Regulation of Mitochondrial Oxidative Metabolism by Tumor Suppressor FLCN

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Background

Birt-Hogg-Dubé (BHD) syndrome is a hereditary hamartoma syndrome that predisposes patients to develop hair follicle tumors, lung cysts, and kidney cancer. Genetic studies of BHD patients have uncovered the causative gene, FLCN, but its function is incompletely understood.

Methods

Mice with conditional alleles of FLCN and/or peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A), a transcriptional coactivator that regulates mitochondrial biogenesis, were crossbred with mice harboring either muscle creatine kinase (CKM)–Cre or myogenin (MYOG)–Cre transgenes to knock out FLCN and/or PPARGC1A in muscle, or cadherin 16 (CDH16)–Cre transgenes to knock out FLCN and/or PPARGC1A in kidney. Real-time polymerase chain reaction, immunoblotting, electron microscopy, and metabolic profiling assay were performed to evaluate mitochondrial biogenesis and function in muscle. Immunoblotting, electron microscopy, and histological analysis were used to investigate expression and the pathological role of PPARGC1A in FLCN-deficient kidney. Real-time polymerase chain reaction, oxygen consumption measurement, and flow cytometry were carried out using a FLCN-null kidney cancer cell line. All statistical analyses were two-sided.

Results

Muscle-targeted FLCN knockout mice underwent a pronounced metabolic shift toward oxidative phosphorylation, including increased mitochondrial biogenesis (FLCN+/+ vs FLCN+/+CKM–Cre: % mitochondrial area mean = 7.8% vs 17.8%; difference = 10.0%; 95% confidence interval = 5.7% to 14.3%; P < .001), and the observed increase in mitochondrial biogenesis was PPARGC1A dependent. Reconstitution of FLCN-null kidney cancer cells with wild-type FLCN suppressed mitochondrial metabolism and PPARGC1A expression. Kidney-targeted PPARGC1A inactivation partially rescued the enlarged kidney phenotype and abrogated the hyperplastic cells observed in the FLCN-deficient kidney.

Conclusion

FLCN deficiency and subsequent increased PPARGC1A expression result in increased mitochondrial function and oxidative metabolism as the source of cellular energy, which may give FLCN-null kidney cells a growth advantage and drive hyperplastic transformation.

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Affected individuals with Birt-Hogg-Dubé (BHD) syndrome are at risk for the development of multifocal chromophobe and hybrid oncocyctic renal cell carcinomas (1,2). The FLCN gene (alias BHD) is a tumor suppressor (3–6), but the mechanism by which FLCN deficiency causes kidney cancer is not completely understood. FLCN encodes folliculin (FLCN), a 64-kDa protein with no known functional or structural domain. FLCN binds to two FLCN-interacting proteins, FNIP1 and FNIP2, both of which interact with 5′-adenosine monophosphate–activated protein kinase (AMPK) (7–9), and several studies have shown that loss of FLCN modulates activity of the mammalian target of rapamycin (mTOR) pathway (3,4,10–12). AMPK is an important energy sensor in cells that negatively regulates mTOR (13,14). Although the function of FLCN remains to be determined, its involvement in a FNIP1/FNIP2/AMPK complex and the modulation of mTOR activity in response to FLCN deficiency suggest that FLCN may play a role in cellular energy and nutrient sensing through interaction with the AMPK–mTOR pathway, a precedent seen with other tumor suppressors involved in cell metabolism (15–19).

FLCN has been reported to interact with several other pathways in addition to the FLCN and AMPK–mTOR pathways already highlighted. The Drosophila FLCN homolog is required for germ line stem cell maintenance through the JAK/STAT pathway (20). FLCN-null embryonic stem cells and the FLCN-null cancer cell line UOK257 show loss of transforming growth factor β–dependent signaling (10,21). Transcriptional activity of TFE3, a member of the MiTF/TFE family, is enhanced by its nuclear localization in FLCN-null cells (22). Although these reports indicate that FLCN...
might be essential for connecting these important pathways, distinct functions of FLCN remain to be elucidated.

To clarify a potential role of FLCN in metabolism, we conducted metabolic assays using FLCN-deficient muscle, which is an important organ for energy production. We also carried out metabolic assays using a FLCN-null kidney cancer cell line, which was reconstituted with wild-type FLCN. We further evaluated the physiological significance of metabolic alterations under FLCN deficiency in the development of FLCN-deficient polycystic enlarged kidneys.

Methods and Materials

Mice
The mice carrying FLCN alleles flanked by loxP sites (floxed, f) were generated as previously described (3). Muscle creatine kinase (CKM)–Cre transgenic mice [FVB–Tg(Ckmm–cre)5Khm/J, stock number: 006405] were obtained from Jackson Laboratories. Cadherin 16 (CDH16)–Cre transgenic mice were described previously (3). The mice carrying PPARGCA alleles flanked by loxP sites (floxed, f) were generous gifts from Dr Bruce Spiegelman at Dana–Farber Cancer Institute, Harvard Medical School (23), and myogenin (MYOG)–Cre transgenic mice were generous gifts from Dr Eric N. Olson at University of Texas Southwestern Medical Center (24). We confirmed that the CKM–Cre, MYOG–Cre, and CDH16–Cre transgenes had no detectable effect on mouse phenotypes. Rapamycin (LC Laboratories, Woburn, MA) was adminis-
tered 2mg/kg daily from postnatal day 7 for 2 months. For muscle-specific FLCN knockout mice, conditional FLCN knock-
out mice (FLCNf/f) were crossed with CKM–Cre transgenic mice and MYOG–Cre transgenic mice to generate FLCNf/f/CKM–Cre and FLCNf/f/MYOG–Cre mice, respectively. For kidney-specific double knockout of FLCN and PPARGCA, conditional FLCN (FLCNf/f) and PPARGCA (PPARGCAf/f) knockout mice were crossed with CKM–Cre transgenic mice and MYOG–Cre transgenic mice to generate FLCNf/f/PPARGCAf/f/CKM–Cre and FLCNf/f/PPARGCAf/f/MYOG–Cre mice, respectively. For kidney-
specific double knockout of FLCN and PPARGCA, conditional FLCN (FLCNf/f) and PPARGCA (PPARGCAf/f) knockout mice were crossed with CDH16–Cre transgenic mice to generate FLCNf/f/PPARGCAf/f/CDH16–Cre mice. All mice that were used in these experiments were housed in the National Cancer Institute animal facilities according to the National Cancer Institute Animal Care and Use Committee guidelines. The aged mice were killed by carbon dioxide asphyxiation, and the white portion of the quadriceps muscle, from cell cultures, and from human kidney specimens (surgically treated at the Urologic Oncology Branch, National Institutes of Health, Bethesda, MD, with written patient permission under National Institutes of Health Institutional Review Board–approved protocol 97-C-0147) using TRIzol reagent (Invitrogen), and total RNA was reverse transcribed to cDNA using a Superscript III reverse transcriptase kit (Invitrogen).

Quantitative Real-Time Polymerase Chain Reaction
Total RNA was isolated from the flash frozen white portion of the quadriceps muscle, from cell cultures, and from human kidney specimens (surgically treated at the Urologic Oncology Branch, National Institutes of Health, Bethesda, MD, with written patient permission under National Institutes of Health Institutional Review Board–approved protocol 97-C-0147) using TRIzol reagent (Invitrogen), and total RNA was reverse transcribed to cDNA using a Superscript III reverse transcriptase kit (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed with the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Fermentas, Glen Burnie, CA) at 1:200 dilution, COX4 at 1:1000 dilution, and Antibodies used included mouse monoclonal FLCN, which was developed by the Monoclonal Antibody Core at the National Cancer Institute, National Institutes of Health, Bethesda, Maryland (7), at 1:1000 dilution, Myoglobin (FL154) at 1:200 dilution, PPARGCA (H300, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution, and COX4 at 1:1000 dilution. Western Blotting, Immunofluorescence Staining, and Antibodies
Frozen muscle pieces and kidney samples were homogenized in radioimmunoprecipitation assay buffer [20mM tris(hydroxymethyl) aminomethane–hydrogen chloride pH 7.5, 150mM sodium chloride, 1mM ethylenediaminetetraacetic acid, 1.0% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate] supplemented with Complete protease inhibitor cocktail and PhosStop phosphatase inhibitor cocktail (Roche, Indianapolis, IN). Immunofluorescence staining of human BHD-associated tumor was done as previously reported (3,4). Antibodies used included mouse monoclonal FLCN, which was developed by the Monoclonal Antibody Core at the National Cancer Institute, National Institutes of Health, Bethesda, Maryland (7), at 1:1000 dilution, Myoglobin (FL154) at 1:200 dilution, PPARGCA (H300, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution, and COX4 at 1:1000 dilution.
was split into equal parts for analysis on the GC, LC+, and LC− platforms. The extracted supernatant was used for metabolite extraction using a proprietary solvent extraction method. The isolated mitochondria were prepared for analysis using Metabolon's UOK257 cells. An XF96 V3 PET plate (Seahorse Bioscience) was coated overnight with 1:15 000 polyethyleneimine solution/assay buffer (137 mM potassium chloride, 2 mM monopotassium phosphate, 2.5 mM magnesium chloride, 20 mM HEPES, 0.5 mM EGTA, 0.2% fatty acid–free BSA). One microgram of mitochondria isolated from entire hind limbs was used for each measurement. The standard Nagarse method (25) was attached to the plate bottom at 956 g for 10 minutes. The plate was warmed at 37°C for 10 minutes and transferred to the Seahorse XF96 analyzer. State 3 respiration (maximum adenosine diphosphate–stimulated oxygen consumption ratio; oxygen consumption ratio under sufficient substrate for mitochondrial complex) of complex I was measured immediately after addition of 5 mM glutamate, 5 mM malate, and 0.5 mM adenosine diphosphate, and complex IV–dependent respiration was measured immediately after addition of 0.5 mM tetramethyl phenylenediamine and 3 mM ascorbic acid.

**Metabolite Analyses**

Metabolite profiling analyses using mass spectrometry platforms of liquid chromatography (LC-MS) and gas chromatography (GC-MS) were carried out in collaboration with Metabolon, Inc (Durham, NC), and 202 compounds were detected, including linolenate, eicosapentaenoate, docosapentaenoate, docosahexaenoate, dihomo-linolenate, palmitoleate, oleate, linoleate, arachidonate, cis-vaccenate, mead acid, stearidionate, 10-nonadecenoate, palmitate, dihomo-linoleate, 10-heptadecenoate, eicosanoate, stearate, margarate, coenzyme A, acetyl coenzyme A, citrate, malate, flavin adenine dinucleotide, ATP, glucose, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-biphosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate, lactate, ribose 5-phosphate, and ribulose 5-phosphate. Six quadriceps muscle tissues of FLCN wild-type mice (FLCN+/+) and six quadriceps muscle tissues of FLCN knockout mice (FLCN−/−/CKM−/Cre) were prepared for analysis using Metabolon’s proprietary solvent extraction method. The extracted supernatant was split into equal parts for analysis on the GC, LC+, and LC− platforms. Signal intensity obtained through LC-MS and GC-MS was normalized to protein concentration determined by Bradford protein assays and described in relative units. Nicotinamide adenine dinucleotide, reduced (NADH) was measured using a nicotinamide adenine dinucleotide/NADH quantitation kit (Biovision, Milpitas, CA).

**Electron Microscopy**

Specimens of human BHD-associated kidney cancer and normal kidney were obtained with intraoperative core needle biopsies at the Urolologic Oncology Branch, National Institutes of Health, Bethesda, Maryland, with written patient permission under National Institutes of Health Institutional Review Board–approved protocol 97-C-0147, and immediately immersed in 4% formaldehyde/2% glutaraldehyde/PBS. Small blocks were then cut, osmicated, and dehydrated before embedding. The sections were mounted and observed in a Jeol 1200 transmission electron microscope equipped with an XR-100 Charge-coupled device camera (Advanced Microscopy Techniques Corporation, Danvers, MA). Percentage of mitochondrial area was measured with Image J (National Institute of Health, Bethesda, MD). One sectional image of muscle was divided into 24 square areas, each of which represented as means and 95% confidence intervals (CIs). The perinuclear area was omitted because of the abundance of mitochondria. At least three pairs of independent sections were analyzed, and similar results were obtained. For percentage of mitochondrial area in kidney cells, the ratios of mitochondrial area to cell area were measured in at least 20 cells and represented as means and 95% confidence intervals.

**Statistical Analysis**

Experimental data are summarized as the mean values with 95% confidence intervals (CIs). Statistical analyses were performed using a two-tailed Student’s t test for in vivo data and analysis of variance for in vitro data (SPSS Statistics version 20, IBM, Armonk, NY), and differences were considered to be statistically significant at a value of P less than .05.

**Results**

To evaluate the potential metabolic role of FLCN, we crossbred mice carrying loxp-flanked FLCN alleles (floxed, f) with muscle creatine kinase (CKM)–Cre transgenic mice, which express Cre recombinase driven by the CKM promoter, thereby deleting FLCN gene sequences, specifically in muscle. Conditional gene expression was necessary because homozygous FLCN knockout mice are embryonic lethal (4). Strikingly, the muscles in the muscle-specific FLCN knockout mice (FLCN−/−/CKM−/Cre) were characterized by distinctive red coloring (Figure 1, A), which was indicative of increased expression of mitochondrial components (26,27). Indeed, FLCN-deficient muscle showed evidence of increased mitochondrial biogenesis, including increased mRNA of mitochondrial genes (FLCN+/+ vs FLCN−/−/CKM−/Cre: n = 6 each; ATP5g mean = 1...
Figure 1. Mitochondrial biogenesis in FLCN-deficient muscle. A) Mice carrying FLCN alleles flanked by loxP sites (floxed, f) were crossed with CKM (muscle creatine kinase)–Cre transgenic mice to generate muscle-specific FLCN knockout mice (FLCN^f/f/CKM–Cre). Pictures show representative images of muscle with indicated genotypes. B) Mitochondrial gene mRNA levels in quadriceps muscle tissue from littermate-matched mice were quantified with the indicated genes by real-time polymerase chain reaction. * Cytochrome C. Mean±95% confidence interval (CI). n=6. Student’s t test (two-sided). C) Lysates of quadriceps muscle from littermate-matched mice were immunoblotted with the indicated antibodies. *Cytochrome C. β-actin served as a loading control. D) Electron microscopy images were obtained from gastrocnemius muscle tissues of littermate matched mice. ×1000 (scale bar: 2 μm) and ×15 000 (scale bar: 500 nm) magnified images. Arrows indicate mitochondria. Right bars represent percentage of mitochondrial area. Mean ± 95% confidence interval. Student’s t test (two-sided).


Table 1. Metabolites related to \(\beta\)-oxidation in FLCN-deficient quadriceps muscle*

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<th>Classification</th>
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<td>.0011</td>
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* Metabolite profiling analyses using mass spectrometry platforms of liquid chromatography and gas chromatography were carried out with extracts from quadriceps muscle, and knock-out (KO) (FLCN \(^{f/f}\)/CKM–Cre)and wild-type (WT) (FLCN \(^{β}β\)) ratios of metabolites related to \(\beta\)-oxidation were obtained. CoA = coenzyme A; IUPAC = International Union of Pure and Applied Chemistry.

† (Number of carbon atoms) : (Number of double bonds) n (Position of double bond).

‡ Student’s \(t\) test (two-sided).

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**vs 2.69, difference = 1.69, 95% CI = 0.64 to 2.73, \(P = .005\); COX5a mean = 1 vs 2.15, difference = 1.15, 95% CI = 0.42 to 1.87, \(P = .006\); CYCS (Cytochrome C) mean = 1 vs 1.89, difference = 0.89, 95% CI = 0.39 to 1.38, \(P = .003\); PDK4 mean = 1 vs 1.79, difference = 0.79, 95% CI = 0.46 to 1.11, \(P < .001\); Ndufs8 mean = 1 vs 1.64, difference = 0.64, 95% CI = 0.42 to 0.86, \(P < .001\); UCP3 mean = 1 vs 1.91, difference = 0.91, 95% CI = 0.32 to 1.51, \(P = .007\) (Figure 1, B), increased expression of mitochondrial proteins (Figure 1, C), and increased mitochondrial area per muscle fiber area (FLCN \(^{f/f}\) vs FLCN \(^{β}β\)/CKM–Cre: percentage of mitochondrial area mean = 7.8% vs 17.8%, difference = 10.0%, 95% CI = 5.7% to 14.3%, \(P < .001\) (Figure 1, D).

To further investigate mitochondrial function in FLCN-deficient muscle, we conducted metabolite profiling analyses using LC-MS and GC-MS. Nutrients for fatty acid \(\beta\)-oxidation (Table 1), metabolites in the tricarboxylic acid cycle (FLCN \(^{f/f}\) vs FLCN \(^{β}β\)/CKM–Cre: n = 6 each; citrate mean = 1 vs 2.78, difference = 1.78, 95% CI = 0.59 to 2.98, \(P = .008\); malate mean = 1 vs 1.80, difference = 0.80, 95% CI = 0.58 to 1.03, \(P < .001\) (Figure 2, A), and coenzymes for the electron transport chain (FLCN \(^{f/f}\) vs FLCN \(^{β}β\)/CKM–Cre: n = 6 each; NADH mean = 2.61 vs 4.24 pmol/µg, difference = 1.63 pmol/µg, 95% CI = 0.80 to 2.46 pmol/µg, \(P = .001\); flavin adenine dinucleotide mean = 1 vs 2.32, difference = 1.32, 95% CI =1.12 to 1.52, \(P < .001\) (Figure 2, B) were increased in FLCN-deficient muscle. These results suggest that FLCN-deficient muscle has a higher flux through the tricarboxylic acid cycle to produce abundant cofactors for the mitochondrial electron transport chain. Furthermore, both the respiratory capacity of isolated mitochondria (FLCN \(^{f/f}\) vs FLCN \(^{β}β\)/CKM–Cre: n = 4 each; complex I state 3 respiration mean = 203.9 vs 316.8 µMoles/minute/g mitochondrial protein, difference = 112.9 µMoles/minute/g mitochondrial protein, 95% CI =76.0 to 150.0 µMoles/minute/g mitochondrial protein, \(P < .001\); complex IV–dependent respiration mean = 240.8 vs 372.0 µMoles/minute/g mitochondrial protein, difference = 131.3 µMoles/minute/g mitochondrial protein, 95% CI = 36.5 to 226.0 µMoles/minute/g mitochondrial protein, \(P = .02\) (Figure 2, C) and the amount of ATP in muscle tissue were higher in FLCN-deficient mice compared with FLCN \(^{β}β\)/control mice (FLCN \(^{f/f}\) vs FLCN \(^{β}β\)/CKM–Cre: n = 6 each; mean = 1 vs 1.70, difference = 0.70, 95% CI = 0.18 to 1.21, \(P = .02\) (Figure 2, D). These results demonstrate that inactivation of FLCN triggers a metabolic shift to mitochondrial oxidative metabolism, resulting in excessive ATP production (Supplementary Table 2 and Supplementary Figure 1, available online). Furthermore, in a doxycycline-inducible, FLCN-null renal tumor cell line model (Supplementary Figure 2, available online), it is noteworthy that the enhanced mitochondrial oxidative functions, including increased expression of mitochondrial genes (Figure 3, A), elevated oxygen consumption ratio (Figure 3, B), and increased membrane potential (Figure 3, C and D) associated with FLCN-deficiency were reversed by doxycycline induction of FLCN expression. Thus, we conclude that FLCN is essential for maintaining a physiologically normal rate of mitochondrial metabolism.

To clarify the molecular mechanism by which FLCN deficiency increases mitochondrial biogenesis and enhances oxidative metabolism, we examined the expression of genes, including transcription factors and coactivators, which had been reported to regulate mitochondrial biogenesis or whose gene-manipulated murine models resulted in a phenotype similar to that found in our FLCN-deficient model that included red-colored muscle and increased mitochondrial biogenesis (26–28). In our screen, we observed increased PPARGC1A mRNA (FLCN \(^{f/f}\) vs FLCN \(^{β}β\)/CKM–Cre:
n = 4 each; PPARGC1A mean = 1 vs 1.99, difference = 0.99, 95% CI = 0.23 to 1.75, P = .02) (Figure 4, A) and increased expression of PPARGC1A protein (Figure 4, B) in FLCN-deficient muscle. Consistent with these results, C2C12 myoblasts showed increased mRNA of PPARGC1A when FLCN was knocked down (Figure 4, A and Supplementary Figure 3, available online). Additionally, FLCN restoration in FLCN-null UOK257 cells suppressed PPARGC1A mRNA (Figure 4, D), suggesting that FLCN

Figure 2. Mitochondrial function in FLCN-deficient muscle tissues. A) Relative tricarboxylic acid cycle metabolite values for citrate and malate were obtained in metabolite profiling analyses on quadriceps muscle tissues of indicated genotypes. Mean ± 95% confidence interval. Quadriceps, n = 6. Student's t test (two-sided). B) Coenzymes for the electron transport chain were measured in quadriceps muscle tissues of indicated genotypes. Nicotinamide adenine dinucleotide, reduced (NADH) was measured using a commercially available kit, and a relative value of flavin adenine dinucleotide was obtained by metabolite profiling analyses. Mean ± 95% confidence interval. Quadriceps, n = 6. Student's t test (two-sided). C) Respiratory capacity of isolated mitochondria was analyzed in muscle tissues of indicated genotypes. State 3 respiration of complex I– and complex IV–dependent respiration was measured by Seahorse XF96 analyzer using mitochondria isolated from the entire hind limb muscle. Mean ± 95% confidence interval. n = 4. Student's t test (two-sided). D) Relative ATP amounts were obtained from metabolite profiling analyses on quadriceps muscle tissues of indicated genotypes. Mean ± 95% confidence interval. Quadriceps, n = 6. Student's t test (two-sided).
Figure 3. Mitochondrial oxidative function of FLCN-null UOK257 renal cancer cells. A) FLCN-null UOK257 renal cancer cells were reconstituted with wild-type FLCN expression. The mRNA of mitochondrial genes was quantified by real-time polymerase chain reaction in FLCN-null UOK257 cells, which express wild-type FLCN in a doxycycline-dependent manner. *Cytochrome C. Mean ± 95% confidence interval of triplicates. Analysis of variance test. B) Baseline respiration and respiratory capacity were analyzed by Seahorse XF96 analyzer on FLCN-inducible UOK257 cells cultured with or without doxycycline. Oxygen consumption ratio of baseline respiration was measured in completely supplemented media, and oxygen consumption ratio of maximum respiration was measured after the addition of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone. Mean ± 95% confidence interval of six wells. Student’s t test (two-sided). C) Representative flow cytometry analysis of JC-1-stained FLCN-inducible UOK257 cells cultured with or without doxycycline. The FL2⁺, FL1⁻ fraction (upper left quadrant) represents cells with high membrane potential, whereas the FL2⁻, FL1⁺ fraction (lower right quadrant) represents cells with low membrane potential. D) Representative flow cytometry analysis of tetramethylrhodamine methyl ester (TMRM)-stained FLCN-inducible UOK257 cells, cultured with or without doxycycline.
Figure 4. PPARGC1A expression under FLCN deficiency. A) The mRNA of transcription factor and coactivator was quantified by real-time polymerase chain reaction on quadriceps of FLCN<sup>f/f</sup> and FLCN<sup>f/f</sup>/CKM-Cre mice. Mean ± 95% confidence interval. Quadriceps, n = 4. Student’s t test (two-sided). B) Lysates from quadriceps muscle of indicated genotypes were immunoblotted with PPARC1A antibody. Quadriceps, n = 3. β-actin served as a loading control. C) FLCN expression was knocked down in C2C12 myoblasts using FLCN small interfering RNA (siRNA), and mRNA of indicated genes was quantified with real-time polymerase chain reaction. * Cytochrome C. Mean ± 95% confidence interval of triplicates. Analysis of variance test. D) PPARC1A mRNA was quantified with real-time polymerase chain reaction on FLCN-inducible UOK257 cells cultured with or without doxycycline. Mean ± 95% confidence interval of triplicates. Analysis of variance test.
modulates PPARGC1A expression levels to produce an adequate level of mitochondrial oxidative phosphorylation for cellular energy needs.

PPARGC1A is a key regulator of mitochondrial function, oxidative metabolism, and energy homeostasis in a variety of tissues (29–34). Notably, muscle-specific PPARGC1A transgenic mice have been shown to develop red-colored muscle with increased mitochondrial biogenesis (27), and PPARGC1A-null mice are characterized by decreased mitochondrial biogenesis and an inability to increase ATP levels (32). Moreover, in vitro bioenergetic analyses have revealed that overexpression of PPARGC1A is associated with an increased membrane potential and respiratory capacity (34–37). Phenotypes of our mouse model in which FLCN inactivation is directed to muscle and FLCN-null in vitro systems are consistent with those of PPARGC1A overexpressing cells and these findings led us to crossbreed muscle-specific FLCN knockout mice with muscle-specific PPARGC1A knockout mice to see whether increased mitochondrial biogenesis under FLCN deficiency is caused by upregulated PPARGC1A. Notably, increased mitochondrial biogenesis, which was associated with FLCN deletion in mouse muscle, was completely reversed by crossbreeding that resulted in deletion of PPARGC1A alleles (Figure 5, A–C). These data strongly support the concept that upregulation of mitochondrial biogenesis caused by FLCN deficiency is PPARGC1A dependent, underscoring a role for FLCN as a physiological negative regulator of PPARGC1A.

To date, few studies have evaluated the role of PPARGC1A in cancer development; therefore, the contribution of PPARGC1A to tumor progression (38,39) or inhibition is unclear (40). In addition to its role in energy homeostasis, PPARGC1A has reported functions that include suppression of reactive oxygen species (41), regulation of angiogenesis (42), and induction of unfolded protein response that mitigates endoplasmic reticulum stress (43), all of which may play a role in tumor development. Both of our muscle-directed FLCN knockout mouse models showed increased mRNA of angiogenic factors such as VEGFb and ANGPT2 and unfolded protein response genes such as ERdj4 and CHOP (FLCN f/f vs FLCN f/f/CKM-Cre: n = 4 each; VEGFb mean = 1 vs 1.97, difference = 0.97, 95% CI = 0.76 to 1.17, P < .001; ANGPT2 mean = 1 vs 1.75, difference = 0.75, 95% CI = 0.17 to 1.33, P = .02; ERdj4 mean = 1 vs 6.13, difference = 5.13, 95% CI = 0.82 to 9.44, P = .03; and FLCN f/f vs FLCN f/f/MYOG-Cre: n = 4 each; VEGFb mean = 1 vs 1.75, difference = 0.75, 95% CI = 0.69 to 0.82, P < .001; CHOP mean = 1 vs 1.66, difference = 0.66, 95% CI = 0.42 to 0.91, P = .001) (Figure 5, D and E). Notably, the increased mRNA of these genes was reversed when both FLCN and PPARGC1A were deleted. These results suggest that FLCN-deficient tissues may have increased angiogenesis and upregulated unfolded protein responses through PPARGC1A. Thus, PPARGC1A may contribute to the development of kidney cancer by enhancing angiogenesis and suppressing endoplasmic reticulum stress during FLCN deficiency.

We previously reported that highly proliferating cystic kidneys in kidney-specific FLCN knockout mice have upregulation of the mTOR and MEK/Erk pathways and that rapamycin treatment statistically significantly reduced the kidney size and extended the lifespan of these mice (3). mTOR has been found to control mitochondrial biogenesis by modulating the interaction between PPARGC1A and YY1, a transcriptional factor whose motif is highly enriched in the promoter region of oxidative phosphorylation genes (28). However, although we treated our muscle-specific FLCN knockout mice with rapamycin, rapamycin was more effective in suppressing expression of mitochondrial genes in the control mouse group (FLCN f/f vehicle vs FLCN f/f rapamycin: n = 5 each; ATP5g mean = 1 vs 0.77, difference = 0.23, 95% CI = 0.16 to 0.30, P < .001; UCP3 mean = 1 vs 0.70, difference = 0.30, 95% CI = 0.01 to 0.58, P = .04) than in the muscle-specific FLCN knockout mouse group (FLCN f/f/CKM–Cre vehicle vs FLCN f/f/CKM–Cre rapamycin: n = 6 each; ATP5g mean = 1.76 vs 1.61, P = .72; UCP3 mean =1.44 vs 1.45, P = .84) (Figure 5, F). This suggests that the increased mitochondrial biogenesis in FLCN-deficient muscle might be due to upregulated PPARGC1A itself rather than enhanced interaction of PPARGC1A–YY1 by mTOR. Notably, mTORC1 was not activated in FLCN-deficient muscle (data not shown).

To investigate a potential role of PPARGC1A in the pathogenesis of human BHD-associated kidney cancer, we examined PPARGC1A mRNA and observed high mRNA levels in BHD-associated kidney cancer relative to normal kidney (normal kidney from BHD patients, n = 4 vs BHD-associated kidney cancer, n = 5: mean = 1.02 vs 4.45, difference = 3.44, 95% CI = 2.13 to 4.75, P < .001) (Figure 6, A). In support of this result, we observed elevated protein levels of PPARGC1A in FLCN-deficient mouse tumors compared with adjacent normal kidney (Figure 6, B). Additionally, we observed increased COX4 staining (Figure 6, C), a downstream target of PPARGC1A, and tumor cells filled with increased numbers of mitochondria in human BHD-associated kidney cancer (Figure 6, D). These results indicate that upregulated PPARGC1A and subsequent increased numbers of mitochondria may play an important role in the growth of FLCN-null tumor cells. We previously reported that kidney-specific FLCN knockout mice develop highly proliferating cystic kidneys by 3 weeks of age (3). In fact, we observed increased PPARGC1A protein in FLCN-deficient kidneys (FLCN f/f vs FLCN f/f/CDH16–Cre: n = 2 each; PPARGC1A mean = 3.17 vs 6.82, difference = 3.65, 95% CI = 2.71 to 4.59, P = .004) (Figure 7, A), and this observation led us to crossbreed kidney-targeted FLCN knockout mice with kidney-targeted PPARGC1A knockout mice. Surprisingly, inactivation of PPARGC1A alleles in this mouse model partially reduced the size of the enlarged kidneys (FLCN f/f/CDH16–Cre vs FLCN f/f/PPARGC1A f/f/CDH16–Cre: n = 8 each; mean of kidney to body weight ratio = 13.13% vs 9.40%, difference = 3.73%, 95% CI = 2.44% to 5.02%, P < .001) (Figure 7, B and C). To clarify the underlying mechanism by which inactivation of PPARGC1A reduced the size of FLCN-deficient kidneys, we examined the histology of FLCN/PPARGC1A doubly inactivated kidneys. Previously, we observed the presence of hyperplastic cells with enlarged eosinophilic, granular cytoplasm in FLCN-deficient kidney (3). Interestingly, we did not observe any hyperplastic cells in the FLCN/PPARGC1A doubly inactivated kidneys (Figure 7, D). Furthermore, by electron microscopy we observed increased numbers of mitochondria in FLCN-deficient kidneys (FLCN f/f vs FLCN f/f/PPARGC1A f/f/CDH16–Cre: n = 24 vs n = 20 cells; mean of percentage of mitochondrial area = 3.44% vs 11.70%, difference = 8.26%, 95% CI = 6.46% to 10.06%, P < .001), and this phenotype was reversed by inactivation of PPARGC1A (FLCN f/f/PPARGC1A f/f/CDH16–Cre) (Figure 7, E and F), consistent with our results in the FLCN-deficient muscle and in vitro systems.
Figure 5. PPARG1A inactivation in FLCN-deficient muscle. A) FLCN<sup>±/±</sup> CKM-Cre mice were crossbred with mice carrying PPARG1A alleles flanked by loxP sites (floxed, <i>f</i>) and FLCN<sup>±/±</sup>PPARG1A<sup>±/±</sup>CKM-Cre mice were generated. A photograph shows representative images of muscle from mice with FLCN<sup>±/±</sup>, FLCN<sup>±/±</sup>/CKM-Cre and FLCN<sup>±/±</sup>/PPARG1A<sup>±/±</sup>CKM-Cre genotypes. B) The mRNA of mitochondrial genes was quantified with real-time polymerase chain reaction on quadriceps samples obtained from FLCN<sup>±/±</sup>, FLCN<sup>±/±</sup>/CKM-Cre and FLCN<sup>±/±</sup>/PPARG1A<sup>±/±</sup>CKM-Cre mice. * Cytochrome C. Mean ± 95% confidence interval. Quadriceps, <i>n</i> = 4. Student’s <i>t</i> test (two-sided). C) Lysates from quadriceps muscle tissues were immunoblotted with the indicated antibodies. α-tubulin served as a loading control. Protein levels in left panel were quantified by Li-Cor Odyssey imager as shown in right panel. * Cytochrome C. Mean ± 95% confidence interval. Quadriceps, <i>n</i> = 2. Student’s <i>t</i> test (two-sided). D) The mRNA of angiogenic factors (VEGFb and ANGPT2) and unfolded protein response gene (ERdj4) was quantified with real-time polymerase chain reaction in quadriceps muscle tissues of indicated genotypes. Mean ± 95% confidence interval. Quadriceps, <i>n</i> = 4. Student’s <i>t</i> test (two-sided). E) A different muscle-specific Cre transgenic strain, myogenin (MYOG)–Cre transgenic mice, was crossbred with mice carrying floxed alleles of FLCN and PPARG1A. The mRNA of PPARG1A, mitochondrial genes (ATP5g, COX5a, and CYCS), angiogenic factor (VEGFb) and unfolded protein response gene (CHOP) was quantified with real-time polymerase chain reaction on quadriceps samples obtained from FLCN<sup>±/±</sup>, FLCN<sup>±/±</sup>/MYOG-Cre and FLCN<sup>±/±</sup>/PPARG1A<sup>±/±</sup>/MYOG-Cre mice. * Cytochrome C. Mean ± 95% confidence interval. Quadriceps, <i>n</i> = 4. Student’s <i>t</i> test (two-sided). F) Mice were treated with rapamycin 2mg/kg daily from postnatal day 7 for 2 months. The mRNA of the indicated genes was quantified with real-time polymerase chain reaction. * Cytochrome C. Mean ± 95% confidence interval. Quadriceps, <i>n</i> = 5 for FLCN<sup>±/±</sup> group and <i>n</i> = 6 for FLCN<sup>±/±</sup>/CKM-Cre group. Student’s <i>t</i> test (two-sided).
Figure 6. PPARGC1A expression in human Birt-Hogg-Dubé (BHD)-associated kidney tumor and FLCN-deficient mouse kidney tumor. A) PPARGC1A mRNA in human BHD-associated kidney cancer was quantified with real-time polymerase chain reaction. Bars represent mean ± 95% confidence interval. Normal kidney from sporadic cases (n = 5) vs normal kidney from BHD patients (n = 4) vs BHD-associated kidney cancer (n = 5). Student's t test (two-sided). B) Lysates of FLCN-deficient mouse kidney tumors, solid tumors that developed in heterozygous FLCN knockout mice at 18–24 months of age, were immunoblotted with the indicated antibodies. β-actin served as a loading control. Normal mouse kidney (n = 4) vs FLCN-deficient mouse kidney tumor (n = 5). C) Human BHD-associated kidney cancer and adjacent normal kidney were stained with an antibody to COX4, a downstream target of PPARGC1A. Green = COX4; Blue = 4',6-diamidino-2-phenylindole. Right panel shows hematoxylin and eosin (H&E) staining of the corresponding area, and insets show higher magnification images. Dotted lines indicate the border between normal kidney and kidney cancer. Four samples of human BHD-associated kidney cancer were stained and similar results were obtained. (Scale bar: 100 µm in low magnified image, 10 µm in high magnified image.) D) Electron microscopy images were obtained from renal medulla of normal kidney from patients with non-BHD (sporadic) and BHD-associated kidney cancer. x500 (scale bar: 10µm) and x5000 (scale bar: 500nm) magnified images. Arrows indicate mitochondria.
Discussion

In this study, we report an important role of FLCN in mitochondrial oxidative metabolism. FLCN-deficient muscle in a mouse model displays a distinctive red color and enhanced mitochondrial oxidative metabolism. PPARGC1A expression was high in FLCN-deficient muscle, and inactivation of PPARGC1A in muscle completely reversed the elevated mitochondrial biogenesis. The UOK257 FLCN-null kidney cancer cell line displayed increased mitochondrial function and an elevated level of PPARGC1A mRNA, which were reversed by reconstitution with wild-type FLCN. Human BHD-associated kidney cancer also displayed an elevated level of PPARGC1A mRNA. Furthermore, inactivation of PPARGC1A in a kidney-targeted FLCN-deficient mouse model...
partially rescued the highly proliferating cystic kidney phenotype. A partial reduction in the size of FLCN-deficient kidneys and complete disappearance of hyperplastic cells that were observed in the FLCN-deficient kidney model (3) underscore the pathological significance of upregulated PPARGC1A in FLCN-null kidney cells.

Although the details of FLCN function have not been completely elucidated, studies have shown that the FLCN pathway interacts with a number of important metabolic pathways, including the AMPK-mTOR (3,4,10–12), TFE3 (22), and HIF–VEGF (44) pathways. In the current study, we demonstrate that FLCN plays a role in mitochondrial homeostasis, further supporting prior work implicating FLCN in metabolism.

In FLCN-deficient muscle, we did not observe the activation of mTORC1, whereas we previously observed the activation of mTORC1 in FLCN-deficient kidney models (3,4). mTORC1 integrates a variety of environmental cues, including energy, nutrient, and growth factors, and, in addition to these inputs, mTORC1 activity in muscle tissues is affected by exercise (45). Therefore, our observation that mTORC1 was not activated in FLCN-deficient muscle may be due to the particular organ context. The precise mechanism by which FLCN deficiency dysregulates the mTOR pathway will require further study.

In 2010, Klomp et al. reported increased mRNA expression of PPARGC1A in human BHD-associated kidney tumors (46). In the current study, we have established PPARGC1A as a key molecule that confers enhanced mitochondrial oxidative metabolism on FLCN-deficient cells. Although aerobic glycolysis (known as the “Warburg effect”) is thought to be the dominant metabolic pathway active in cancer cells, Otto Warburg originally developed his theory based on the efficiency of ATP production from one molecule of glucose through mitochondrial oxidative phosphorylation (n = 29–30 ATP) compared with glycolysis (n = 2 ATP) (47,48), which is a requirement for proliferating cells to maximize energy production (18). Our data indicating that upregulation of PPARGC1A in response to FLCN deficiency contributes to hyperplastic cell formation in the FLCN-deficient mouse kidneys strongly support the idea that mitochondria are important organelles that can fuel tumor suppressor-null cells to initiate tumorigenesis or acquire a growth advantage driving tumor progression.

Numerous electron microscopy studies have demonstrated increased numbers of mitochondria in sporadic chromophobe renal cell carcinoma and renal oncocytoma compared with normal kidney (49–51), and expression profiling analyses of both sporadic (52–56) and familial (46) chromophobe renal cell carcinoma and renal oncocytoma revealed the increased expression of mitochondrial genes or transcriptional regulators for mitochondrial components. For many years, these observations have raised questions among researchers about the underlying mechanisms by which mitochondria increase in these types of tumors. On the other hand, genetic studies of sporadic chromophobe renal cell carcinoma and renal oncocytoma have reported the rearrangement of mitochondrial DNA in these types of tumors (57–60). Taken together, these findings have led researchers to speculate that increased mitochondrial expression might be a compensatory consequence of impaired mitochondrial function. However, our findings that loss of FLCN enhances ATP production and mitochondrial respiratory function support the concept that the increased number of mitochondria observed in BHD-associated kidney cancer is a primary consequence of upregulated PPARGC1A resulting from FLCN deficiency and that FLCN-null cells augment mitochondrial oxidative metabolism by increasing mitochondrial number. Although it is not known whether the FLCN pathway is dysregulated in sporadic chromophobe renal cell carcinoma and renal oncocytoma, increased numbers of mitochondria in these tumor types would suggest the potential for attenuation of a pathway involving FLCN.

In mouse kidneys in which FLCN and PPARGC1A were both inactivated, we no longer detected the cosinophilic, hypertrophic cells with increased numbers of mitochondria that were observed in the FLCN-deficient mouse kidneys. Therefore, it is possible that the increased expression of PPARGC1A and its target genes leading to excess mitochondrial ATP production under FLCN deficiency may contribute to tumorigenesis and/or tumor progression. However, one obvious limitation of this study is that the kidney-targeted FLCN knockout mice die due to renal failure around 3 weeks of age without progression of the hyperplastic cells to malignancy (1). To address this limitation, we have recently developed another FLCN knockout mouse model in which one FLCN allele is deleted in the germline. Subsequent loss of heterozygosity of the remaining wild-type FLCN allele in kidney cells leads to development of chromophobe and hybrid oncocytic tumors at 18–24 months of age, thereby mimicking the human BHD-associated renal tumor paradigm (4). Although this in vivo tumor model of BHD requires a longer time frame, it would be interesting to see the consequence of PPARGC1A inactivation on tumor formation in this heterozygous FLCN knockout mouse model. In addition to its role in mitochondrial homeostasis, PPARGC1A regulates angiogenesis (42), neutralizes reactive oxygen species, and induces expression of unfolded protein response proteins that mitigate endoplasmic reticulum stress (43). Because these functions may be important for cancer cells to grow, the PPARGC1A pathway could be a potential therapeutic target in patients with BHD-associated kidney cancer.

Our findings support a role for tumor suppressor FLCN as a physiologically important negative regulator of PPARGC1A, a key regulator of energy homeostasis, and demonstrate that loss of FLCN leads to increased mitochondrial oxidative metabolism with subsequent energy overproduction. Furthermore, PPARGC1A enhances the development of hyperplastic cells and enlarged cystic kidneys in the kidney-targeted FLCN-null mouse model, suggesting that upregulation of PPARGC1A and subsequent increased mitochondrial oxidative metabolism in FLCN-null kidney cells may contribute to renal tumor initiation and/or progression in BHD syndrome. These findings provide a foundation for the management of BHD patients and the development of targeted therapeutic approaches for patients with BHD-associated kidney cancer.

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Notes

H. Hasumi, M. Baba, L. S. Schmidt, and W. M. Linehan conceived the project,
analyzed the data, and wrote the manuscript. L. S. Schmidt organized mice pro-
jects, and M. Baba provided FLCN knockout mice. Y. Hasumi, Y. Huang, and
H. Oh performed mice dissection, quantitative real-time PCR, and immuno-
blotting. M. Baba and R. M. Hughes generated a UOK257 doxycycline-
inducible cell line. H. Oh and M. E. Klein performed PCR genotyping of mice.
S. Takikita performed mice metabolic assays. W. M. Linehan decided to submit the manuscript. All
the authors discussed the results and commented on the manuscript.

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