HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2021 June 11.

Published in final edited form as:

Biochem Biophys Res Commun. 2020 February 19; 522(4): 931–938. doi:10.1016/j.bbrc.2019.11.184.

FLCN alteration drives metabolic reprogramming towards nucleotide synthesis and cyst formation in salivary gland

Yasuhiro Isono[a], **Mitsuko Furuya**[b], **Tatsu Kuwahara**[a], **Daisuke Sano**[a], **Kae Suzuki** [c], **Ryosuke Jikuya**[c], **Taku Mitome**[c], **Shinji Otake**[c], **Takashi Kawahara**[c], **Yusuke Ito**[c], **Kentaro Muraoka**[c], **Noboru Nakaigawa**[c], **Yayoi Kimura**[d], **Masaya Baba**[e], **Kiyotaka Nagahama**[f], **Hiroyuki Takahata**[g], **Ichiro Saito**[h], **Laura S. Schmidt**[i,j], **W. Marston Linehan**[i], **Tatsuhiko Kodama**[k], **Masahiro Yao**[c], **Nobuhiko Oridate**[a,**], **Hisashi Hasumi**[c,*]

[a]Department of Otorhinolaryngology, Yokohama, 236-0004, Japan

[b]Department of Molecular Pathology, Yokohama, 236-0004, Japan

[c]Department of Urology, Yokohama, 236-0004, Japan

[d]Advanced Medical Research Center, Yokohama City University, Yokohama, 236-0004, Japan

[e]International Research Center for Medical Sciences, Kumamoto University, Kumamoto, 860-0811, Japan

[f]Department of Pathology, Graduate School of Medical Sciences, Kyorin University, Mitaka, Tokyo, 181-8611, Japan

[g]Department of Pathology, Shikoku Cancer Center, Matsuyama, Ehime, 791-0280, Japan

[h]Department of Pathology, Tsurumi University School of Dental Medicine, Yokohama, 230-8501, Japan

[i]Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[j]Basic Science Program, Frederick National Laboratory for Cancer Research, Frederick, MD, USA

[k]Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, Tokyo, 153-8904, Japan

Abstract

FLCN is a tumor suppressor gene which controls energy homeostasis through regulation of a variety of metabolic pathways including mitochondrial oxidative metabolism and autophagy. Birt-Hogg-Dubé (BHD) syndrome which is driven by germline alteration of the *FLCN* gene, predisposes patients to develop kidney cancer, cutaneous fibrofolliculomas, pulmonary cysts and less frequently, salivary gland tumors. Here, we report metabolic roles for *FLCN* in the salivary

*Corresponding author. **Corresponding author. Yokohama City University, 3-9 Fuku-ura, Kanazawa-ku, Yokohama, 2360004, Japan. noridate@yokohama-cu.ac.jp (N. Oridate), hasumi@ yokohama-cu.ac.jp (H. Hasumi).

Declaration of competing interest

Authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.11.184.

gland as well as their clinical relevance. Screening of salivary glands of BHD patients using ultrasonography demonstrated increased cyst formation in the salivary gland. Salivary gland tumors that developed in BHD patients exhibited an upregulated mTOR-S6R pathway as well as increased GPNMB expression, which are characteristics of *FLCN*-deficient cells. Salivary gland-targeted *Flcn* knockout mice developed cytoplasmic clear cell formation in ductal cells with increased mitochondrial biogenesis, upregulated mTOR-S6K pathway, upregulated TFE3-GPNMB axis and upregulated lipid metabolism. Proteomic and metabolite analysis using LC/MS and GC/MS revealed that *Flcn*-inactivation in salivary gland triggers metabolic reprogramming towards the pentose phosphate pathway which consequently upregulates nucleotide synthesis and redox regulation, further supporting that *Flcn* controls metabolic homeostasis in salivary gland. These data uncover important roles for *FLCN* in salivary gland; metabolic reprogramming under *FLCN* deficiency might increase nucleotide production which may feed *FLCN*-deficient salivary gland cells to trigger tumor initiation and progression, providing mechanistic insight into salivary gland tumorigenesis as well as a foundation for development of novel therapeutics for salivary gland tumors.

Keywords

Birt-hogg-dubé (BHD) syndrome; FLCN; Salivary gland tumor; Mitochondria; mTOR-TFE3 pathway

1. Introduction

Salivary gland tumor development is a complex disease which exhibits more than 30 histologies [1]. A variety of fusion genes involving transcription factors have been found in each histology; adenoid cystic carcinoma (ACC) harbors *MYB-NFIB* gene fusion; mucoepidermoid carcinoma (MEC) harbors *CRTC1-MAML2* gene fusion; mammary analogue secretory carcinoma (MASC) harbors *ETV6-NTRK3* gene fusion [2,3]. In addition, several histological types of tumors exhibit dysregulation of the PI3K-AKT-mTOR pathway. ACC harbors somatic mutations in *PIK3CA* and salivary duct carcinoma (SDC) harbors mutations in *PIK3CA* and *PTEN*, suggesting that an altered PI3K-AKT-mTOR pathway may play a role in the development of a subset of salivary gland tumors [1].

Folliculin (FLCN) is the causative gene for Birt-Hogg-Dubé (BHD) syndrome, a hamartoma syndrome which predisposes patients to develop hair follicle tumors, lung cysts, spontaneous pneumothorax, kidney cancer and less frequently, salivary gland tumors. *FLCN* is a tumor suppressor which controls a wide variety of metabolic pathways including the AMPK-mTOR pathway, and *PGC1α*-driven mitochondrial metabolism through the interaction with its two binding partners, folliculin-interacting proteins 1 and 2 (FNIP1 and FNIP2) [4–8]. Expression of *FLCN*, *FNIP1* and *FNIP2* in human tissues is high in metabolic organs including muscle, fat and liver and prominently in the salivary gland [9]. Notably, several cases of salivary gland tumors have been reported in BHD patients, suggesting that the FLCN/FNIP1/FNIP2 complex may have an important role in tumor suppression of salivary gland tumors [10–12].

Here, we uncover important roles for *FLCN* in the salivary gland. To characterize salivary gland tumors that developed in BHD patients, we performed immunohistochemistry using antibodies against molecules associated with the *FLCN* pathway. To understand the roles of *FLCN* in the human salivary gland, we screened salivary glands of BHD patients using ultrasonography. To further clarify metabolic roles for *FLCN* in salivary gland, we generated salivary gland-targeted *Flcn* knockout mouse models. We examined these models using a variety of methodologies including proteomic profiling analysis and metabolite analysis.

2. Material and methods

Material and methods are available at BBRC online.

3. Results

3.1. Molecular characteristics of a salivary gland tumor from a BHD patient

Several cases of salivary gland tumor development in BHD patients have been reported. To clarify molecular characteristics of the salivary gland tumor with oncocytic features that developed in a BHD patient, we performed immunohistochemistry using antibodies against molecules which have been reported to be associated with the *FLCN* pathway [10–18]. Immunohistochemistry of the BHD-associated clear cell oncocytoma of the salivary gland exhibited ubiquitous staining of mTOR, strong staining of phospho-mTOR (Ser2448) in outer edge of the tumor, diffuse staining of phospho-S6R (Ser235/236), strong staining of COX IV, one of the mitochondrial components, nuclear staining of TFE3 and diffuse staining of GPNMB in outer edge of the tumor (Fig. 1). These data suggest that alteration of metabolic pathways resulting from loss of *FLCN* may trigger salivary gland tumorigenesis in BHD patients.

3.2. Increased incidence of cyst development in salivary glands of BHD patients

To further clarify the role of *FLCN* in salivary gland homeostasis, we screened salivary glands of BHD patients using ultrasonography. Interestingly, BHD patients exhibited an increased incidence of salivary gland cysts (Table 1), whereas none of 8 non-BHD volunteers showed cyst formation in their salivary glands (data not shown). These data further indicate important roles for *FLCN* in salivary gland metabolism and suggest that metabolic alterations resulting from *FLCN* deficiency may result in cyst development in BHD patients.

3.3. Destruction of salivary gland duct in salivary gland under Flcn-deficiency

To further elucidate the role of *FLCN* in salivary gland homeostasis, we established salivary gland-targeted *Flcn* knockout mice by crossing *Flcn* conditional knockout mice with *mouse mammary tumor virus (MMTV)-Cre* transgenic mice which express Cre recombinase in salivary gland, mammary epithelial cells, skin and lymphocytes [19,20]. Strikingly, *Flcn f/f*; *MMTV-Cre* mice (*Flcn* KO) showed red-colored salivary glands compared to *Flcn f/f* mice (*Flcn* WT) (Fig. 2a). Histological analyses revealed that salivary ducts in *Flcn* KO mice were replaced by cells with clear cytoplasm in an age-dependent manner (Fig. 2b). At 24 weeks of age, the acinar glands were subsequently affected and replaced by cells with clear cytoplasm

as well at 100% penetrance. Indeed, we observed a similar phenotype in another salivary gland-targeted *Flcn* knockout mouse model using *Lama (PSP)-Cre* transgenic mice which express Cre recombinase under the *parotid secretory protein (PSP)* promoter, a specific promoter for salivary gland and lacrimal gland (Supplemental Fig.1). These data suggest that metabolic alterations resulting from *Flcn* deficiency might lead to dysfunction of salivary ducts, leading to replacement of salivary glands by cells with clear cytoplasm.

3.4. Upregulation of the mTOR-TFE3 axis in Flcn-deficient salivary gland

Results from studies in several murine models support the upregulation of the mTOR-S6K pathway under *Flcn*-deficiency, leading to aberrant proliferation of kidney cells or cardiac hypertrophy [19,21]. Consistent with those reports, *Flcn*-deficient salivary glands showed upregulation of the mTOR-S6K pathway (Fig. 3a). Also, previous reports demonstrated that TFE3 is translocated into the nucleus under *FLCN*-deficiency and *FLCN* relieves mTOR-dependent cytoplasmic retention of TFE3 in adipose tissue [22,23]. Consistent with those reports, Tfe3 was translocated into the nucleus and its downstream target, Gpnmb was overexpressed in *Flcn*-deficient salivary gland (Fig. 3b). These data suggest that the mTOR-TFE3 axis might also be upregulated in *Flcn*-deficient salivary gland.

3.5. Increased mitochondrial biogenesis in Flcn-deficient salivary gland

To further understand the molecular composition of the *Flcn*-deficient salivary gland, we performed proteomic analysis using LC/ MS. Gene ontology of the proteomic analysis showed that more than half of the proteins that were increased in *Flcn*-deficient salivary glands are associated with mitochondrial metabolism (Table 2). Previously, we and others showed that *FLCN* controls mitochondrial homeostasis through regulation of *PGC1a*, an important co-activator for mitochondrial biogenesis [14,24]. Indeed, we observed increased expression of Pgc1a and its downstream genes including Cox IV and *Ucp3* in *Flcn*-deficient salivary gland (Fig. 3c and d), suggesting that *Flcn* might control mitochondrial homeostasis in salivary gland.

3.6. Upregulated pentose phosphate pathway and nucleotide synthesis in Flcn-deficient salivary gland

To further understand the role of *FLCN* in salivary gland homeostasis, we conducted a metabolomics study of *Flcn*-deficient salivary gland using LC/MS. Glycolytic metabolites including glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-phosphate, dihydroxyacetone phosphate (DHAP) and lactic acid were increased in *Flcn*-deficient salivary gland (Fig. 4a). Metabolites in the pentose phosphate pathway including 6-phosphogluconic acid, ribose 5-phosphate, sedoheptulose 7-phosphate and phosphoribosyl diphosphate were increased in *Flcn*-deficient salivary gland (Fig. 4b). Importantly, nucleotides including ADP and GDP were increased in *Flcn*-deficient salivary gland, suggesting that nucleotide synthesis might be upregulated in *Flcn*-deficient salivary gland (Fig. 4c). In addition to regulation of mitochondrial oxidative metabolism, *FLCN* has been reported to regulate glycolysis; loss of *FLCN* leads to upregulated glycolysis, increased glycogen biosynthesis and increased ATP production [25–27]. Taken together, loss of *Flcn* in salivary gland might upregulate glycolysis and the pentose phosphate pathway, and

consequently upregulate nucleotide synthesis that is necessary for salivary gland proliferation and may drive initiation and progression of salivary gland tumors.

4. Discussion

In this study, we report an important role for *FLCN* in salivary gland metabolism. We have shown that BHD patients have an increased risk of cyst formation in salivary gland and demonstrated upregulation of the *mTOR-TFE3* axis in a salivary gland tumor from a BHD patient, which is characteristic of the altered metabolism seen under *FLCN* deficiency. *Flcn* inactivation in murine salivary gland leads to destruction of ductal structures which was subsequently replaced by cells with clear cytoplasm. *Flcn*-deficient salivary glands exhibited increased mitochondrial biogenesis, upregulated glycolysis and upregulated pentose phosphate pathway metabolites which may provide *FLCN*-deficient salivary cells with increased nucleotide synthesis to support proliferation of cells in these *Flcn*- deficient salivary gland.

A variety of fusion genes involving various transcription factors have been identified in salivary gland tumors. A murine model overexpressing *PLAG1* proto-oncogene that develops salivary gland tumors displaying various pleomorphic adenoma features identified the fusion gene, *PLAG1* fusion, as a driver for salivary gland tumorigenesis [28]. In addition, murine models overexpressing oncogenes including *AKT3*, *HER-2/neu* and *K-Ras* have been generated which develop ACC, acinic cell adenocarcinoma and squamous cell carcinoma (SCC), respectively [29–31]. Somatic mutations in *PTEN*, *PIK3CA*, *NOTCH1/2*, chromatin remodeling genes, genes in the *FGFR* pathway, genes in DNA-damage/checkpoint signaling pathways and *PRKD1* have been identified in salivary gland tumors [1]. Among these somatic mutations, two murine models of salivary tumorigenesis driven by *Pten* inactivation have been reported. Deletion of both *Pten* and *Apc* in mice results in the development of salivary gland tumors similar to human acinic cell carcinoma, and deletion of both *Pten* and *Smad* in mice results in the development of pleomorphic adenoma in the salivary gland [32,33]. Therefore, compared to murine models overexpressing oncogenes, inactivation of at least two tumor suppressor genes may be necessary for the development of murine salivary gland tumors. *FLCN* has been shown to be a classic two-hit tumor suppressor gene for renal tumorigenesis [34–36]. Heterozygous *Flcn* knockout mice develop kidney cancer at 18 months of age, suggesting that additional genetic alterations may be necessary for renal tumorigenesis based on the concept of multistep carcinogenesis [36]. In support of this idea, whole-exome sequencing of BHD-associated kidney cancer was conducted and somatic mutations were identified in a variety of additional genes including chromatin remodeling genes, implying that those mutations may drive renal tumorigenesis in cooperation with *FLCN* alteration [37]. Because *Flcn* inactivation in murine salivary gland did not develop salivary gland tumors in mice aged to 2 years (data not shown), it would be interesting to see whether inactivation of *Flcn* along with other tumor suppressor genes in murine salivary glands will develop salivary gland tumors.

Catalogue of Somatic Mutations In Cancer (COSMIC) showed *FLCN*, *FNIP1* and *FNIP2* mutations in 2 of 190, one of 80 and none of 80 samples of salivary gland tumors, respectively, indicating that the majority of salivary gland tumors do not harbor genetic

alterations in the *FLCN/FNIP1/FNIP2* pathway. In this study, inactivation of Flcn in salivary gland leads to metabolic reprograming towards nucleotide synthesis, which is important for tumor initiation and progression. Therefore, transcriptional levels of *FLCN*, *FNIP1* and *FNIP2* and post-translational modification of FLCN, FNIP1, and FNIP2 proteins should be carefully assessed in sporadic salivary gland tumors.

BHD patients develop a variety of renal tumors including chromophobe renal cell carcinoma, oncocytoma and hybrid oncocytic/chromophobe tumor, and ultrastructural analyses of those oncocytic cells display numerous mitochondria in their cytoplasm [14]. This phenotype is thought to be associated with *FLCN* dysfunction since *FLCN* maintains mitochondrial homeostasis through regulation of *PGC1a*, an important co-activator for mitochondrial biogenesis [14]. In addition to salivary gland oncocytoma, many cases of salivary gland tumors or hyperplasia display oncocytic changes including oncocytic mucoepidermoid carcinoma (OMEC) and papillary cystadenocarcinoma with oncocytic epithelial lining [38–44], suggesting that oncocytosis may be implicated in a subset of salivary gland tumors. Historically, electron microscopy and immunohistochemistry using anti-mitochondrial antibodies in those tumors have shown numerous mitochondria in the tumor cell cytoplasm [45,46]. Because *Flcn*-deficient salivary glands exhibited increased mitochondrial biogenesis, it would be interesting to investigate *FLCN* status in those salivary gland tumors with oncocytosis.

Our finding delineates an important role for *FLCN* in regulation of metabolic homeostasis in salivary gland. A *FLCN*-deficient salivary gland tumor that developed in a BHD patient showed upregulation of the mTOR-S6R pathway as well as increased GPNMB expression, which are molecular characteristics of *FLCN* deficiency and may be involved in salivary gland tumorigenesis. Our murine model of *Flcn* inactivation targeted to salivary gland exhibited metabolic alterations including increased mitochondrial biogenesis, upregulated glycolysis and increased nucleotide synthesis, which may potentially confer *FLCN*-deficient salivary gland cells with an increased potential for proliferation. These findings provide a foundation for the management of BHD patients as well as the development of targeted therapeutic approaches for salivary gland tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Tomoko Akiyama at Advanced Medical Research Center, Yokohama City University for her excellent work in proteomic profiling analyses. We thank Ms. Hiromi Soeda at Department of Molecular Pathology, Yokohama City University and Dr. Reiko Tanaka at Medical Mycology Research Center, Chiba University for their excellent work in Sanger sequencing. We thank outside institutes which provided clinical information of BHD patients.

Funding

Authors were supported by JSPS KAKENHI Grant Number as following; N.O. by 18K09382, D.S. by 16K11240, M.F. by 17K08745, Y.K. by 16H05230, T.K. by 16K20152, K.N. by 16K08698, M.B. by 18H02938/18K19619 and H.H. by 16K11020. This work was also supported by the Intramural Research Program of the National Institutes of Health (NIH), National Cancer Institute (NCI), Center for Cancer Research. This project was funded in part with

federal funds from the Frederick National Laboratory for Cancer Research, NIH, under Contract HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the US Government.

Abbreviations

FLCN
Folliculin

BHD syndrome
Birt-Hogg-Dubé syndrome

***MMTV-Cre* transgenic mouse**
mouse mammary tumor virus-Cre transgenic mouse

***Lama (PSP)-Cre* transgenic mouse**
Lama parotid secretory protein- Cre transgenic mouse

References

[1]. Andersson MK, Stenman G, The landscape of gene fusions and somatic mutations in salivary gland neoplasms - implications for diagnosis and therapy, Oral Oncol. 57 (2016) 63–69. [PubMed: 27101980]

[2]. Seethala RR, Salivary gland tumors: current concepts and controversies, Surg. Pathol. Clin. 10 (2017) 155–176. [PubMed: 28153132]

[3]. Lawal AO, Adisa AO, Kolude B, Adeyemi BF, Olajide MA, A review of 413 salivary gland tumours in the head and neck region, J. Clin. Exp. Dent. 5 (2013) 218–222.

[4]. Baba M, Hong SB, Sharma N, Warren MB, Nickerson ML, Iwamatsu A, Esposito D, Gillette WK, Hopkins RF 3rd, Hartley JL, et al., Folliculin encoded by the BHD gene interacts with a binding protein, FNIP1, and AMPK, and is involved in AMPK and mTOR signaling, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 15552–15557. [PubMed: 17028174]

[5]. Nagashima K, Fukushima H, Shimizu K, Yamada A, Hidaka M, Hasumi H, Ikebe T, Fukumoto S, Okabe K, Inuzuka H, Nutrient-induced FNIP degradation by SCFbeta-TRCP regulates FLCN complex localization and promotes renal cancer progression, Oncotarget 8 (2017) 9947–9960. [PubMed: 28039480]

[6]. Schmidt LS, Marston Linehan W, FLCN: the causative gene for Birt-Hogg-Dube syndrome, Gene 640 (2018) 28–42. [PubMed: 28970150]

[7]. Hasumi H, Baba M, Hasumi Y, Furuya M, Yao M, Birt-Hogg-Dube syndrome: clinical and molecular aspects of recently identified kidney cancer syndrome, Int. J. Urol. 23 (2016) 204–210. [PubMed: 26608100]

[8]. Hasumi H, Baba M, Hasumi Y, Lang M, Huang Y, Oh HF, Matsuo M, Merino MJ, Yao M, Ito Y, et al., Folliculin-interacting proteins Fnip1 and Fnip2 play critical roles in kidney tumor suppression in cooperation with Flcn, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) E1624–E1631. [PubMed: 25775561]

[9]. Hasumi H, Baba M, Hong SB, Hasumi Y, Huang Y, Yao M, Valera VA, Linehan WM, Schmidt LS, Identification and characterization of a novel folliculin-interacting protein FNIP2, Gene 415 (2008) 60–67. [PubMed: 18403135]

[10]. Liu V, Kwan T, Page EH, Parotid oncocytoma in the Birt-Hogg-Dube syndrome, J. Am. Acad. Dermatol. 43 (2000) 1120–1122. [PubMed: 11100034]

[11]. Lindor NM, Kasperbauer J, Lewis JE, Pittelkow M, Birt-Hogg-Dube syndrome presenting as multiple oncocytic parotid tumors, Hered. Cancer Clin. Pract. 10 (2012) 13. [PubMed: 23050938]

[12]. Yoshida K, Miyagawa M, Kido T, Ide K, Sano Y, Sugawara Y, Takahata H, Monden N, Furuya M, Mochizuki T, Parotid oncocytoma as a manifestation of birt-hogg-dube syndrome, Case Rep. Radiol. 2018 (2018) 6265175. [PubMed: 29971177]

[13]. Hasumi H, Hasumi Y, Baba M, Nishi H, Furuya M, Vocke CD, Lang M, Irie N, Esumi C, Merino MJ, et al., H255Y and K508R missense mutations in tumour suppressor folliculin (FLCN) promote kidney cell proliferation, Hum. Mol. Genet. 26 (2017) 354–366. [PubMed: 28007907]

[14]. Hasumi H, Baba M, Hasumi Y, Huang Y, Oh H, Hughes RM, Klein ME, Takikita S, Nagashima K, Schmidt LS, Linehan WM, Regulation of mitochondrial oxidative metabolism by tumor suppressor FLCN, J. Natl. Cancer Inst. 104 (2012) 1750–1764. [PubMed: 23150719]

[15]. Baba M, Endoh M, Ma W, Toyama H, Hirayama A, Nishikawa K, Takubo K, Hano H, Hasumi H, Umemoto T, et al., Folliculin regulates osteoclastogenesis through metabolic regulation, J. Bone Miner. Res. 33 (2018) 1785–1798. [PubMed: 29893999]

[16]. Baba M, Toyama H, Sun L, Takubo K, Suh HC, Hasumi H, Nakamura-Ishizu A, Hasumi Y, Klarmann KD, Nakagata N, et al., Loss of folliculin disrupts hematopoietic stem cell quiescence and homeostasis resulting in bone marrow failure, Stem Cells 34 (2016) 1068–1082. [PubMed: 27095138]

[17]. Baba M, Keller JR, Sun HW, Resch W, Kuchen S, Suh HC, Hasumi H, Hasumi Y, Kieffer-Kwon KR, Gonzalez CG, et al., The folliculin-FNIP1 pathway deleted in human Birt-Hogg-Dube syndrome is required for murine B-cell development, Blood 120 (2012) 1254–1261. [PubMed: 22709692]

[18]. Furuya M, Hong SB, Tanaka R, Kuroda N, Nagashima Y, Nagahama K, Suyama T, Yao M, Nakatani Y, Distinctive expression patterns of glycoprotein non-metastatic B and folliculin in renal tumors in patients with Birt-Hogg-Dube syndrome, Cancer Sci. 106 (2015) 315–323. [PubMed: 25594584]

[19]. Baba M, Furihata M, Hong SB, Tessarollo L, Haines DC, Southon E, Patel V, Igarashi P, Alvord WG, Leighty R, et al., Kidney-targeted Birt-Hogg-Dube gene inactivation in a mouse model: Erk 1/2 and Akt-mTOR activation, cell hyperproliferation, and polycystic kidneys, J. Natl. Cancer Inst. 100 (2008) 140–154. [PubMed: 18182616]

[20]. Wagner KU, Wall RJ, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, Li M, Furth PA, Hennighausen L, Cre-mediated gene deletion in the mammary gland, Nucleic Acids Res. 25 (1997) 4323–4330. [PubMed: 9336464]

[21]. Hasumi Y, Baba M, Hasumi H, Huang Y, Lang M, Reindorf R, Oh HB, Sciarretta S, Nagashima K, Haines DC, et al., Folliculin (Flcn) inactivation leads to murine cardiac hypertrophy through mTORC1 deregulation, Hum. Mol. Genet. 23 (2014) 5706–5719. [PubMed: 24908670]

[22]. Hong SB, Oh H, Valera VA, Baba M, Schmidt LS, Linehan WM, Inactivation of the FLCN tumor suppressor gene induces TFE3 transcriptional activity by increasing its nuclear localization, PLoS One 5 (2010), e15793. [PubMed: 21209915]

[23]. Wada S, Neinast M, Jang C, Ibrahim YH, Lee G, Babu A, Li J, Hoshino A, Rowe GC, Rhee J, et al., The tumor suppressor FLCN mediates an alternate mTOR pathway to regulate browning of adipose tissue, Genes Dev. 30 (2016) 2551–2564. [PubMed: 27913603]

[24]. Yan M, Audet-Walsh E, Manteghi S, Dufour CR, Walker B, Baba M, St-Pierre J, Giguere V, Pause A, Chronic AMPK activation via loss of FLCN induces functional beige adipose tissue through PGC-1 alpha/ERRalpha, Genes Dev. 30 (2016) 1034–1046. [PubMed: 27151976]

[25]. Possik E, Ajisebutu A, Manteghi S, Gingras MC, Vijayaraghavan T, Flamand M, Coull B, Schmeisser K, Duchaine T, van Steensel M, et al., FLCN and AMPK confer resistance to hyperosmotic stress via remodeling of glycogen stores, PLoS Genet. 11 (2015), e1005520. [PubMed: 26439621]

[26]. Possik E, Jalali Z, Nouet Y, Yan M, Gingras MC, Schmeisser K, Panaite L, Dupuy F, Kharitidi D, Chotard L, et al., Folliculin regulates ampk-dependent autophagy and metabolic stress survival, PLoS Genet. 10 (2014), e1004273. [PubMed: 24763318]

[27]. Yan M, Gingras MC, Dunlop EA, Nouet Y, Dupuy F, Jalali Z, Possik E, Coull BJ, Kharitidi D, Dydensborg AB, et al., The tumor suppressor folliculin regulates AMPK-dependent metabolic transformation, J. Clin. Investig. 124 (2014) 2640–2650. [PubMed: 24762438]

[28]. Declercq J, Van Dyck F, Braem CV, Van Valckenborgh IC, Voz M, Wassef M, Schoonjans L, Van Damme B, Fiette L, Van de Ven WJ, Salivary gland tumors in transgenic mice with targeted PLAG1 proto-oncogene overexpression, Cancer Res. 65 (2005) 4544–4553. [PubMed: 15930271]

[29]. Zboray K, Mohrherr J, Stiedl P, Pranz K, Wandruszka L, Grabner B, Eferl R, Moriggl R, Stoiber D, Sakamoto K, et al., AKT3 drives adenoid cystic carcinoma development in salivary glands, Cancer Med. 7 (2018) 445–453. [PubMed: 29282901]

[30]. Diodoro MG, Di Carlo E, Zappacosta R, Iezzi M, Coletti A, Modesti A, D'Antuono T, Forni G, Musiani P, Salivary carcinoma in HER-2/neu transgenic male mice: an angiogenic switch is not required for tumor onset and progression, Int. J. Cancer 88 (2000) 329–335. [PubMed: 11054659]

[31]. Raimondi AR, Vitale-Cross L, Amornphimoltham P, Gutkind JS, Molinolo A, Rapid development of salivary gland carcinomas upon conditional expression of K-ras driven by the cytokeratin 5 promoter, Am. J. Pathol. 168 (2006) 1654–1665. [PubMed: 16651631]

[32]. Cao Y, Liu H, Gao L, Lu L, Du L, Bai H, Li J, Said S, Wang XJ, Song J, et al., Cooperation between Pten and Smad 4 in murine salivary gland tumor formation and progression, Neoplasia 20 (2018) 764–774. [PubMed: 29958137]

[33]. Diegel CR, Cho KR, El-Naggar AK, Williams BO, Lindvall C, Mammalian target of rapamycin-dependent acinar cell neoplasia after inactivation of Apc and Pten in the mouse salivary gland: implications for human acinic cell carcinoma, Cancer Res. 70 (2010) 9143–9152. [PubMed: 21062985]

[34]. Furuya M, Yao M, Tanaka R, Nagashima Y, Kuroda N, Hasumi H, Baba M, Matsushima J, Nomura F, Nakatani Y, Genetic, epidemiologic and clinicopathologic studies of Japanese Asian patients with Birt-Hogg-Dube syndrome, Clin. Genet. 90 (2016) 403–412. [PubMed: 27220747]

[35]. Hasumi H, Yao M, Hereditary kidney cancer syndromes: genetic disorders driven by alterations in metabolism and epigenome regulation, Cancer Sci. 109 (2018) 581–586. [PubMed: 29325224]

[36]. Hasumi Y, Baba M, Ajima R, Hasumi H, Valera VA, Klein ME, Haines DC, Merino MJ, Hong SB, Yamaguchi TP, et al., Homozygous loss of BHD causes early embryonic lethality and kidney tumor development with activation of mTORC1 and mTORC2, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 18722–18727. [PubMed: 19850877]

[37]. Hasumi H, Furuya M, Tatsuno K, Yamamoto S, Baba M, Hasumi Y, Isono Y, Suzuki K, Jikuya R, Otake S, et al., BHD-associated kidney cancer exhibits unique molecular characteristics and a wide variety of variants in chromatin remodeling genes, Hum. Mol. Genet. 27 (2018) 2712–2724. [PubMed: 29767721]

[38]. Schwarz S, Stiegler C, Muller M, Ettl T, Brockhoff G, Zenk J, Agaimy A, Salivary gland mucoepidermoid carcinoma is a clinically, morphologically and genetically heterogeneous entity: a clinicopathological study of 40 cases with emphasis on grading, histological variants and presence of the t(11;19) translocation, Histopathology 58 (2011) 557–570. [PubMed: 21371076]

[39]. Sato S, Kishino M, Ogawa Y, Nakatsuka S, Hoshida Y, Ogawa I, Hattori K, Takata T, Toyosawa S, Multifocal nodular oncocytic hyperplasia of bilateral parotid glands: a case report with a histological variant of clear cells, Pathol. Res. Pract. 207 (2011) 452–455. [PubMed: 21689893]

[40]. Kwon H, Lim W, Choi Y, Nam J, Han C, Kim J, Ko Y, Kim I, Kim S, Kim M, et al., High-grade oncocytic mucoepidermoid carcinoma of the minor salivary gland origin: a case report with immunohistochemical study, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 109 (2010) 72–77. [PubMed: 19926499]

[41]. Kumar R, Natarajan S, Sneha KS, Chitra NS, Boaz K, Manaktala N, Oncocytes in mucoepidermoid carcinoma of the Palate: diagnostic challenges, Case Rep. Dent. 2017 (2017) 5741821. [PubMed: 29445552]

[42]. Goyal R, Ahuja A, Gupta N, Singh G, Vaiphei K, Multifocal nodular oncocytic hyperplasia in parotid gland: a case report, Acta Cytol. 51 (2007) 621–623. [PubMed: 17718138]

[43]. Fujimaki M, Fukumura Y, Saito T, Mitani K, Uchida S, Yokoyama J, Yao T, Ikeda K, Oncocytic mucoepidermoid carcinoma of the parotid gland with CRTC1-MAML2 fusion transcript: report of a case with review of literature, Hum. Pathol. 42 (2011) 2052–2055. [PubMed: 21676434]

[44]. Capone RB, Ha PK, Westra WH, Pilkington TM, Sciubba JJ, Koch WM, Cummings CW, Oncocytic neoplasms of the parotid gland: a 16-year institutional review, Otolaryngol. Head Neck Surg. 126 (2002) 657–662. [PubMed: 12087334]

[45]. Johns ME, Regezi JA, Batsakis JG, Oncocytic neoplasms of salivary glands: an ultrastructural study, The Laryngoscope 87 (1977) 862–871. [PubMed: 865204]

[46]. Shintaku M, Honda T, Identification of oncocytic lesions of salivary glands by anti-mitochondrial immunohistochemistry, Histopathology 31 (1997) 408–411. [PubMed: 9416480]

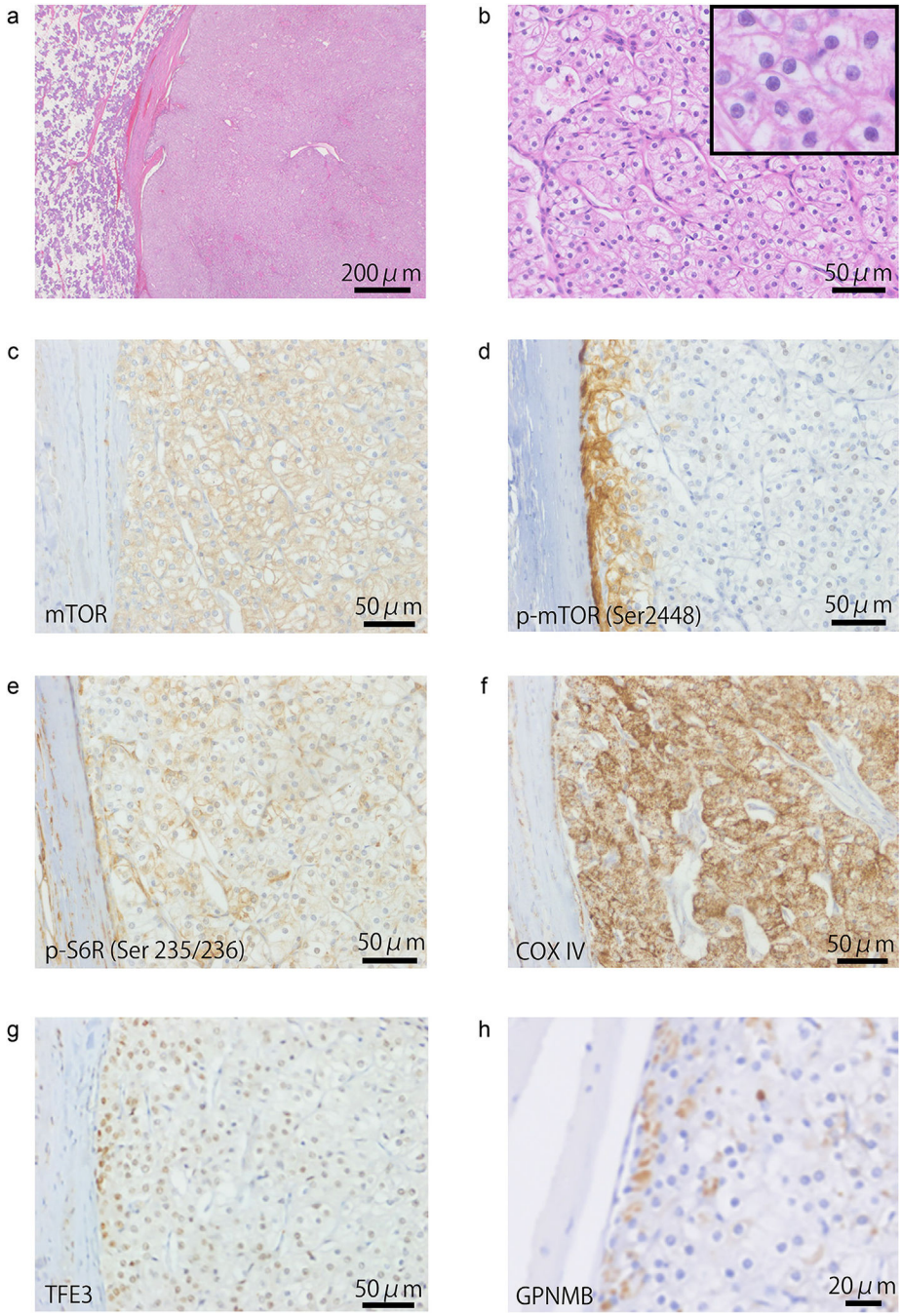

Fig. 1. Histopathology of BHD-associated salivary gland tumor.

Hematoxylin and eosin staining of clear cell oncocytoma of the salivary gland in a BHD patient. A clearly demarcated solid tumor is shown (a). The tumor is composed of oncocytic cells with peri-nuclear halo. Inset; higher magnification (b). Immunostaining for mTOR (c), phospho-mTOR (Ser2448) (d), phospho-S6R (Ser235/236) (e), COX IV, one of the mitochondrial components (f), TFE3 (g), and its downstream target, GPNMB (h).

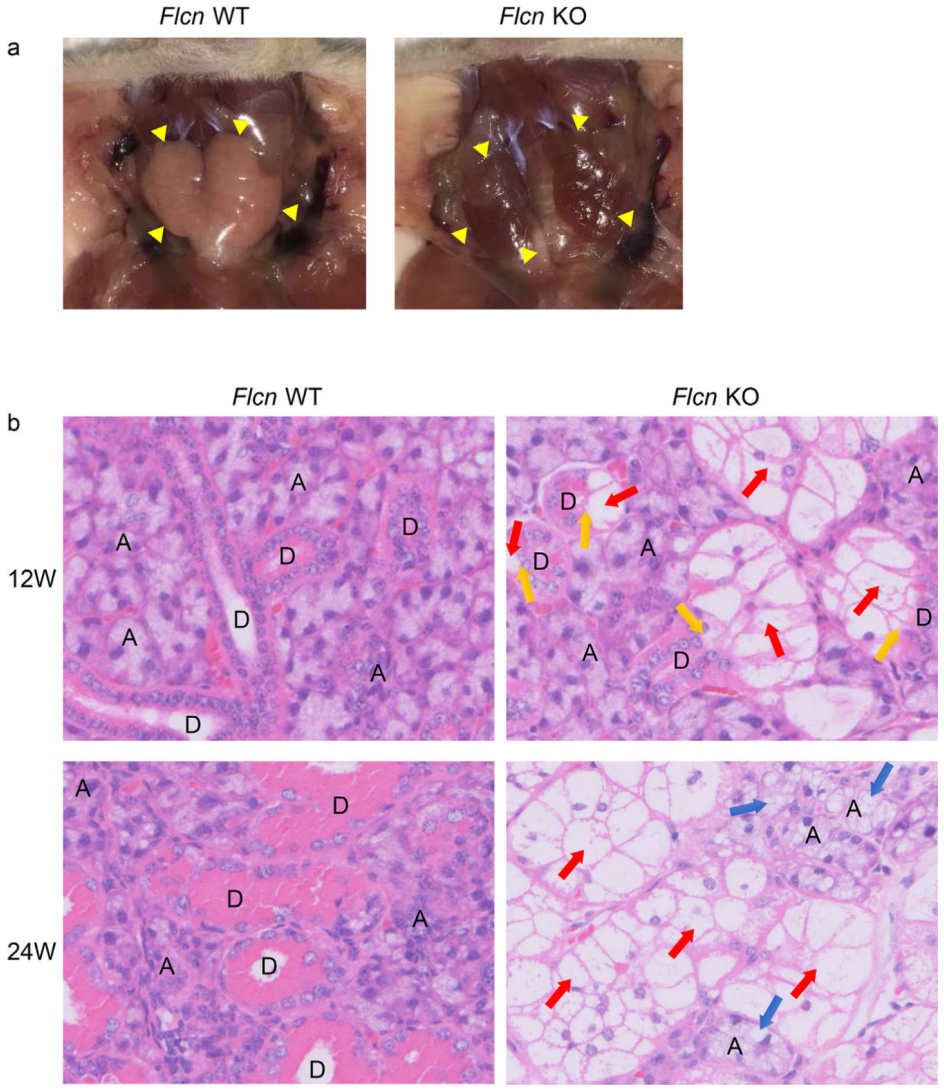

Fig. 2. Destruction of salivary gland duct in salivary gland-targeted *Flcn* knockout mouse.

Salivary gland-targeted *Flcn* knockout mouse showed red-colored salivary gland. Yellow triangles indicate salivary glands (a). H&E staining of salivary gland showed salivary ducts are replaced by cells with clear cytoplasm. Red arrows indicate salivary ducts replaced by cells with clear cytoplasm. Yellow arrows indicate borders between salivary ductal cells and cells with clear cytoplasm. Blue arrows indicate salivary acinar glands subsequently replaced by cells with clear cytoplasm (b). *Flcn* WT: *Flcn f/f; Flcn* KO: *Flcn f/f, MMTV-Cre*(+). A, salivary acinar gland; D, salivary duct. 12 W (12 weeks), 24 W (24 weeks).

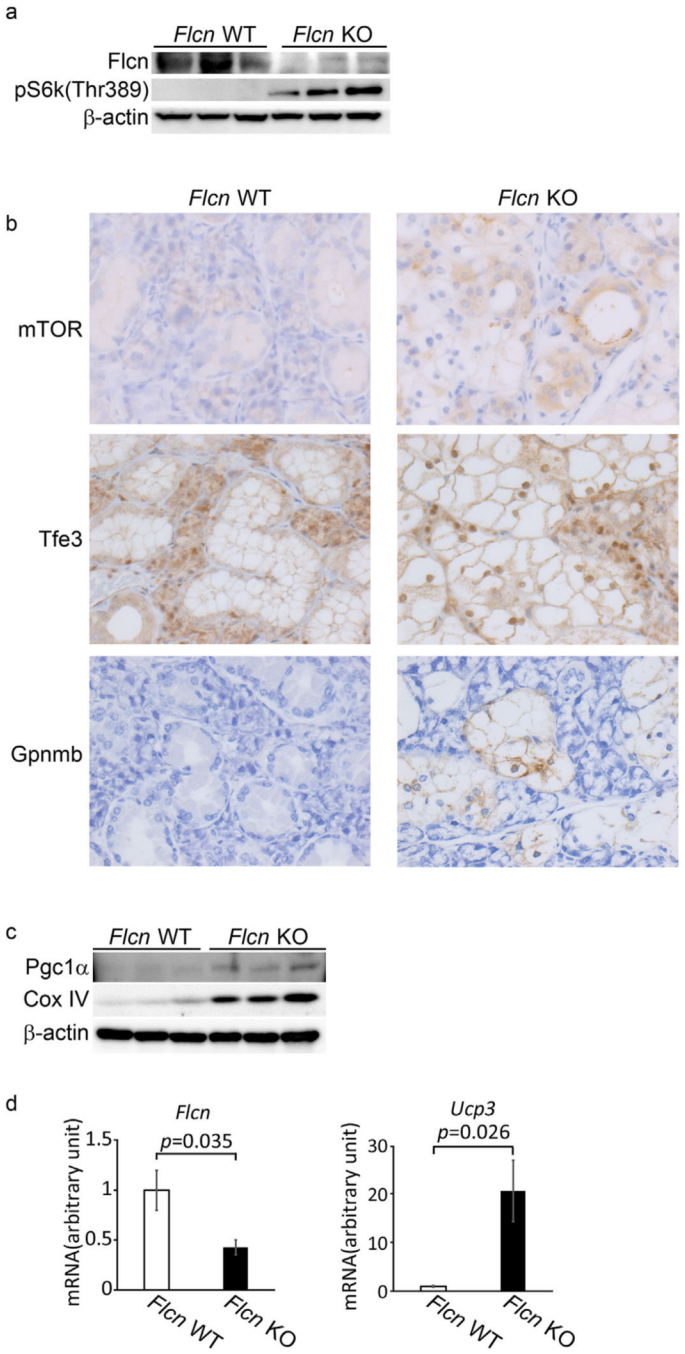

Fig. 3. Dysregulation of mTOR-TFE3 axis and increased mitochondrial biogenesis in *Flcn*-deficient salivary gland.

Western blotting showed increased phosphorylation in S6K at Thr389 in *Flcn*-deficient salivary gland (a). Immunohistochemistry exhibited increased expression of mTOR, translocation of Tfe3 into nucleus and increased expression of Gpnmb, a downstream target of the Tfe3 transcription factor in *Flcn*-deficient salivary gland duct (b). Western blotting of *Flcn*-deficient salivary glands exhibited increased expression of Pgc1a and Cox IV (c). Realtime PCR of *Flcn*-deficient salivary glands showed increased expression of *Ucp3*, one

of the mitochondrial components. n = 6 for each genotype (d). *Flcn* WT: *Flcn f/f; Flcn* KO: *Flcn f/f, MMTV-Cre*(+).

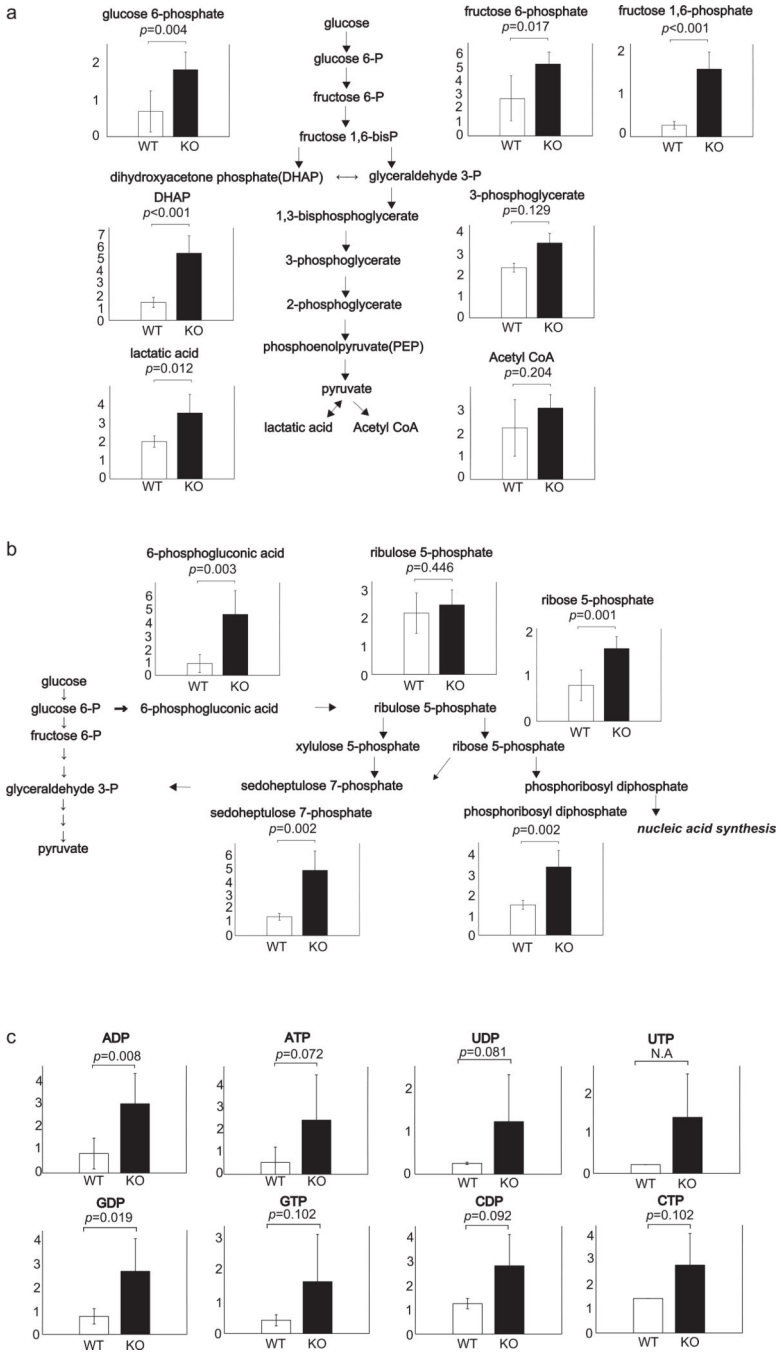

Fig. 4. Upregulated glycolysis and pentose phosphate pathway in *Flcn*-deficient salivary gland.

Metabolomic analyses of *Flcn*-deficient salivary glands using LC/MS exhibited upregulated glycolysis, pentosephosphate pathway and nucleotide synthesis. WT: *Flcn f/f;* KO: *Flcn f/f, MMTV-Cre*(+). n = 6 for each genotype.

Table 1

Screening of salivary gland using ultrasonography demonstrated that BHD patients have an increased risk of cyst formation in the salivary gland.

BHD patient	Sex	Age	Number of cysts	Size	*FLCN* mutation
1	F	43	1	4.4 mm × 3.6 mm	Exon 4 c199dupG
2	M	41	1	4.0 mm × 2.4 mm	Exon 7 c769–771 delTCC
3	M	55	1	5.3 mm × 4.5 mm	Exon11 c1285dupC
4	F	55	2	2.7 mm × 2.0 mm, 2.2 mm × 1.5 mm	Exon11 c1285dupC
5	F	46	1	3.6 mm × 1.9 mm	Exon11 c1285dupC
6	F	36	2	6.2 mm × 3.5 mm, 5.2 mm × 4.7 mm	Exon11 c1285dupC
7	F	52	1	2.6 mm × 1.4 mm	Exon 12 c1347–1353 dupCCACCCT
8	F	54	3	3.6 mm × 2.5 mm, 2.8 mm × 1.7 mm, 2.6 mm × 1.6 mm	Exon 13 c1522–1524 delAAG

Table 2

GO ontology of proteomic analysis of *Flcn*-deficient salivary gland revealed that half of cellular components increased in *Flcn*-deficient salivary gland were associated with mitochondrial metabolism.

Category	Term	Count	%	P-Value	Benjamini
mitochondria	mitochondrion	217	45.6	1.90E-25	7.90E-23
mitochondria	mitochondrial inner membrane	88	18.5	8.30E-13	1.70E-10
cytosol	cytosol	149	31.3	2.40E-06	3.20E-04
neuron	myelin sheath	61	12.8	3.60E-05	3.60E-03
mitochondria	mitochondrial proton-transporting ATP synthase complex	14	2.9	1.40E-04	1.10E-02
proteasome	proteasome complex	24	5	2.10E-04	1.40E-02
mitochondria	mitochondrial matrix	36	7.6	6.60E-04	3.80E-02
proteasome	proteasome core complex	10	2.1	1.60E-03	7.90E-02
peroxisome	peroxisome	18	3.8	1.90E-03	8.20E-02
mitochondria	mitochondrial membrane	15	3.2	4.20E-03	1.60E-01
mitochondria	mitochondrial outer membrane	17	3.6	9.90E-03	3.10E-01
mitochondria	respiratory chain	21	4.4	1.90E-02	4.80E-01
mitochondria	mitochondrial proton-transporting ATP synthase complex, coupling factor F(o)	7	1.5	2.20E-02	5.00E-01
mitochondria	mitochondrial respiratory chain complex III	7	1.5	2.20E-02	5.00E-01
proteasome	proteasome accessory complex	9	1.9	3.90E-02	6.90E-01
mitochondria	mitochondrial proton-transporting ATP synthase complex, catalytic core F(1)	5	1.1	4.90E-02	7.50E-01
mitochondria	proton-transporting ATP synthase complex, catalytic core F(1)	5	1.1	4.90E-02	7.50E-01
mitochondria	proton-transporting ATP synthase complex, coupling factor F(o)	6	1.3	5.00E-02	7.30E-01
peroxisome	peroxisomal matrix	6	1.3	5.00E-02	7.30E-01
proteasome	proteasome core complex, alphasubunit complex	6	1.3	5.00E-02	7.30E-01
membrane	integral component of membrane	78	16.4	6.80E-02	8.20E-01
proteasome	cytosolic proteasome complex	6	1.3	9.60E-02	9.00E-01
proteasome	proteasome regulatory particle, base subcomplex	6	1.3	9.60E-02	9.00E-01
perinuclear	perikaryon	9	1.9	9.90E-02	8.90E-01