Cumhuriyet Sci. J., 45(2) (2024) 235-239
DOI: https://doi.org/10.17776/csj.1398415

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Investigating the Impact of Birt–Hogg–Dubé Syndrome Associated Folliculin (FLCN) and Retinitis Pigmentosa 2 (RP2) Loss on Cilia Function and Morphology

Oktay İsmail Kaplan [1,a]

[1] *Rare Disease Laboratory, School of Life and Natural Sciences, Abdullah Gul University, Kayseri, Türkiye.*
**Corresponding author*

Research Article

History
Received: 30/11/2023
Accepted: 13/06/2024

ABSTRACT

Folliculin (FLCN), a GTPase-activating protein (GAP), has been linked to Birt–Hogg–Dubé syndrome, the mTORC1 signaling pathway and cilia. Disruptions in cilia structure and function lead to a group of diseases known as ciliopathies. Birt-Hogg-Dubé syndrome is one of 35 different ciliopathy diseases and there are more than 250 genes that cause ciliopathy diseases. FLCN interacts with kinesin-2 along cilia. The specific role of FLCN in regulating Kinesin-IFT trafficking has, however, remained unclear. In the current study, we investigated the effects of *flcn-1* loss (the human ortholog of FLCN) on kinesin and IFT trafficking in *C. elegans*. The loss of *flcn-1* alone did not result in any apparent alterations to kinesin or IFT trafficking within the cilia. However, when we combined the deletion of *flcn-1* with the deletion of Retinitis Pigmentosa 2 (RP2), another GAP protein, the ciliary entry of a non-ciliary membrane protein TRAM-1 (Translocation Associated Membrane Protein 1) occured. Additionally, although cilia length was unaltered, our analysis of double mutants revealed the extra branch in wing AWB cilia morphology but not the single rod-like PHA/PHB cilia. In summary, our study reveals the previously unknown functions of FLCN in ciliary gating and cilia morphology in *C. elegans*.

Keywords: Folliculin, Cilia, Retinitis pigmentosa 2, Ciliary gate.

[a] *oktay.kaplan@agu.edu.tr* *https://orcid.org/0000-0002-8733-0920*

Introduction

Cilia were the first cellular organelles discovered by scientists and are characterized by the presence of microtubules as a fundamental structure [1]. The terms "motile" and "non-motile" (primary cilium) refer to two functionally and structurally distinct forms of cilia [2]. In unicellular organisms, cilia serve a crucial function in enabling the mobility of the whole organism. In higher multicellular organisms, it has been demonstrated that cilia are important not only for cellular motility and sensation but also for crucial embryonic developmental processes [2,3]. Both the motile and primary cilia are involved in a variety of rare diseases, collectively known as ciliopathies. More than 50 cilia-related disorders exist, with more than 4000 distinct symptoms [4].

A bi-directional movement process spanning the entire length of cilia was initially discovered in 1993 in the single-celled green alga *Chlamydomonas reinhardtii*, and this phenomenon was termed intraflagellar transport (IFT) [5, 6]. Further analysis, including the biochemical isolation of IFT components and genomic comparisons across various species, revealed that IFT components and motor proteins, such as cytoplasmic dynein and kinesin, are conserved in ciliated organisms [7, 8]. Subsequent work revealed that the cilia host different types of proteins, including signaling molecules, small GTPase, GTPase-activating proteins (GAP) proteins, and membrane. Folliculin (FLCN) is a protein that functions as a tumor suppressor and acts as a GTPase-activating protein (GAP). Specifically, FLCN activates the GTPase enzymes RagC and RagD. FLCN plays essential roles in a range of cellular activities, including cilia function, apoptosis, cell proliferation, and autophagy, and was implicated in Birt–Hogg–Dubé syndrome, a cancer-related syndrome [9-13]. Notably, it has been reported to reside within cilia, where it forms interactions with kinesin-2 (the anterograde kinesin motor responsible for IFT) [9, 13, 14]. The ciliary localization of FLCN is dependent on its binding with kinesin-2. Furthermore, FLCN has been associated with the regulation of mTORC1 signaling, and the loss of FLCN leads to aberrant mTORC1 signaling [9, 15]. However, the role of FLCN in the IFT trafficking remains unknown.

In our study, we obtained a null allele of *C. elegans* FLCN (flcn-1) and observed that the loss of *flcn-1* resulted in a normal length of cilia, which is consistent with findings in mammals. There are no notable IFT trafficking defects in *flcn-1* mutants. However, we observed defects in cilia morphology when FLCN deletion was coupled with the GTPase activating protein RP2 (Retinitis pigmentosa 2), but cilia length was not affected. Notably, in *flcn-1;rpi-2* double mutants, a non-cilia localizing transmembrane protein was observed to enter cilia, suggesting a potential role for FLCN in regulating ciliary gate control. In summary, our analysis of null mutants has unveiled previously undisclosed functions of FLCN in cilia.

Materials and Methods

Materials

In the preparation of Nematode Growth Medium (NGM), we used the following reagents and materials: NaCl, Agar, Peptone, cholesterol (5 mg/ml in ethanol), KH_2PO_4, K_2HPO_4, $MgSO_4$, H_2O, and 6 cm Petri plates. The medium was sterilized in an autoclave, cooled, and then

placed on Petri plates under carefully controlled conditions. These petri plates were maintained at room temperature for 48 hours, after which they were evenly coated with OP50, an *Escherichia coli* (*E. coli*) derivative.

Genetics Cross and Worm Maintenance

We employed forward and reverse primers to track mutations in both *flcn-1(ok975)* and *rpi-2(ok1863)* to carry out genetic crosses with the wild type. Specifically, strains GOU2362, ift-74(cas499[ift-74::gfp]), N2;Ex[OSM-3::GFP + pRF4], N2;Ex[MKS-2::GFP + TRAM-1::tdTOMATO + pRF4], or str-1pro::mCherry were crossed with flcn-1(ok975), rpi-2(ok1863) single mutants, as well as flcn-1(ok975); rpi-2(ok1863) double mutants [10, 16].

Primers

flcn-1(ok975)F 5'GGACCTTGCCTTCTGTCAAATG 3' (OK698)
flcn-1(ok975)R 5'GGTAGAATCCTCGCGCTTTC 3' (OK699)
rpi-2(ok1863)F 5'GAGACGCAGACATCTCATCTG 3' (OK589)
rpi-2(ok1863)R 5' CAGGTCGTTCTCGGACATCAC3' (OK590)

Microscopy Analysis

Transgenic strains expressing ift-74(cas499[ift-74::gfp], N2;Ex[MKS-2::GFP + TRAM-1::tdTOMATO + pRF4], or *str-1pro::mCherry* were imaged with the Zeiss LSM 900 confocal microscope as previously described [16].

Statistical Significance

When comparing the statistical significance of cilia lengths, we used the Kruskal-Wallis test in the R programming ("R version 4.2.2 (2022-10-31)").

Results and Discussion

A Transmembrane Protein from outside of Cilia Enters Cilia in the loss of both Folliculin (FLCN) and Retinitis Pigmentosa 2 (RP2)

Our previous work implicated GTPase-activating proteins (GAP) proteins, including RP2 (Retinitis pigmentosa 2) and ELMOD3 (ELMO Domain Containing 3) in restricting the ciliary entry of TRAM-1 (Translocation Associated Membrane Protein 1) [16]. TRAM-1 (Translocation Associated Membrane Protein 1) is a transmembrane protein that localizes to the periciliary membrane compartment but does not normally enter into cilia. We investigated the role of a GAP protein FLCN in limiting access of TRAM-1 to cilia. We obtained a previously characterized mutant of *flcn-1*(ok975) from the Caenorhabditis Genetics Center (CGC), Minnesota, USA, and established a PCR (Polymerase Chain Reaction) based genotyping strategy. We next crossed N2;Ex[MKS-2::GFP + TRAM-1::tdTOMATO + pRF4] into *flcn-1*(ok975) to visualize both TRAM-1 and the transition zone marked with MKS-2::GFP (Fig. 1a and b.). Interestingly, TRAM-1::tdTOMATO was present in the cilium of the double mutants *flcn-1* (the human ortholog of FLCN);*rpi-2* (the human ortholog of RP2) but lacking in the single mutants and wild type, suggesting FLCN and RP2 may play a role in restricting entry of non-ciliary transmembrane protein into cilia.

Cilia Morphology is Impacted by the loss of both Folliculin (FLCN) and Retinitis Pigmentosa 2 (RP2)

We next investigated the role of folliculin and RP2 in the transport of intraflagellar transport (IFT), a bidirectional motility process along the cilia. We especially wanted to find out the effects of the absence of both RPI-2 and FLNC-2 on IFT transport. We chose the human ortholog of kinesin motor KIF17 (OSM-3), and an IFT-A complex subunit, namely intraflagellar transporter protein 140 (IFT140) (the human ortholog of IFT140). Subsequently, we generated single and double mutants with them. Normally, fluorescence tagged OSM-3 and IFT-140 localize to the cilia and translocate along the cilia. Our confocal microscopy revealed that the localizations of the kinesin motor and IFT-140 are unaffected in both single and double mutants (Fig. 2a.).

Furthermore, we measured the cilia length in wild type, single and double mutants, and found that the length of cilia is comparable with that of wild type (Fig. 2b.). However, the cilia morphology of AWB cilia, Y-like cilia structure, has more extra branches in the *flcn-1;rpi-2* double mutants (Fig. 3a and b.).

Conclusions

Our current study provides the first evidence for the potential role of Birt–Hogg–Dubé syndrome associated folliculin (FLCN) in controlling the ciliary gate. Our previous work had already implicated the two GAP proteins (Retinitis Pigmentosa 2 and ELMO Domain Containing protein) in this process [16]. Surprisingly, in both the FLCN and RP2 single and double mutants, the length of the cilia is unchanged, but the existence of extra branches in the AWB cilia morphology of the flcn-1;rpi-2 double mutants raises the possibility that FLCN and RP2 are involved in the regulation of structural integrity of the cilia.

The role of folliculin in regulating the ciliary gate control is undeniably intriguing. There are, in fact, two distinct possibilities that could potentially explain the observed ciliary gate defects in the *flcn-1;rpi-2* double mutants. Firstly, it is widely recognized that the transition zone (TZ) plays a key role in governing ciliary gating, with proteins residing in the TZ known to influence this process [17]. FLCN and RP2 could potentially impact the function or localization of proteins within the transition zone, potentially facilitating the entry of non-membrane proteins into the cilia due to their loss. Alternatively, the disruption in the intraflagellar transport (IFT) process could be responsible for the ciliary entry of membrane proteins in the *flcn-1;rpi-2* double mutants. However, the lack of FLCN and RP2 did not affect the ciliary localization of the kinesin motor KIF17 (OSM-3) and the IFT-A complex member IFT140. Thus, a defect in IFT trafficking may not explain the potential mechanism behind the ciliary leakiness of non-ciliary membrane proteins. Further in-depth analysis is warranted to unravel this intriguing phenomenon. Taken together, our work expands our understanding of FLCN and RP2 in cilia biology, offering valuable insights into their contributions to ciliary gate control and morphology. Further studies may uncover the precise mechanisms underlying these intriguing observations and their implications for cellular processes.

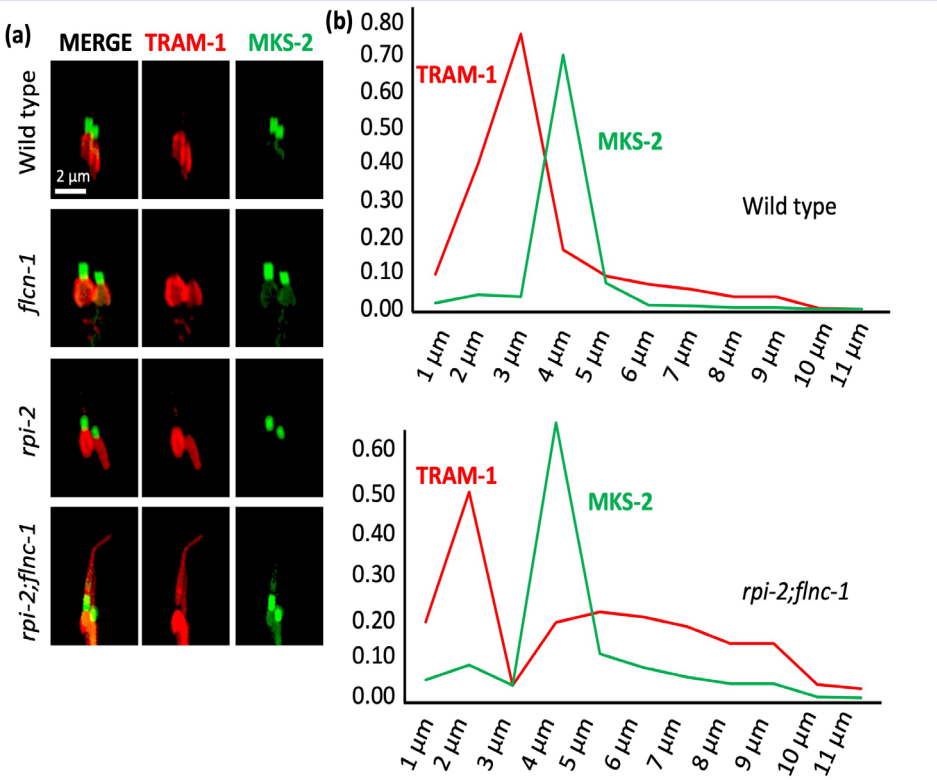

Figure 1. (a) Shown are localizations of MKS-2::GFP (green) and TRAM-1::tdTOMATO (red) in the corresponding mutants and wild type. (b) The line plot displays normalized measurements of MKS-2::GFP and TRAM-1::tdTOMATO along the distal dendrite to the cilia.

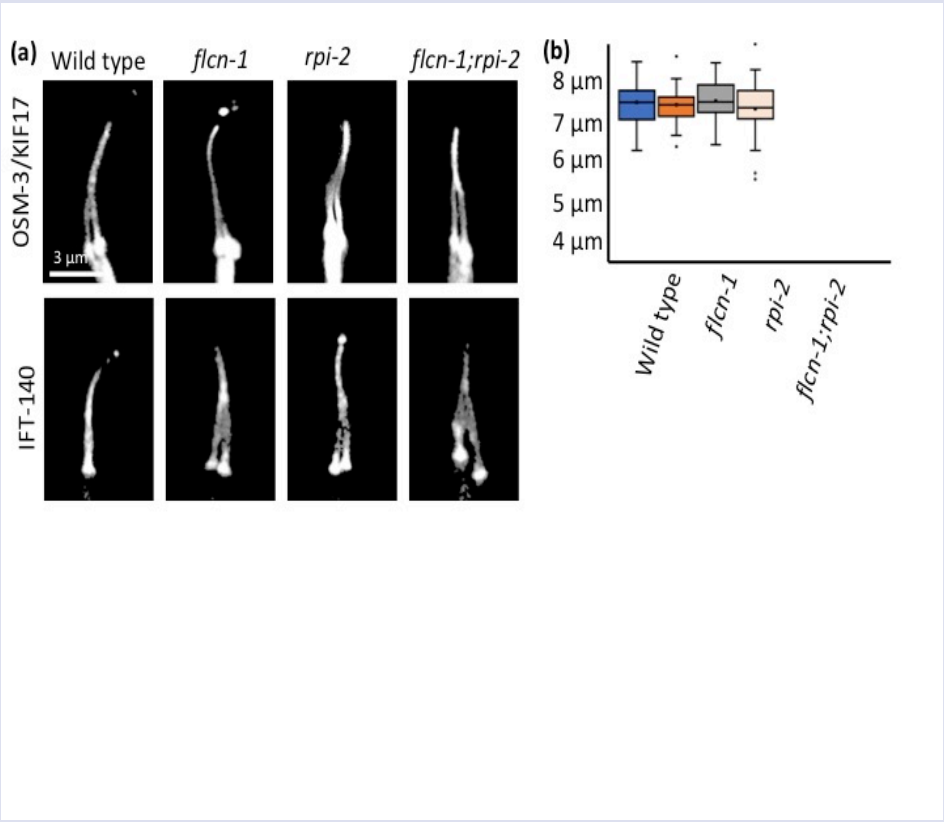

Figure 2. (a) The localization of IFT-140::GFP and OSM-3::GFP (human KIF17) were shown in the wild type and respective mutants. (b) The cilia length measurements were presented for the wild type and indicated mutants. There is no noticeable statistical significance in the cilia length between any of the mutants and the wild type.

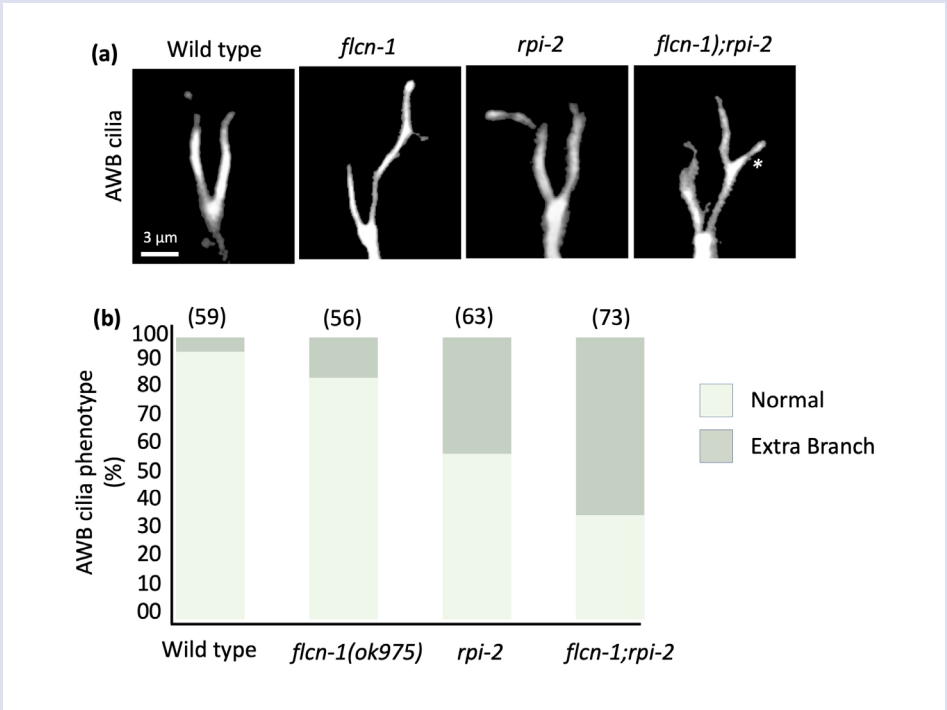

Figure 3. (a) Images for AWB cilia were displayed for wild type and indicated mutants. (b) The percentage of AWB cilia with branches and mutants displaying normal morphology was visualized in a stacked bar plot for both the wild type and the indicated mutant strains. The number of AWB cilia counted is displayed at the top of the plot within parentheses.

Acknowledgements

We thank Ali Sina Arslan and Sebiha Cevik for their technical assistance.

Conflicts of Interest

The authors declare that, there are no conflicts of interest in this work.

References

[1] Satir P, Christensen ST., Overview of Structure and Function of Mammalian Cilia, *Annu Rev Physiol*., 69(1) (2007) 377-400.

[2] Mitchison HM, Valente EM. Motile and non-motile cilia in human pathology: from function to phenotypes: Motile and non-motile ciliopathies, *J. Pathol*., 241(2) (2017) 294-309.

[3] Shah AS, Ben-Shahar Y, Moninger TO, Kline JN, Welsh MJ., Motile Cilia of Human Airway Epithelia Are Chemosensory, *Science*, 325(5944) (2009) 1131-1134.

[4] Turan MG, Orhan ME, Cevik S, Kaplan OI., CiliaMiner: an integrated database for ciliopathy genes and ciliopathies, *Database.*, (2023) baad047.

[5] Kozminski KG, Johnson KA, Forscher P, Rosenbaum JL., A motility in the eukaryotic flagellum unrelated to flagellar beating, *Proc Natl Acad Sci.,* 90(12) (1993) 5519-5523.

[6] Kozminski KG, Beech PL, Rosenbaum JL., The Chlamydomonas kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane, *J Cell Biol.,* 131(6) (1995) 1517-1527.

[7] Rosenbaum JL, Witman GB., Intraflagellar transport, *Nat Rev Mol Cell Biol.,* 3(11) (2002) 813-825.

[8] Blacque OE, Cevik S, Kaplan OI., Intraflagellar transport: from molecular characterisation to mechanism, *Front Biosci.*, 13(13) (2008) 2633.

[9] Zhong M, Zhao X, Li J, Yuan W, Yan G, Tong M, Guo S, Zhu Y, Jiang Y, Liu Y, Jiang Y, Tumor Suppressor Folliculin Regulates mTORC1 through Primary Cilia, *J Biol Chem*., 291(22) (2016) 11689-11697.

[10] Possik E, Jalali Z, Nouët Y, Yan M, Gingras MC, Schmeisser K, Panaite L, Dupuy F, Kharitidi D, Chotard L, Jones RG, Hall DH, Pause A., Folliculin Regulates Ampk-Dependent Autophagy and Metabolic Stress Survival, *PLoS Genet*., 10(4) (2014) e1004273.

[11] Baba M, Toyama H, Sun L, Takubo K, Suh HC, Hasumi H, Nakamura-Ishizu A, Hasumi Y, Klarmann KD, Nakagata N, Schmidt LS, Linehan WM, Suda T, Keller JR. Loss of *Folliculin* Disrupts Hematopoietic Stem Cell Quiescence and Homeostasis Resulting in Bone Marrow Failure, *Stem Cells*., 34(4) (2016) 1068-1082.

[12] Dunlop EA, Seifan S, Claessens T, Behrends C, Kamps MA, Rozycka E, Kemp AJ, Nookala RK, Blenis J, Coull BJ, Murray JT, van Steensel MA, Wilkinson S, Tee AR., FLCN, a novel autophagy component, interacts with GABARAP and is regulated by ULK1 phosphorylation, *Autophagy*., 10(10) (2014) 1749-1760.

[13] Luijten MN, Basten SG, Claessens T, Vernooij M, Scott CL, Janssen R, Easton JA, Kamps MA, Vreeburg M, Broers JL, van Geel M, Menko FH, Harbottle RP, Nookala RK, Tee AR, Land SC, Giles RH, Coull BJ, van Steensel MA., Birt–Hogg–Dubé syndrome is a novel ciliopathy, *Hum Mol Genet.,* 22(21) (2013) 4383-4397.

[14] Zhang Y, Liu Y, Dai Y, Ren Y, Bao G, Ai B, Jiang Y., Ciliary localization of folliculin mediated via a kinesin-2-binding

motif is required for its functions in mTOR regulation and tumor suppression, *FEBS Lett.,* 595(1) (2021) 123-132.

[15] Hasumi Y, Baba M, Hasumi H, Huang Y, Lang M, Reindorf R, Oh HB, Sciarretta S, Nagashima K, Haines DC, Schneider MD, Adelstein RS, Schmidt LS, Sadoshima J, Marston Linehan W., Folliculin (Flcn) inactivation leads to murine cardiac hypertrophy through mTORC1 deregulation, *Hum Mol Genet*., 23(21) (2014) 5706-5719.

[16] Cevik S, Peng X, Beyer T, Pir MS, Yenisert F, Woerz F, Hoffmann F, Altunkaynak B, Pir B, Boldt K, Karaman A, Cakiroglu M, Oner SS, Cao Y, Ueffing M, Kaplan OI., WDR31 displays functional redundancy with GTPase-activating proteins (GAPs) ELMOD and RP2 in regulating IFT complex and recruiting the BBSome to cilium, *Life Sci Alliance*., 6(8) (2023) e202201844.

[17] Williams CL, Li C, Kida K, Inglis PN, Mohan S, Semenec L, Bialas NJ, Stupay RM, Chen N, Blacque OE, Yoder BK, Leroux MR., MKS and NPHP modules cooperate to establish basal body/transition zone membrane associations and ciliary gate function during ciliogenesis, *J Cell Biol*., 192(6) (2011) 1023-1041.