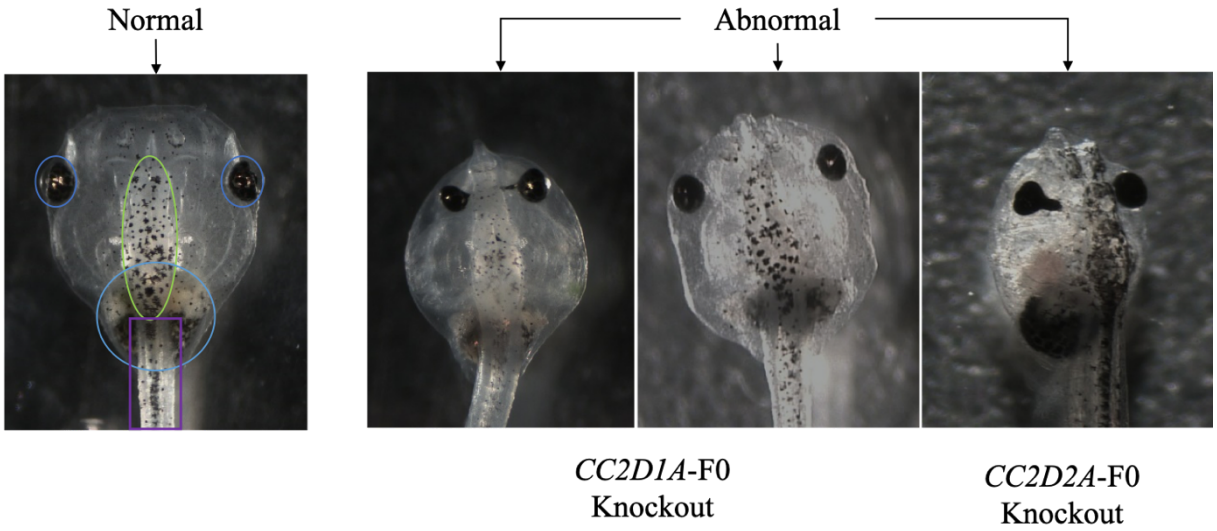

Figure 5. 3. Craniofacial analysis of control and CC2D1A knockout X.tropicalis
Dorsal views of gross craniofacial morphology of wild type, *CC2D1A* and *CC2D2A* knockout *X.tropicalis* obtained by light microscopy is shown in the figure.

5.5. Discussion

Analysis on epidermal cilia of *X.tropicalis* tadpoles revealed altered function as well as altered structure in *CC2D1A* knockout models when compared to wild type controls.

Knockout animals represented reduced ciliary flow over epidermal surface, which indicates a functional problem. The reduction was even more than the CC2D2A knockout models, which were used as positive controls as *CC2D2A* is an established ciliopathy gene. Immunohistochemical staining of epidermal multiciliated cells revealed reduced number of cilia per area in *CC2D1A* knockout animals, compared to wild types.

Further investigations to analyze gross craniofacial features of *X.tropicalis* tadpoles revealed that loss of CC2D1A protein results in certain alterations in craniofacial structures of the tadpoles, similar to the changes created by the loss of CC2D2A protein.

It is likely that this protein has a functional role during ciliogenesis that its absence resembles the phenotypic features created by the absence of a known ciliopathy gene.

CHAPTER SIX: DISCUSSION AND CONCLUSION

Ciliopathies are complex genetic disorders that are caused by defects in cilia structure or function. Identification of non-motile cilia that present on almost all cell types in eukaryotic organisms and their involvement in numerous cellular processes greatly accelerated the research and data-flow in the field within the last two decades (Mitchison and Valente 2017). Advances in molecular genetic research, such as development of highly sensitive next generation sequencing technologies and genome editing techniques led to the establishment of several novel genes associated with cilia and identification of genetic variations resulting in ciliopathy-related phenotypes.

One of the greatest leaps in genome editing technology was the adaptation of CRISPR-Cas9 system to be used in eukaryotic cells. It allowed the creation of model organisms for evaluation of the function of novel genes or effect of detected variants in various systems (Ma and Liu 2015).

In this project, we designed model systems to analyze the effect of the *CC2D1A* gene in ciliogenesis and cilia function upon identification of homozygous variants in *CC2D1A* gene in three patients representing ciliopathy-related symptoms. Additionally, protein localization was analyzed by creating transgenic *C.elegans* model to have an insight about the function of the protein in cilia.

6.1. Detection of *CC2D1A* Gene Variants in Patients with Ciliopathy Phenotype

Patient 1, who was the first patient participated in this research study had clinical symptoms suggestive of Joubert syndrome, later supported by the MRI results. The patient is a 17-year-old male and was born to non-consanguineous healthy parents. He also has a non-affected healthy sister.

Further evaluation of this patient by a child and adolescent psychiatrist because of physciatric symptoms started two years ago and characterized by repetitive questions, extreme anxiety and anger when non-answered by his mother and worrying about getting sick revealed that he has severe obsessive-compulsive disorder co-occuring

with pure JS. Even though behavioral problems such as hyperactivity, temper tantrums, inattention and depression were reported in Joubert syndrome patients before, our patient represents emotional and behavioral acts more suggestive of obsessive compulsive disorder, which was not reported to co-occur with JS in the literature previously (Farmer et al. 2006; Fennell et al. 1999; Parisi and Glass 2017). Molecular genetic analysis of patient 1 revealed two homozygous variations inherited from heterozygous parents; *AHI1* c.2106G>A p.(Thr702=) (rs1276908141) and *CC2D1A* c.1739C>T p.Thr580Ile (rs202057391).

AHI1 is an established Joubert syndrome gene and pathogenic variations in the gene are common in patients. However, *AHI1* c.2106G>A rs1276908141 is a silent change in the 702nd codon with no effect on the amino acid sequence. According to the ClinVar database, the variant was only reported in one patient with Joubert syndrome previously *in trans* with a pathogenic *AHI1* variant (p.Pro560Thrfs*5) (ClinVar accession RCV000534772.2) (Dixon-Salazar et al. 2004; Valente, Brancati, et al. 2006). It was thought that this variation might be contributing to the disease phenotype observed in the patient, however there is no experimental evidence. In this context, the variation was classified as likely-pathogenic in the ClinVar database. To our knowledge, this patient was the first case reported to the literature carrying homozygous rs1276908141 variant with clinical features consistent with JS.

It was noted in the ClinVar submission that splice site prediction algorithms predict that this variation may create or strengthen a new splice site. However, when we utilized the NNSplice prediction tool, which is a neural network modeling program, no changes in acceptor or donor site predictions were shown.

Previous reports have indicated that the type of the two recessive variants can determine the severity of ciliopathy disorders. Nonsense mutations are classified as strong mutations, whereas missense and synonymous mutations are referred as weak. Presence of two strong mutations result in more severe, early-onset developmental disorders with a broad-range organ involvement as in Meckel's syndrome. Presence of at least one weak mutation, however, causes a milder, late-onset degenerative

disorder such as a mild form of JS (Hildebrandt et al. 2011). We think that the pure JS phenotype co-occurring with severe OCD observed in patient 1 cannot be explained by the presence of two 'weak' synonymous *AHI1* variations.

CC2D1A c.1739C>T, p.Thr580Ile (rs202057391), on the other hand, is a missense variation resulting in the substitution of threonine amino acid in position 580 of the amino acid sequence with isoleucine. Although there are conflicting interpretations of pathogenicity in the ClinVar database, the c.1739C>T (rs202057391) variant detected in our patient was previously reported to be associated with Smith-Magenis syndrome-like disorder and was classified as pathogenic in one submission (Loviglio et al. 2016). However, there are no reports in the databases associating *CC2D1A* gene with Joubert syndrome or any other cilia related disorders and molecular mechanisms by which the CC2D1A protein may be involved in these neuropsychiatric-related behaviors are not explained. Only a recent study by Ma *et.al.* revealed that loss-of-function of *CC2D1A* in zebrafish model results in ciliary dysfunction and defective left-right patterning, suggesting that the loss of this gene may be associated with ciliary dysfunction (Ma et al. 2020).

On the contrary, this variant was classified as benign or likely-benign in other ClinVar submissions and there are five healthy homozygote individuals reported in the GnomAD database for this variant.

In the light of these information and the results obtained from patient 1 we considered that the presence of homozygous *CC2D1A* c.1739C>T, p.Thr580Ile missense variation may enhance the effect of the homozygous synonymous *AHI1* variant in our patient. It is known that involvement of mutations in other modifier genes or the combined effect of two or more recessive genes with heterozygous mutations (true oligogenicity) are important in determination of genotype-phenotype correlations of ciliopathy phenotype (Hildebrandt et al. 2011; Leitch et al. 2008). Shortly after, detection of a novel homozygous *CC2D1A* c.1186C>T (p.Arg396*) variant in two siblings with symptoms of ciliopathy disorder including obesity, renal cysts and autism further supported our hypothesis.

The siblings were born to healthy consanguineous parents. Patient 2 is 10 years old female and patient 3 is a 6-year-old male patient. Neurological and neuropsychiatric evaluation of patients revealed symptoms of autism spectrum disorder and non-syndromic mental retardation (NMR). Patient 2 has obesity, whereas patient 3 had left renal cysts suggestive of a ciliopathy phenotype. There were no abnormalities in electroencephalogram (EEG) and MRI brain tests of both siblings, eliminating JS and other common ciliopathies.

Different from patient 1, there were no other homozygous inherited or *de novo* mutations detected in WES analysis of the siblings, suggesting that the ciliopathy phenotype observed is resulted from the variation detected in *CC2D1A*. *CC2D1A* c.1186C>T (p.Arg396*) variant results in the production of

As previously mentioned, the involvement of the CC2D1A protein in ciliogenesis or cilia functioning was not shown before. Therefore, we decided to generate animal models to establish a role of this protein in cilia.

6.2. Functional and Expressional Analysis of CC2D1A in *C.elegans*

C.elegans were selected to analyze the effect of *CC2D1A* gene knock-out in cilia due to their ease of maintenance, quick manipulation and analysis time as well as the fact that their genome was entirely sequenced and there is a comprehensive map of their neurons and sensory cilia readily available. Likely, they have exactly 959 somatic cells of which entire lineage has been traced. Cell lineages of *C.elegans* are entirely invariant between organisms (Gilbert 2000). Any defect in cilia and related neurons and structures can be observed and analyzed easily.

After the genetic manipulation of *C.elegans* to disturb *CC2D1A* by CRISPR-Cas9 technique, even though heterozygote animals were established, no homozygous mutants could be obtained. It is likely that the complete loss of this protein is lethal in lower eukaryotes, such as *C.elegans*. In the literature, the role of the CC2D1A protein was shown to be studied in model organisms including *Droshophila melanogaster*,

mouse and human cells, however there are no reports of the use of *C.elegans* (Al-Tawashi et al. 2012; Gallagher and Knoblich 2006; Lopes et al. 2010; Yang et al. 2019; Zhao et al. 2011). This may be explained by our observation of lethality of the absence of the protein in this organism.

In many eukaryotic organisms, cilia are involved in developmental processes such as cell fate decisions and establishment of left-right asymmetry. These are achieved by the fluid flow created by motile cilia within the embryonic node as well as by functioning as signaling centers (Drummond 2012). However, in *C.elegans* the only ciliated cell type is the sensory neuron. 60 out of 302 neurons in an adult hermaphrodite *C.elegans*, have cilia at the ends of the dendrites and none of these are motile. Rather, developmental processes and cell fates are largely determined by cell-cell interactions (Inglis et al. 2007).

In this context, no role in early embryonic development of *C.elegans* is attributed to the cilia. The main function of ciliary cells in the nematode is as sensory organs mostly for mechano- chemo- and oxygen-sensation. A small proportion is involved in mating behaviours.

Also, it is known that cilia proteins have extraciliary roles in various processes such as cell cycle, trafficking, cytoskeleton (Hua and Ferland 2018). Previous studies have shown that CC2D1A protein is involved in transcriptional regulation of some proteins such as NF-κB and in regulation of spindle pole localization of the cohesin subunit SCC1/RAD21, mediating centriole cohesion during mitosis (Nakamura et al. 2008, 2009; Zhao et al. 2010). Complete loss of protein may be resulting in disturbed cell division during embryonic development of the nematode, or abnormal neuronal development may be resulting in death of mutants. Abnormal neuronal development and function was observed in mutant *droshophila melanogaster* models potentially due to disturbed endosomal trafficking of Notch signaling pathway (Gallagher and Knoblich 2006). Notch signaling was shown to be a key pathway for cilia length control in deltaD zebrafish mutants (Lopes et al. 2010). However, there is no

information about the role of CC2D1A in the Notch signal pathway and cilia length control.

A mouse model that produces a truncated form of CC2D1A protein, which lack the C2 domain and three DM14 domains, showed that the protein is important in differentiation of neurons by transducing signals to the cAMP-protein kinase A (PKA) pathway (Al-Tawashi et al. 2012). A recent study indicated that conditional deletion of *CC2D1A* from excitatory neurons of male mouse impair cognitive function through hyperactivation of Rac1, which may implicate Rac1 inhibitors in the treatment of intellectual disability caused by *CC2D1A* mutations (Yang et al. 2019).

Further analysis was performed to detect the localization of CC2D1A protein in *C.elegans* by generation of a transgenic model expressing fluorescently labelled CC2D1A. Results indicated that the protein is localized in the dendrites and cilia regions of *C.elegans* phasmid cilia. To our knowledge, this is the first time that the CC2D1A was shown to be localized in cilia. Cilia proteins have very diverse roles and protein localization can provide precious information about the protein function. Presence of the protein in the dendrites and cilia suggest an involvement in ciliogenesis.

6.3. Functional Analysis of CC2D1A in *Xenopus* Model

Another model organism used to analyse the CC2D1A protein function in cilia was *Xenopus tropicalis*. Presence of multiciliated cells on the larval epidermal surface and established methods to analyse cilia function effectively made it an ideal model organism for this research project. As in *C.elegans*, CRISPR-Cas9 genome editing technique was used to knock-out the *CC2D1A* gene in *X.tropicalis*. Even though the percentage was lower than usual, homozygous knock-out animals were successfully established.

Disturbed fluid flow over the ciliated skin of *X.tropicalis* larvae in the *CC2D1A* knockout animals clearly represent disturbed function of cilia compared to wild type animals. Cilia-driven fluid flow is crucial for multiple processes including left-right

patterning, mucus clearance, gamete transport in the oviduct and cerebrospinal fluid circulation (Huang and Choma 2015). Previous studies indicated that cilia-driven leftward flow precedes asymmetric *nodal* gene expression in *Xenopus* embryo, determining laterality. Failure of leftward flow results in laterality defects in the tadpoles. Heart looping defect, where the heart bends towards the left (L-loop) side rather than right (D-loop), is among these laterality defects observed in tadpoles with inhibited ciliary flow (Schweickert et al. 2007). In the light of this information we decided to analyze the heart looping patterns of *CC2D1A* knockout tadpoles and preliminary data suggests a significantly increased number of tadpoles with L-loop heart compared to wild types. Cardiac malformations are frequent in ciliopathy disorders and a central role for cilia in congenital heart disease has been established (Elbedour et al. 1994; Klena, Gibbs, and Lo 2017).

A recent study by Ma *et.al.* revealed that loss-of-function of *CC2D1A* in zebrafish model, created by TALENs, results in ciliary dysfunction and heterotaxy suggesting that the loss of this gene may be associated with ciliary dysfunction (Ma et al. 2020). Our results support their findings and also provides an explanation to their observation. Defective cilia caused by loss-of-function of CC2D1A result in ciliopathy-related phenotypes, which include defective left-right patterning during early developmental stages. Likely, our results indicated that absence of CC2D1A cause aberrations in the craniofacial features of *Xenopus* tadpoles.

Loss of skin epithelial cilia on *CC2D1A* knockout tadpole skin explains the disrupted function observed by OCT. It is likely that the protein is involved in ciliogenesis process. Localization data further supports this hypothesis as it is localized in the dendrite and cilia, however its molecular function should further be assessed.

6.4. Conclusion

This study provides experimental evidence that CC2D1A protein encoded by the *CC2D1A* gene in humans is involved in ciliary function, for the first time in the literature. Loss-of-function mutations in the gene are likely to cause severe ciliopathy-

related phenotypes as ciliogenesis is disturbed, resulting in reduced number of cilia in studied model systems.

Additionally, here we report a patient with pure Joubert syndrome that also present severe obsessive-compulsive disorder symptoms carrying a synonymous homozygous *AHI1* variation. It is likely that the phenotype is enhanced by the presence of the homozygous missense variation in the *CC2D1A* gene in this patient. This observation was strengthened by the detection of the novel homozygous missense mutation in the *CC2D1A* gene in two siblings with ciliopathy-related phenotypes.

Identification of novel genes and gene variants in complex genetic diseases is of great importance for development of effective diagnosis and therapy options for the patients. We believe that this work will make a good contribution to the literature and provide valuable information for further studies in the field.

6.5. Future Remarks

Immunocytochemical and mRNA expression analysis in patient-derived fibroblast cells will enable us to detect the differences caused by the *CC2D1A* variations present in our patients compared to healthy, wild type controls.

Further research for molecular characterization, identification of protein-protein interactions and the pathways that the protein is involved in will be planned to widen our knowledge about the molecular mechanisms underlying the functional and structural defects reported in the absence of CC2D1A expression.

Additionally, creation of variant-specific models will enable us to further analyze the effect of variations detected in our patients and identify genotype-phenotype associations. This is particularly important for for the confirmation of predicted combined effect of the two variations in *AHI1* and *CC2D1A* genes in patient 1.

These works will contribute to shed a light on the path of the development of personalized medicine opportunities for patients, especially suffering from rare genetic disorders.

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ENCLOSURES

ENCLOSURE 1: CURRICULUM VITAE

1. PERSONAL INFORMATION

NAME, SURNAME: DATE of BIRTH and PLACE:	Gulten Tuncel 11.11.1991, Nicosia/Cyprus
CURRENT OCCUPATION: Research Assistant, PhD Candidate/ Near East University ADDRESS of CORRESPONDENCE: Near East University/DESAM Institute TELEPHONE: GSM 0533 875 45 00 E-MAIL: gulten.tuncel@yahoo.com	

2. EDUCATION

YEAR	GRADE	UNIVERSITY	DEPARTMENT
2017-now	Postgraduate	Near East University	Medical Biology, Molecular Medicine
2014-2016	Masters	Middle East Technical University	Biology, Cancer Genetics
2009-2013	Undergraduate	University of Manchester	Genetics
2009-2013	High School	19 Mayis Turk Maarif Koleji	-

3. EXPERIENCE

PERIOD	TITLE	DEPARTMENT	INSTITUTION
2018-now	Research Assistant	DESAM Institute	Near East University

4. FOREIGN LANGUAGES

LANGUAGE	READING	SPEAKING	WRITING
English	Excellent	Excellent	Excellent

5. PUBLICATIONS

Peer-reviewed articles

Kashoura, Y., Serakinci, N., Beleva, N., Kaçamak, N. I., Tuncel, G., & Oz, U. (2021). *"WNT signaling pathway genes expression profile in isolated hypodontia."* Applied Nanoscience (Switzerland), 1, 3. https://doi.org/10.1007/s13204-021-01850

Tuncel G, Akcan N, Gul S, Sag SO, Bundak R, Mocan G, Temel SG, Ergoren MC. (2021) *"Identification of a Novel De Novo COMP Gene Variant as a Likely Cause of Pseudoachondroplasia."* Appl Immunohistochem Mol Morphol. https://doi.org/10.1097/PAI.0000000000000914. PMID: 33595934.

MC, Ergoren, Paolacci S, Manara E, Dautaj A, Dhuli K, Anpilogov K, Camilleri G, Suer HK, Sayan M, Tuncel G, Sultanoglu N, Farronato M, Tartaglia GM, Dundar M, Farronato G, Gunsel IS, Bertelli M, and Sanlidag T. (2020) *"A pilot study on the preventative potential of alpha-cyclodextrin and hydroxytyrosol against SARS-CoV-2 transmission."* Acta bio-medica : Atenei Parmensis 91 (13-S). https://doi.org/10.23750/abm.v91i13-S.10817

Ergoren, M. C., Tuncel, G., Sag, S. O., & Temel, S. G. (2020) "*A rare case of fructose-1,6-bisphosphatase deficiency: a delayed diagnosis story,"* Turkish Journal of Biochemistry, 45(5), 613-616. doi: https://doi.org/10.1515/tjb-2019-0473

Ergoren, M.C., E. Manara, S. Paolacci, H. Cobanogullari, G. Tuncel, M. Betmezoglu, M. Bertelli, and T. Sanlidag. (2020) *"The Biennial report: The collaboration between MAGI Research, Diagnosis and Treatment Center of Genetic and Rare Diseases and Near East University DESAM Institute."* The EuroBiotech Journal 4 (4). https://doi.org/10.2478/ebtj-2020-0020.

R, Emekli, İsmailoğullari S, Bayram A, Akalin H, Tuncel G, and Dündar M. (2020) *Comparing expression levels of PERIOD genes PER1, PER2 and PER3 in chronic insomnia patients and medical staff working in the night shift.* Sleep medicine 73. doi: 10.1016/j.sleep.2020.04.027.

E, Kandemis, Tuncel G, Asut O, Temel SG, and Ergoren MC. (2020) *Strong Association between Serotonin Transporter 5-HTTVNTR Variant and Psychoactive Substance (Nicotine) Use in the Turkish Cypriot Population. Current drug metabolism*. doi: 10.2174/1389200221666200620201348.

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Gelener, P., Tuncel, G., Manara, E., Paolacci, S., Tuzlali, H., Severino, M., Bertelli, M., Ergoren, M.C. (2020) *A Rare and An Unusual Presentation of Asymptomatic Glutaric Aciduria Type I In A 35-Year-Old Woman*. Neurogenetics 1-8**.**

Tuncel, G. and M.C. Ergoren (2019) *Functional coding and non-coding variants in human BRCA1 gene and their use in genetic screening.* Med Oncol. 36(8): p. 71.

Tuncel, G., Temel S.G., and Ergoren M.C. (2019) *Strong association between VDR FokI (rs2228570) gene variant and serum vitamin D levels in Turkish Cypriots.* Mol Biol Rep, 46(3): p. 3349-3355.

Tuncel, G. and Kalkan, R. (2019) *Importance of m N(6)-methyladenosine (m(6)A) RNA modification in cancer.* Med Oncol. 36(4): p. 36.

Tuncel, G. and Kalkan, R. (2018) *Receptor Tyrosine Kinase-Ras-PI3 Kinase-Akt Signalling Network In Glioblastoma Multiforme*, Med Oncol. Aug 4;35(9): p. 122.

Book Chapters

"Precision Nutrition: Mediterranean Diet and Genetic Susceptibility" Chapter

Ergören, M.C. and Tuncel, G.

The Mediterranean Diet, 2nd Edition. Elsevier, 2019

ENCLOSURE 2: ETHICAL APPROVAL

NAER EAST UNIVERSITY

SCIENTIFIC RESEARCH ETHICS COMMITTEE

05.05.2021

Dear Assoc. Prof. Dr. Mahmut Cerkez Ergoren

Your application titled **"New Candidate Gene For Joubert Syndrome: *CC2D1A*"** with the application number NEU/2019/68-796 has been evaluated by the Scientific Research Ethics Committee and granted approval.

Prof. Dr. Rüştü Onur

Near East University

Scientific Research Ethics Committee Director

ENCLOSURE 3: TURNITIN REPORT

PhD Thesis

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