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Molecular Genetics and Clinical Features of Birt-Hogg-Dubé-Syndrome

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Abstract

Birt-Hogg-Dubé (BHD) syndrome is an inherited renal cancer syndrome in which affected individuals are at risk to develop benign, cutaneous fibrofolliculomas, bilateral pulmonary cysts and spontaneous pneumothoraces, and kidney tumors. Bilateral multifocal renal tumors that develop in BHD syndrome are most frequently hybrid oncocytic tumors and chromophobe renal carcinoma, but may present with other histologies. Germline mutations in the *FLCN* gene on chromosome 17 are responsible for BHD syndrome. BHD-associated renal tumors show inactivation of the wild-type *FLCN* allele by somatic mutation or chromosomal loss, confirming that *FLCN* is a tumor suppressor gene that fits the classic two-hit model. FLCN interacts with two novel proteins, FNIP1 and FNIP2, and with AMPK, a negative regulator of mTOR. Studies with *FLCN*-deficient cell and animal models support a role for FLCN in modulating the AKT-mTOR pathway. Emerging evidence links FLCN with a number of other molecular pathways and cellular processes important for cell homeostasis that are frequently deregulated in cancer, including regulation of TFE3/TFEB transcriptional activity, amino acid-dependent mTOR activation through Rag GTPases, TGF-β signaling, PGC1α-driven mitochondrial biogenesis, and autophagy. Currently, surgical intervention is the only therapy available for BHD-associated renal tumors. Further understanding of the FLCN pathway will hopefully lead to the development of effective forms of therapy for this disease.

Keywords

Birt-Hogg-Dubé syndrome; BHD; FLCN; folliculin; FNIP1; FNIP2; inherited renal cancer syndrome; chromophobe renal cancer; fibrofolliculoma; mTOR

Introduction

Renal cell carcinoma (RCC) is not a single entity but a group of diseases originating in the kidney epithelium that are characterized by different histologies, gene alterations and clinical courses.[1] Substantial insight into the molecular genetics of RCC has come from

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studies of families with inherited renal cancer syndromes. These studies have led to the identification of the *von-Hippel-Lindau* (*VHL*) tumor suppressor gene mutated in the germline of patients with the inherited clear cell RCC disorder, von-Hippel Lindau disease [2] , and in the majority of sporadic clear cell renal carcinomas [3,4]. Subsequent RCC family studies have led to the discovery of germline activating *MET* oncogene mutations in patients with hereditary papillary renal carcinoma type 1, [5] and to the identification of mutations in the tricarboxylic acid cycle gene *fumarate hydratase (FH)* in the germline of patients with hereditary leiomyomatosis renal cell carcinoma[6] who are predisposed to develop type 2 papillary renal tumors. In this review we describe the clinical presentation, molecular genetics, and management of patients with Birt-Hogg-Dubé syndrome (BHD; OMIM 135150), a hereditary cancer syndrome caused by germline mutations in the *FLCN* (*folliculin*) tumor suppressor gene that predispose to chromophobe RCC, hybrid oncocytic renal tumors, and non-malignant renal oncocytomas.[7]

In 1977 Birt, Hogg and Dubé described small, pale, dome-shaped skin tumors, " a hereditary pilar hamartoma", on the face, neck and upper torso of 15 members of a large Canadian kindred[8] that appeared at about the age of 25 in association with trichodiscomas[9] and acrochordons. Histologically, these lesions, termed fibrofolliculomas, consisted of specialized connective tissue surrounding an abnormal hair follicle with epithelial strands extending into the connective tissue mantle. Two years earlier Hornstein and Knickenberg had reported perifollicular fibromatosis cutis, a lesion nearly indistinguishable clinically and histologically from fibrofolliculoma, in a small two-generation kindred.[10] The segregation pattern in these early kindreds suggested that these cutaneous fibrofolliculomas were inherited in an autosomal dominant manner, and the disease was subsequently named Birt-Hogg-Dubé (BHD) syndrome. Reports published more than a decade later noted the presence of multiple lung cysts in BHD patients contributing to an increased frequency of spontaneous pneumothorax that cosegregated with the cutaneous lesions.[11-13] In 1993, bilateral multifocal chromophobe renal carcinoma was reported in a BHD patient[14] and a few years later the cosegregation of renal tumors and fibrofolliculomas was identified in members of three renal cancer kindreds seen at the National Cancer Institute.[13] The association of kidney neoplasia with BHD syndrome was confirmed in a subsequent study evaluating the risk factors for renal tumors in 111 affected BHD patients and 112 unaffected family members.[15]

Clinical manifestations of BHD syndrome

Cutaneous manifestations

Fibrofolliculomas, trichodiscomas and acrochordons are considered the hallmark cutaneous lesions in BHD syndrome although acrochordons (skin tags) are not diagnostic for BHD given their frequent appearance in the general population. Fibrofolliculomas are the most common phenotypic features of BHD syndrome occurring in greater than 85% of BHD patients over the age of 25.[16-19] These smooth, dome shaped, white to flesh colored papules are 2-4mm in diameter, and can occur singly or in numbers greater than 100 that can sometimes coalesce in a plaque (**Figure 1A**). They tend to occur on the face, neck, upper torso, and less frequently on ear lobes or oral mucosa.[13,19] Fibrofolliculomas, which develop

after puberty, are generally neither painful nor pruritic. Biopsies of these lesions reveal multiple anastomosing strands of 2-4 epithelial cells that emanate from a central aberrant hair follicle and frequently are continuous with sebaceous glands (**Figure 1B**).[13,20] Schulz *et al.* have suggested that trichodiscomas and fibrofolliculomas are the same lesions but sectioned in different planes giving rise to artificial differences in histologic interpretation. [21]

Pulmonary manifestations

Patients affected with BHD syndrome are at risk for the development of pulmonary cysts and pneumothorax. Multiple bilateral pulmonary cysts are highly penetrant manifestations in BHD syndrome , detected on high resolution computed tomography (CT) of the chest in 70-84% of BHD affected individuals, and occur at a median age of 30-40 years (**Figure 1C, D**).[16,17,19, 22, 23] BHD-associated lung cysts tend to be located in the basilar and mediastinal regions of the lung in contrast to other cystic lung diseases.[24,25] Cysts that develop in BHD patients are predominantly irregular shaped with sharply demarcated thin walls that do not enhance on CT, and vary in size and number. [25-27] Despite the presence of pulmonary cysts, lungs generally function normally or show only mild airway obstruction in BHD patients, but cystic lung disease may be more severe in BHD-affected individuals who are or were smokers.[25,27]

In a study of 198 BHD patients, a statistically significant association between the presence of lung cysts and history of spontaneous pneumothorax was found.[24] BHD-affected individuals have a 50-fold greater risk (after adjusting for age) of developing spontaneous pneumothorax than unaffected family members.[15] Approximately 30% of BHD patients report a history of spontaneous pneumothorax, generally prior to the age of 40 years, with diagnoses in children as young as 7 years of age.[16,17,19,28] Although a single episode of pneumothorax may occur, a history of multiple pneumothoraces was reported by Toro *et al.* in 75% of their BHD cohort (n=198) [24] and in 25 of 29 (86%) BHD patients by Kunogi *et al.*[22]

Renal manifestations

Patients affected with BHD syndrome are at risk for the development of bilateral, multifocal renal tumors. Renal neoplasms develop in 12-34% of individuals affected with BHD syndrome with a mean age of onset of 46-52 years (**Figure 2**), but have been reported in patients as young as 20 years of age.[16,17,29,30,31] The risk of developing renal tumors was 7-fold greater in BHD affected individuals when compared with their unaffected siblings.[15] In contrast to other inherited renal cancer syndromes such as VHL (clear cell RCC) or hereditary papillary renal carcinoma (type 1 papillary RCC) that predispose to a single histologic tumor subtype, BHD-associated renal tumors may present with multiple histologies. The most frequent histologic tumor type in BHD affected individuals is the hybrid oncocytic tumor (50%) with features of chromophobe RCC and renal oncocytoma.[32] Additionally, BHD patients are at risk to develop chromophobe RCC (35%), clear cell RCC (9%) and, less frequently, renal oncocytoma (5%) (**Figure 3**).[32-34]

An additional feature of the kidneys of BHD patients is the presence of renal "oncocytosis",[32] defined as small microscopic foci of oncocytic cells scattered throughout the "normal" kidney parenchyma[35] that are presumed to be precursor lesions to malignant tumors. Since these foci occur in the kidneys of BHD patients regardless of their tumor histology, it has been suggested that renal oncocytic cells may be precursors of the various histologic subtypes of BHD-associated renal tumors.[32]

Other manifestations

A number of other clinical features have been reported in BHD patients including lipomas[12,13,16,19] , parathyroid adenomas [12,19], thyroid nodules[19] and cancer[17,22], and parotid oncocytomas[16,17 ,36,37,38], but whether these are incidental findings or true phenotypic features of BHD syndrome is unclear.

Early case reports linked the presence of colon polyps and/or colorectal cancer with BHD syndrome,[10,11,31, 39,40] but a risk assessment performed in 111 BHD patients seen at the National Institutes of Health did not find a significant increase in colon related manifestations when BHD patients were compared with their 112 unaffected siblings.[15] In contrast, the risk of developing colonic neoplasms assessed in 149 BHD patients in a University of Birmingham study was found to be 20% by age 80 compared to 4.9% in the general U.K. population.[41] Differences in environmental exposures or genetic modifiers in the two cohorts may also contribute to these divergent results and will require additional study to confirm or refute a link between colon neoplasia and BHD syndrome.

Diagnostic criteria for BHD syndrome

BHD syndrome may be phenotypically heterogeneous within members of a family or between families with the same *FLCN* mutation (**Figure 4**). Any combination of the cutaneous, pulmonary or renal manifestations in an individual or multiple members of a BHD family can be considered part of the phenotypic spectrum. Therefore, the following diagnostic criteria are suggestive for BHD syndrome: 1) at least 2 cutaneous papules clinically consistent with fibrofolliculoma/trichodiscoma and at least 1 histologically confirmed fibrofolliculoma; 2) multiple bilateral pulmonary cysts located mainly in the basilar regions of the lung with or without a history of spontaneous pneumothorax that develops prior to age 40, but especially with a family history of these pulmonary manifestations; 3) bilateral, multifocal chromophobe renal carcinomas or hybrid oncocytic tumors especially with a family history of renal tumors or early age (<50 years) of onset; 4) a combination of these cutaneous, pulmonary or renal manifestations presenting in a patient or members of his family. A definitive diagnosis of BHD syndrome is confirmed with a diagnostic genetic test that is positive for a germline *FLCN* mutation.(**BOX 1**)

Molecular genetics of BHD syndrome

The *FLCN* gene

In 1998, a rare genetic disorder, termed familial renal oncocytoma (FRO), was described[42] in which individuals within a family presented with multiple bilateral renal oncocytomas. Further evaluation of these patients resulted in the identification of cutaneous facial papules

that, when biopsied, were found to be consistent with fibrofolliculomas.[13] Recruitment of additional BHD families provided the basis for genetic linkage analysis, which led to mapping of the disease locus to chromosome 17p11.2 [43,44] and the subsequent cloning of a novel gene, *folliculin* (*FLCN*), which was found to be mutated in the germline of BHD-affected individuals.[7] A *FLCN* exon 11 insertion/deletion mutation in a tract of 8 cytosines (c.1285dupC or c.1285delC) was reported by Nickerson *et al.* in 44% of their BHD patient cohort[7] and appears to be a mutation "hot spot" in other cohorts as well.[16-18, 29, 30] To date, 149 unique *FLCN* germline mutations spanning all 14 exons have been identified in BHD syndrome and familial spontaneous pneumothorax families, and catalogued in the Leiden Open Variation Database.[45,46] They are predominantly mutations (insertion/deletion, nonsense, splice site) that result in premature protein truncation and presumed loss of FLCN function. In addition, 9 large intragenic deletions or duplications expected to severely disrupt protein structure or, at a minimum, to delete the last exon (**Figure 5**),[22,29,47,48] and 7 missense mutations resulting in amino acid substitutions have been documented. Nahorski *et al.* evaluated four of these *FLCN* missense mutations and found that p.Arg239Cys and p.His255Pro *FLCN* mutants demonstrated reduced protein stability *in vitro* and the latter mutation supported colony formation in soft agar, whereas p.Val400Ile and p.Lys508Arg mutant proteins demonstrated stability similar to wild-type FLCN and suppressed anchorage independent growth *in vitro*.[49] Additional biochemical studies are needed to confirm the pathogenicity of the *FLCN* missense mutations reported in BHD patients. New technologies including high throughput sequencing and multiplex ligation-dependent probe amplification (MLPA) have improved the ability to detect sequence variants and large deletions/duplications resulting in *FLCN* mutation detection rates that approach 90%.[16,17,29]

Genotype-phenotype correlations

To date, no clear correlation between type of mutation or location within the *FLCN* gene and any of the phenotypic manifestations has been identified, although some interesting trends have been noted .[16,17,18,29] *FLCN* mutations appeared to occur more frequently (23/30) in the 3' end of the gene in a Japanese BHD cohort in which lung cysts and history of pneumothorax were the predominant phenotypic features.[22] In support of this association, Toro *et al.* found a trend toward more episodes of pneumothorax in BHD patients with *FLCN* mutations in exons 9 and 12, and a statistically significant association between mutations in exon 9 and a greater number of lung cysts.[24] Another report by Schmidt *et al.* noted that BHD affected individuals with the c.1285delC mutation in the exon 11 mutation "hot spot" had fewer renal tumors (1/26) compared to BHD affected individuals with the c. 1285dupC mutation (13/56), although the sample size was too small for significance[16] and this was not replicated in an additional 50 BHD families from the same institution.[17]

Although Zbar *et al.* did not find a significantly greater risk for colorectal neoplasia in a U.S. BHD cohort,[15] a University of Birmingham study in the U.K. compared BHD patients with two recurrent *FLCN* mutations and found a significantly higher risk of colorectal neoplasia in patients with the c.1285dupC mutation (n=37) versus the c.610delGCinsTA mutation (n=32; χ^2=5.78, p=0.016).[41] These findings raise the possibility that different allelic variants of *FLCN* may predispose to a greater or lesser risk of colon neoplasia in BHD syndrome. *FLCN* insertion/deletion mutations in the exon 11 C_8 tract have also been

reported in 16-23 % of sporadic colorectal tumors with demonstrated microsatellite instability (MSI)[41,50] suggesting that the polycytosine tract in *FLCN* may be an effective target of MSI.

Loss of tumor suppression under *FLCN* deficiency

FLCN is a novel tumor suppressor gene that fits the two-hit model for tumor suppression.[51] In addition to an inactivating germline *FLCN* mutation, Khoo *et al.* found a somatic "second hit" *FLCN* mutation in a chromophobe renal tumor from a BHD patient.[31] Vocke *et al.* found somatic *FLCN* mutations (predominantly frameshift) in 53% of 77 renal tumors from BHD patients harboring germline *FLCN* mutations, and loss of heterozygosity on chromosome 17p in 17% of the remaining tumors (**Figure 6**).[52] Naturally-occurring canine and rat models of BHD syndrome also develop kidney tumors as a consequence of inheriting germline mutations in the *FLCN* homolog with subsequent loss of the remaining *FLCN* allele in the tumors.[53,54]

Yang *et al.* established a cell line from a BHD-associated renal tumor that carries only the germline *FLCN* mutation of the patient from which it was derived indicating that the tumor had lost the wild type *FLCN* allele.[55] This cell line was tumorigenic in immunocompromised mice, [55,56] but lost its tumorigenic properties when the normal copy of *FLCN* was restored.[56] Further support for *FLCN* as a tumor suppressor is provided by Hudon *et al.* who showed that *FLCN* knockdown in the clear cell renal tumor cell line ACHN resulted in formation of significantly larger tumors in athymic nude mice, whereas overexpression of *FLCN* in 786-O cells, which are deficient for the tumor suppressor *VHL* and tumorigenic *in vivo*, led to a decrease in *VHL*-deficient xenograft growth.[57] Taken together, these data underscore a tumor suppressor function for FLCN in normal kidney cells and demonstrate loss of tumor suppression when FLCN is inactivated *in vivo*.

Paucity of *FLCN* mutations in sporadic tumors

The *VHL* gene that is mutated in the germline of clear cell RCC patients with the inherited multisystem disorder von-Hippel Lindau disease[2] is also inactivated by mutation, hypermethylation or loss in >90% of sporadic clear cell kidney cancer.[4,58] These findings led investigators to evaluate sporadic renal tumors, especially those with chromophobe and oncocytic histologies, for *FLCN* mutations. However, very few *FLCN* mutations were found in these tumors. *FLCN* mutations were identified in 5/46 chromophobe renal tumors (although 3/5 were likely germline) and 1/18 renal oncocytomas by Gad *et al.*[59] Khoo *et al.* found only one *FLCN* mutation (lacking matched normal) in 39 sporadic renal tumors[60] and Fernandez da Silva *et al.* found only 2 missense mutations in 30 sporadic renal tumors and cell lines,[61] whereas Nagy *et al.* found no mutations in 16 chromophobe tumors and renal oncocytomas.[62] A recent report from the Cancer Genome Atlas (TCGA) project confirmed the absence of *FLCN* mutations in 66 sporadic chromophobe renal tumors by whole exome sequencing.[63] Taken together these findings would suggest that somatic *FLCN* mutations play only a minor role in the development of sporadic counterpart renal tumors.

FLCN functional studies and interacting proteins

FLCN and FNIP1

Following the cloning of the *FLCN* gene and identification of disease-causing mutations in the germline of BHD families, efforts were focused on elucidating folliculin (FLCN) protein function to understand how loss of function leads to kidney cancer, fibrofolliculomas and pulmonary cysts. The first protein-protein interaction studies led to the identification of a novel folliculin interacting protein, FNIP1, which interacts with the carboxy-terminus of FLCN and with AMP-activated protein kinase (AMPK), an important energy sensing enzyme that monitors cellular energy status and is a negative regulator of mechanistic target of rapamycin (mTOR), the master controller of protein synthesis and cell growth.[64,65] These and subsequent studies demonstrated that FLCN could exist in multiple phosphorylated forms, which were differentially affected by FNIP1 binding and by inhibitors of AMPK and mTOR.[64,66]

FLCN and FNIP2

A second folliculin interacting protein, FNIP2,[67] also referred to as FNIPL[68] or MAPO1[69], was identified by bioinformatics searches for FNIP1 homologous proteins. FNIP2 has 49% homology with FNIP1 and, like FNIP1, binds to the carboxy-terminus of FLCN and interacts with AMPK.[67] FNIP1, FNIP2 and FLCN can form homo- and heteroduplexes suggesting that FNIP1 and FNIP2 may work cooperatively with FLCN or independently. Their functions may be unique in some tissues based on tissue-specific expression of one compared to the other, but both FNIP1 and FNIP2 are similarly expressed in kidney suggesting a potential functional redundancy.[67] Evidence for overlapping growth suppressive functions for FNIP1 and FNIP2 in kidney was provided by the fact that kidney-targeted *Fnip1*/*Fnip2* double knockout mice developed enlarged polycystic kidneys, and *Fnip1* heterozygous/*Fnip2* homozygous knockout mice produced kidney tumors, whereas neither *Fnip1* knockout mice nor *Fnip2* knockout mice demonstrated a kidney phenotype.[70,71] These findings point to redundant roles for FNIP1 and FNIP2 in their interaction with the FLCN tumor suppressor pathway in kidney. An additional function for Fnip1 in B-cell development is supported by the finding that B-cell defective phenotypes developed in two independent *Fnip1* knockout mouse models.[70,72]

Potential pathways in BHD tumorigenesis

Modulation of the AKT-mTOR pathway

Initial clues to understanding FLCN function were obtained from studies of a number of *Flcn*-deficient animal models. Kidney-targeted inactivation of *Flcn* in a mouse model using Cre-lox technology produced a multicystic kidney phenotype that led to renal failure and shortened lifespan, and displayed activation of the AKT-mTOR pathway in the cystic kidneys. This phenotype was markedly reduced by treatment with the mTOR inhibitor, rapamycin.[73,74] Heterozygous *Flcn* knockout mice develop late-onset solid renal tumors with histologies similar to human BHD tumors[75,76] and show loss of the wild type *Flcn* allele[75]. Hasumi *et al.* showed that *Flcn* +/− mouse tumors as well as renal tumors from BHD patients displayed activated AKT, mTORC1 and mTORC2, supporting a role for

FLCN in down-regulating the AKT-mTOR pathway[75]. On the other hand, Hartman *et al.* demonstrated reduced mTOR activity in *Flcn +/−* spontaneous renal tumors, and in renal tumors with reduced latency that developed in a *Flcn*-heterozygous mouse model subjected to N-ethyl-N-nitrosourea (ENU) mutagenesis [76] in agreement with *in vitro FLCN*-knockdown cell line results and yeast homolog *bhd* mutant data from the same group.[77] In a third *Flcn* heterozygous model, both mTOR activation (elevated phospho-S6 in large cysts) and mTOR inactivation (reduced phospho-S6 in small cysts) were seen.[57] The conflicting results presented in these *in vitro* and *in vivo* systems have led to the hypothesis that the effect of *FLCN* deficiency on mTOR activation or inhibition may depend on the cell type or context in which it occurs (**Figure 7**).

mTOR activation by amino acids through Rags

mTORC1 is a master regulator of cell growth and senses the energy and nutrient needs of the cell to regulate anabolic and catabolic processes. Emerging evidence has shown that mTORC1 senses and is activated by amino acids at the lysosome surface through a multimeric protein complex that includes Rag GTPases (RagA/B and RagC/D), whose bound nucleotide state (GDP/GTP) determines the recruitment of mTORC1 to the lysosome. [78] Tsun *et al.* have shown a role for FLCN, in complex with FNIP1/2, as a GTPase activating protein (GAP) for RagC/D. GDP loading of RagC/D facilitates mTORC1 binding to the lysosome for activation in an amino-acid sensitive manner. [79] Evidence by Petit *et al.* also supports a role for FLCN in amino acid-dependent mTORC1 activation on the lysosome surface by a somewhat different mechanism. Their findings support FLCN with protein partner, FNIP1, as a guanine exchange factor (GEF) for RagA (and predicted for RagB), resulting in GTP loading of RagA/B and recruitment and activation of mTOR. [80] Recently the crystal structure of the carboxy-terminus of FLCN was solved and was found to be distantly related to differentially expressed in normal cells and neoplasia (DENN) proteins that are characterized by their Rab GEF function. [81] Nookala *et al.* was able to show *in vitro* that FLCN had GEF activity against another Rab GTPase, Rab35. Together these studies provide strong support for a role of FLCN in facilitating mTOR activation at the lysosome surface in an amino-acid sensitive manner through the Rab GTPases (Figure 7). However, these studies do raise the question of why FLCN, a tumor suppressor, is a positive effector of the mTOR pathway. One idea put forth in both of these reports is that suppression of mTORC1 activity resulting from FLCN deficiency may push other pathways into overdrive that could more than compensate for mTOR inhibition. Additional studies will be necessary to clarify the mechanistic details involved in modulation of mTOR signaling by the FLCN/FNIP complex.

TFE3/TFEB transcriptional activation

The first evidence that FLCN controlled the transcriptional activity of basic-helix-loop-helix transcription factor TFE3, a member of the microphthalmia transcription (MiT) family, was reported by Hong *et al.*, who showed that TFE3 was sequestered in the cytoplasm by FLCN, but moved to the nucleus where it became transcriptionally active under FLCN deficiency. They demonstrated that TFE3 nuclear localization was correlated with decreased TFE3 phosphorylation and increased activity in a number of *FLCN*-deficient human and mouse renal tumors and cell lines.[82] Petit *et al.* investigated FLCN-facilitated TFE3

phosphorylation in more detail. Earlier work from this group demonstrated that mTORC1-dependent phosphorylation of the MiT family member TFEB (also TFE3) at serine 211 triggered its interaction with 14-3-3 proteins, thereby preventing nuclear accumulation when the lysosome was functioning efficiently.[83] In recent work they showed that FLCN and its interacting partner FNIP1 were required for mTOR recruitment to the lysosome for amino acid-stimulated activation, and that mTOR-dependent TFEB phosphorylation at serine 211 was diminished and nuclear localization of TFEB was enhanced in *FLCN* knockdown cells.[80] Taken together, these studies support a role for FLCN in regulating TFE3/TFEB transcriptional activity through mTOR-dependent phosphorylation of these transcription factors.

Finally, Betschinger *et al.* performed a functional screen of mouse embryonic stem cells (ESC) to identify genes required for exit from ground-state pluripotency to cell lineage differentiation. In further support of a role for FLCN in controlling TFE3 transcriptional activity, they found that Flcn, in complex with both binding partners Fnip1 and Fnip2, drives ESC differentiation by cytoplasmic sequestering of Tfe3, thereby inhibiting transcriptional activation of the Tfe3 target gene, *Esrrb*, an orphan nuclear receptor and central pluripotency factor (**Figure 7**).[84]

Cell-cell adhesions and RhoA signaling

Two independent studies using yeast two hybrid screens have identified p0071 (plakophilin-4) as a FLCN interacting protein. This armadillo repeat-containing protein binds E-cadherin at adherens junctions, which are important for maintenance of cell architecture in epithelial tissues, and regulates RhoA activity in the cytoplasm. *FLCN* loss resulted in increased cell-cell adhesion, disruption of cell polarity and dysregulated Rho A signaling.[85,86] Increased cell-cell adhesions were reported in *FLCN*-deficient lung cell lines *in vitro* [87] and marked reduction in E-cadherin expression at adherens junctions and increased alveolar apoptosis were observed in lungs of mice with lung-targeted *Flcn* inactivation.[88] These findings support a role for FLCN in maintaining critical cell-cell adhesions for maintenance of lung and kidney epithelial cell integrity (**Figure 7**).

Additional pathways in which FLCN interacts

Recent reports support a role for FLCN in other cancer-associated pathways. Hong *et al.*[56], using a gene expression microarray approach, demonstrated downregulation of genes involved in TGF-β activation and signaling in *FLCN*-deficient *in vitro* and *in vivo* models, including BHD-associated renal tumors. Using *Flcn*-deficient mouse ESCs, Cash *et al.*[89] found defects in cell-intrinsic apoptosis that correlated with reduced TGF-β-mediated transcription of the proapoptotic BH3-only gene *Bim;* Bim expression was also downregulated in BHD renal tumors. These studies provide support for a role of FLCN in regulation of TGF-β signaling.

Several reports identified FLCN functions in autophagy. Possik *et al.*[90] found that loss of *Flcn* in a *Caenorhabditis elegans* model led to constitutive AMPK activation and induction of autophagy. Dunlop *et al.*[91], however, showed that autophagy was impaired in *FLCN*-deficient kidney cell lines and BHD renal tumors. This report supports a mechanism by

which FLCN, regulated by ULK (unc-51 like autophagy activating kinase 1) and in complex with FNIP1/2, positively regulates autophagy through interaction with GABARAP (GABA(A) receptor-associated protein), a component of the autophagosome machinery. Interaction of FLCN in TGF-β signaling and autophagy pathways suggests a fundamental role for FLCN in maintenance of cellular homeostasis.

Management of BHD patients and families

Surveillance of at-risk BHD family members

Individuals are at risk for developing renal tumors if they or their family members present with manifestations of BHD syndrome including fibrofolliculomas or pulmonary cysts with or without a history of spontaneous pneumothorax, a family history of bilateral, multifocal, hybrid oncocytic or chromophobe renal tumors, or if they test positive for a germline *FLCN* mutation. Since renal tumors have been reported in BHD family members as young as 20 years of age,[30,31] diagnostic genetic testing for *FLCN* is recommended with informed consent starting at age 20 or 21.[92,93] It is recommended that at-risk BHD family members undergo abdominal imaging at least every 36 months and lifelong surveillance is warranted. Renal ultrasonography may not detect small or isoechoic hybrid oncocytic and chromophobe tumors [94] so abdominal computed tomography(CT) imaging is recommended, or magnetic resonance imaging (MRI) with intravenous contrast to provide the best anatomical detail of the kidney and distinguish cystic and solid lesions. MRI imaging may be preferred for long-term surveillance to reduce radiation exposure.[92] Once a renal mass is detected, imaging intervals are determined by the size and growth rate of the renal tumors.

Management of BHD-associated renal tumors

Renal tumors that develop in the setting of BHD syndrome are most commonly hybrid oncocytic tumors and chromophobe RCC, which tend to be more indolent than, for example, the clear cell RCC that occurs in the setting of VHL disease or the type 2 papillary renal tumors found in HLRCC patients.[95-97] Therefore, patients are managed by active surveillance until the largest tumor reaches the 3 cm threshold. At that point, nephron-sparing surgery is recommended. BHD patients are at risk to develop bilateral, multifocal renal tumors and may require multiple surgeries during their lifetime; therefore, it is important to utilize surgical procedures that preserve renal function whenever possible. Generally very small margins of normal kidney parenchyma are taken during tumor enucleation of more indolent BHD tumors; however, wider margins may be used for renal tumors with more aggressive histology, such as clear cell RCC. Other factors such as tumor growth rate, size and location may affect the appropriateness of the “3 cm rule” for surgical intervention. Prior to surgery, pulmonary assessment should be performed to evaluate the presence of pulmonary cysts, and excessive intraoperative positive pressure ventilation should be avoided to reduce the chance of a cyst rupture leading to a possible pneumothorax.[92,33]

In cases where tumors measuring >3 cm are detected in both kidneys, staged surgical procedures are recommended. Surgery on the kidney requiring the least invasive resection

may be performed first, followed by surgical management of the contralateral kidney only after complete recovery from the initial surgical procedure.[92]

Although it has been suggested that ablation methods such as radiofrequency ablation (RFA) or cryoablation therapy may be appropriate treatment for small renal tumors, [92] our experience is to not recommend these procedures in healthy patients who are good surgical candidates. BHD patients will more often have multiple lesions that require extensive surgical intervention to remove all accessible tumors; previous ablation may complicate subsequent surgical procedures and also can make post-ablation imaging difficult to interpret.[92,98,99] Cryotherapy or ablative procedures may, however, be options for elderly patients or those with comorbidities that would preclude surgical intervention.

Since BHD patients tend to develop more indolent types of renal tumors including hybrid oncocytic tumors and chromophobe RCC, metastases are seen less often in BHD syndrome than, for example, in VHL, where patients are at risk to develop clear cell renal tumors that are more aggressive and likely to metastasize. Benusiglio *et al.* reported metastases in 4 of 33 BHD patients but only one died, and of an unrelated cause.[30] In contrast, 5 of 14 patients with BHD-associated renal tumors described by Houweling *et al.* developed metastases; 4 of the 5 patients died within one year of diagnosis despite surgical, radiological, chemotherapeutic and/or immunotherapeutic treatments (**Table 1**).[29] Two of 10 BHD patients with renal tumors reported by Pavlovich *et al.* developed metastases and died.[33] In these and other cases where metastases led to patient mortality, the predominant tumor histology was the more aggressive clear cell type,[29,31,33, 100] in contrast to the more indolent chromophobe and hybrid tumors that developed in the metastatic cases reporting longer term survival.[30]

Differential diagnosis of BHD tumors

It is important to be able to distinguish BHD-associated renal tumors from sporadic hybrid oncocytic and chromophobe renal tumors. Therefore, there is a need to develop biomarkers for the differential diagnosis of BHD-associated tumors and their sporadic counterparts. In that regard, Hong *et al.* found that the expression of glycoprotein nonmetastatic B (GPNMB), a transcriptional target of TFE3, was high in *FLCN*-deficient kidney tumors from BHD patients as well as in *FLCN*-deficient renal tumor cell lines and tumors from *Flcn*-deficient *in vivo* mouse models.[82] Furuya *et al.* have evaluated this biomarker in 19 BHD renal tumors including 6 hybrid oncocytic, 9 chromophobe, 2 clear cell and 2 papillary tumors, and compared GPNMB expression to that of sporadic counterpart tumors. They found strong GPNMB staining in the BHD chromophobe and oncocytic hybrid tumors, but none or minimal staining in the sporadic tumors, suggesting that GPNMB may serve as a biomarker to distinguish BHD and sporadic hybrid oncocytic and chromophobe renal tumors, and could potentially provide a therapeutic target for treatment of BHD renal cancer.[100]

Both BHD-associated and sporadic renal oncocytomas and chromophobe RCC are characterized by the presence of large numbers of mitochondria. [33,101] Sporadic renal oncocytomas are associated with somatic mutations in mitochondrial DNA (mtDNA) that

inactivate subunits of mitochondrial Complex I of the electron transport chain,[102] but no mtDNA sequencing data has been reported for BHD-associated renal oncocytomas. Klomp *et al.* performed gene expression microarray analysis of BHD-associated renal oncocytomas and chromophobe RCC and found clear genetic differences from sporadic counterpart tumors.[103] Specifically, BHD-associated tumors lacked the chromosomal abnormalities typically seen in sporadic renal oncocytoma (loss of chromosome 1 or chromosome 11q13 translocation) and chromophobe RCC (losses of chromosomes 1, 2, 6, 10 and 17). Importantly, however, although both BHD and sporadic counterpart tumors showed a prominent mitochondrial gene expression phenotype, BHD-associated tumors displayed a unique upregulation of two transcription factor genes involved in mitochondrial biogenesis, *PGC-1α /PPARGC1A* (peroxisome proliferator-activated receptor γ, coactivator 1 α) and *TFAM* (transcription factor A, mitochondrial); a clear inverse correlation between *FLCN* expression and a set of genes known to be transcriptionally activated by PGC-1α was also observed.[103] Hasumi *et al.* confirmed that loss of *FLCN* leads to upregulation of *PGC-1α / PPARGC1A* gene expression in BHD-associated kidney tumors, a BHD renal tumor cell line, and *Flcn*-deficient murine kidney, heart and muscle tissues.[104,105] Taken together these data suggest that a unique FLCN-PGC-1α-TFAM signaling axis exists in BHD-associated renal tumors that may be absent from the sporadic counterpart tumors and could be useful in the differential diagnosis of BHD-associated tumors from their sporadic counterparts.

Potential therapeutic options for BHD

The long term goal of the efforts that led to the identification of the gene for BHD (*FLCN*) is focused on elucidating the mechanistic details of the FLCN pathway to provide the foundation for the development of an effective form of therapy for patients with metastatic as well as localized BHD-associated kidney cancer, cutaneous fibrofolliculomas and pulmonary cysts. Although the development of advanced disease is uncommon in BHD, BHD-associated renal tumors are malignant and can metastasize, and there are currently no approved therapeutic options available. To date, the FDA has approved seven agents that target the VEGF and mTOR pathways (sorafenib, sunitinib, temsirolimus, bevacizumab, everolimus, pazopanib, and axitinib) for treatment of metastatic renal cell carcinoma. However, only a few cases of metastatic chromophobe RCC have been described in the literature, and experimental evidence for the use of VEGF and mTOR pathway inhibitors in this histologic subtype is lacking. Shuch *et al.* summarized four case reports of patients with sporadic metastatic chromophobe RCC who had progressed on VEGF inhibitors sorafenib or sunitinib, but continued to have stable disease 1 to 4 years after initiating mTOR pathway targeted therapy.[106] Everolimus provided a longer term effect than VEGF inhibitors against metastatic papillary RCC in a BHD patient reported by Nakamura *et al.*[107] Treatment with the mTOR inhibitor rapamycin resulted in partial regression of the early onset multicystic kidney phenotype of two preclinical *Flcn*-deficient animal models[73,74] although this model may or may not be an accurate representation of the mTOR status of BHD-associated solid tumors. Given the conflicting data concerning whether FLCN is a positive or negative regulator of mTOR, clinical trials will be needed to determine the efficacy of agents which inhibit the mTOR pathway in patients with BHD-associated renal cell carcinoma.

In addition to targeting the mTOR pathway, studies have uncovered other potential targets and therapeutic approaches for BHD-associated renal cancer that are being pursued. Using the COMPARE algorithm to analyze public data on FLCN expression and response of the NCI-60 cancer cell line panel to anticancer drugs, Lu *et al.* identified mithramycin as 10-fold more cytotoxic against the BHD patient renal tumor cell line UOK257 when compared to the *FLCN*-restored companion line.[108] *Flcn*-deficient cells treated with mithramycin were growth arrested in the S and G2-M phases of the cell cycle, and this effect was enhanced by low dose rapamycin. In a subsequent study by Lu *et al.*, a synthetic-lethal screen using a phosphatase siRNA library approach revealed that knockdown of Slingshot 2 (SSH2) serine phosphatase induced caspase3/7 activation and apoptotic death in *FLCN*-deficient human cell lines but not *FLCN*-restored counterpart lines.[109]

Work by Preston *et al.* has suggested another potential pathway for targeting in BHD. These researchers have shown high levels of hypoxia-inducible factor (HIF) activity and its transcriptionally upregulated genes in *FLCN*-deficient renal tumor cell lines under both hypoxia and normoxia, and re-expression of *FLCN* reversed these effects.[110] Evaluation of the metabolic profile of these cells revealed a shift under *FLCN* deficiency to aerobic glycolysis (the so-called Warburg effect) with a dependency on glucose. Findings in a follow up study by these researchers suggest that FLCN loss leads to activation of AMPK resulting in increased PGC1-α, upregulated mitochondrial biogenesis, and increased ROS production leading to elevated HIF transcriptional activity, thereby driving Warburg metabolic reprogramming.[111] In their earlier study, Preston *et al.* were able to inhibit growth of *FLCN*-deficient tumor cells by blocking glycolysis with 2-deoxyglucose,[110] suggesting that targeting the glycolytic pathway may be a potential therapeutic approach for treatment of BHD patients. The results reported in these studies support the need for follow-up research efforts to pursue these potential novel targets for BHD-associated renal cancer.

Conclusions

The clinical phenotype in BHD syndrome is now very well characterized, and the hybrid oncocytic tumors that develop in the setting of BHD are unique among hereditary renal cancer syndromes. In the decade or more since the cloning of the *FLCN* gene, we have obtained a broader understanding of the function of this tumor suppressor and how inactivation of FLCN drives the development of BHD-associated renal tumors, cutaneous fibrofolliculomas and pulmonary cysts. Interacting proteins FNIP1 and FNIP2 have been identified that link FLCN to mTOR through AMPK, and evidence supporting a role for FLCN in modulating the AKT-mTOR pathway is accumulating, although the question of whether FLCN is involved in mTOR activation or inhibition may be related to cell type or context. A role for FLCN in other pathways and cell processes known to be deregulated in cancer is supported by work from a number of research labs. Currently surgical intervention is the only therapy available to BHD patients. Development of effective targeted therapies to treat BHD-associated renal tumors will depend on a more detailed understanding of the primary pathways in which FLCN functions as a tumor suppressor, which when deregulated under FLCN deficiency may drive renal tumorigenesis.

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Box 1. Diagnostic criteria for Birt-Hogg-Dubé syndrome

These diagnostic criteria are suggestive for BHD syndrome:

- At least 2 cutaneous papules clinically consistent with fibrofolliculoma/trichodiscoma and at least 1 histologically confirmed fibrofolliculoma
- Multiple bilateral pulmonary cysts located mainly in the basilar regions of the lung with or without a history of spontaneous pneumothorax that develops prior to age 40, but especially with a family history of these pulmonary manifestations
- Bilateral, multifocal chromophobe renal carcinomas or hybrid oncocytic tumors especially with a family history of renal tumors or diagnosed at an age <50 years
- A combination of these cutaneous, pulmonary or renal manifestations presenting in the patient or members of his family

A diagnosis of BHD is confirmed:

- Diagnostic genetic test positive for a germline *FLCN* mutation

Key Points

- Birt-Hogg-Dubé (BHD) syndrome is an autosomal dominant inherited renal cancer disorder that predisposes at-risk individuals to benign, cutaneous fibrofolliculomas, pulmonary cysts, spontaneous pneumothoraces and increased risk for renal cell carcinoma
- Renal tumors that develop in the setting of BHD syndrome are most often bilateral, multifocal hybrid oncocytic tumors and chromophobe renal cell carcinomas, but may present with other histologies
- Germline mutations in the *FLCN* gene predicted to prematurely truncate the protein predispose to BHD syndrome; renal tumors show somatic inactivation/loss of the remaining *FLCN* allele confirming a tumor suppressor function
- FLCN interacts with the novel proteins, FNIP1 and FNIP2, as well as AMPK, a negative regulator of mTOR, and acts to modulate the AKT-mTOR pathway
- Other pathways in which FLCN may play a role include regulation of TFE3/TFEB transcriptional activity, amino acid-dependent mTOR activation through Rag GTPases, TGF-β signaling, PGC1α-driven mitochondrial biogenesis, and autophagy
- Surgical intervention is currently the only available therapy for BHD-associated renal tumors. Elucidation of FLCN-interacting pathways that are deregulated in *FLCN*-deficient renal cancer will hopefully enable the development of effective targeted therapies.

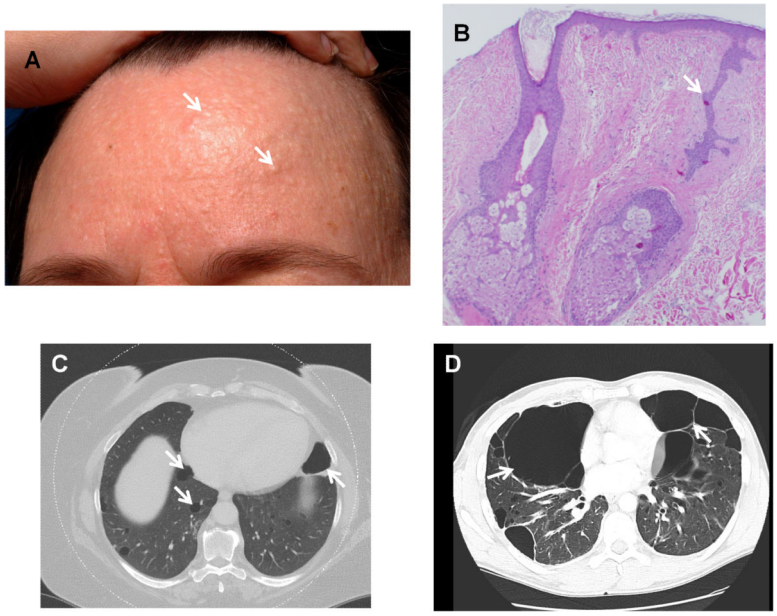

Figure 1. Cutaneous and pulmonary manifestations of Birt-Hogg-Dubé syndrome

(A) Multiple fibrofolliculomas on the face of a BHD patient (arrow). (B) H&E staining of a fibrofolliculoma showing strands of epithelial cells surrounded by fibrous stroma (arrow) with adjacent hair follicle (left). (4X). (C, D) Chest CT scans of BHD patients showing bilateral multiple pulmonary cysts of various sizes. (Panel A and B images are provided courtesy of Dr. Mary Eid and Dr. Edward Cowen, Dermatology Branch, National Cancer Institute, NIH).

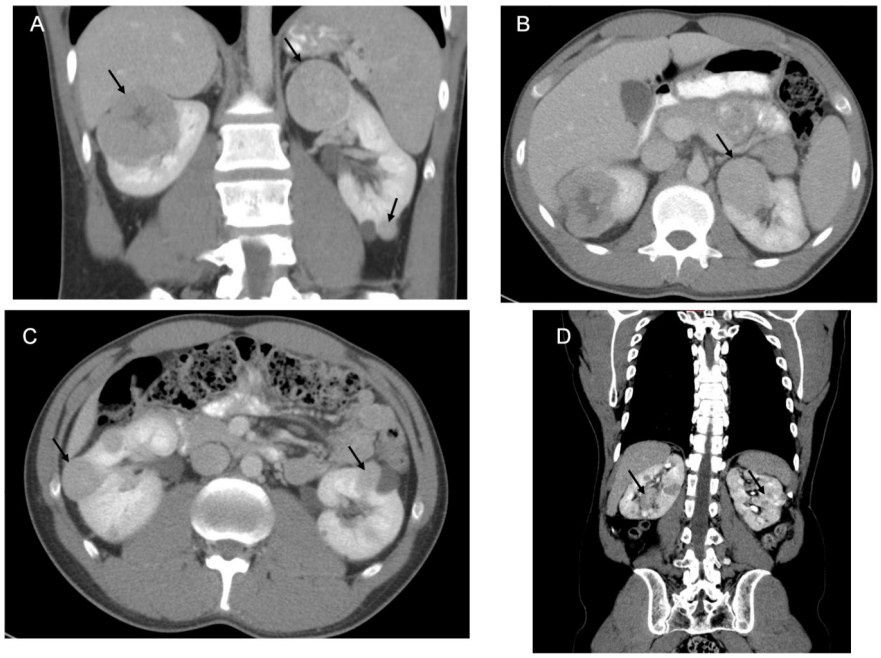

Figure 2. Renal manifestations of Birt-Hogg-Dubé syndrome

Abdominal coronal CT scans of BHD renal tumors (A,D) demonstrating bilateral multifocal tumors in BHD patients. Axial CT scans demonstrating multifocal bilateral tumor presentation (B,C).

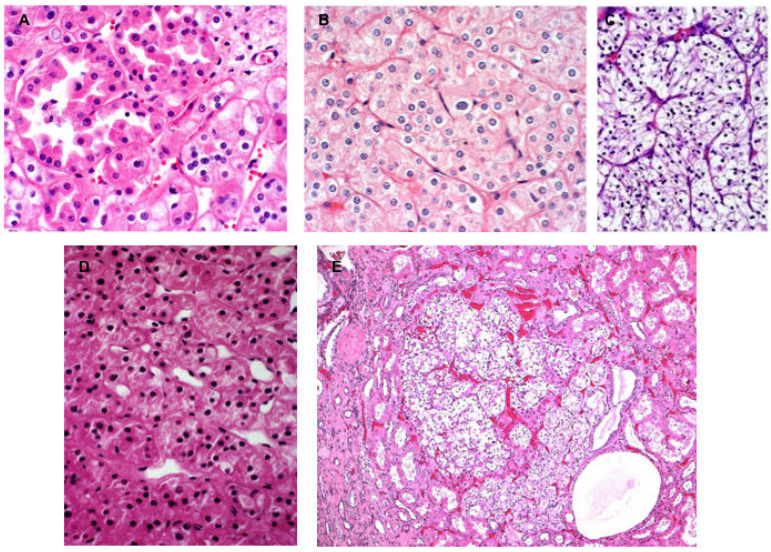

Figure 3. BHD renal tumor histology

A) Hybrid oncocytic tumor (150x). (B) Chromophobe renal tumor with characteristic perinuclear halos (150X). (C) Clear cell renal tumor. (D) Renal oncocytoma in a BHD patient (150x). (E) Renal oncocytosis in normal kidney parenchyma of a BHD patient (100X). (Images are provided courtesy of Dr. Maria J. Merino, Laboratory of Pathology, National Cancer Institute, NIH).

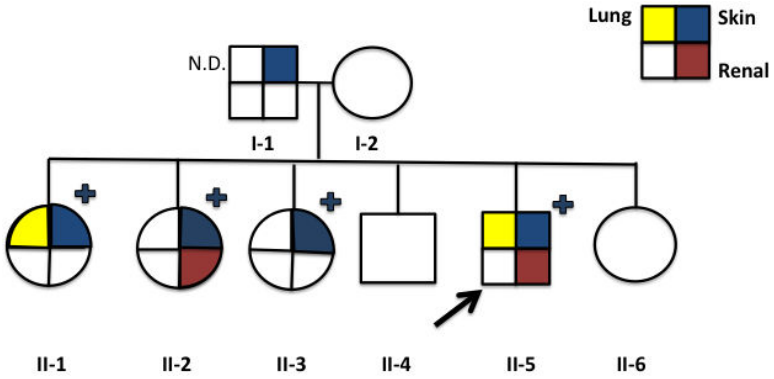

Figure 4. Phenotypic heterogeneity in a family affected with BHD syndrome

A BHD kindred in which affected family members who inherit the same germline mutation present with different phenotypes. Blue= fibrofolliculoma; red=renal tumor; yellow=pulmonary cysts/pneumothorax. Square, male; circle, female; arrow, proband; mutation carrier, +; N.D., not determined.

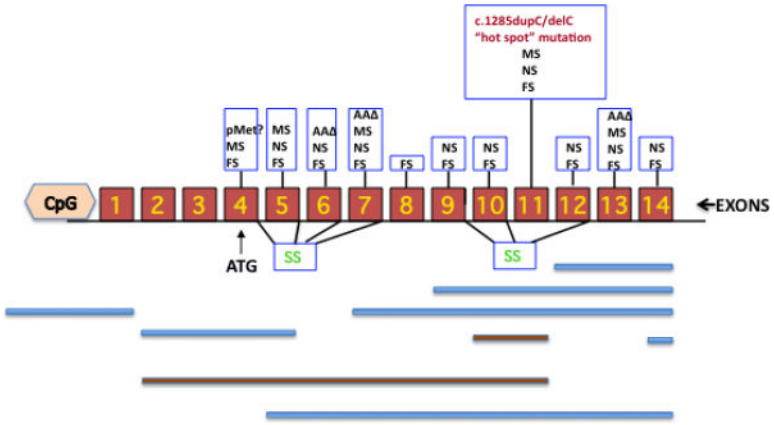

Figure 5. Germline *FLCN* mutations responsible for BHD syndrome

FLCN exon structure showing spectrum of mutation types and their location in all coding exons. FS, frameshift; MS, missense; NS, nonsense; AAΔ, amino acid deletion inframe; pMet1?, proposed deletion of initiator codon; SS, splice site. Blue bar, intragenic deletion; red bar, intragenic duplication. ATG, initiator codon. CpG, putative promoter region. References: sequence variant information from LOVD Gene Homepage for *FLCN*[46]; ex 5-14 del and ex12-14 del, Houweling *et al*.[29]; ex 1 del, ex 2-5 del, ex 7-14 del and ex 10-11 dup, Benhammou *et al*.[47]; ex 9-14 del and ex 14, Kunogi *et al*.[22]; ex 14 del, Sempau *et al*.[48]

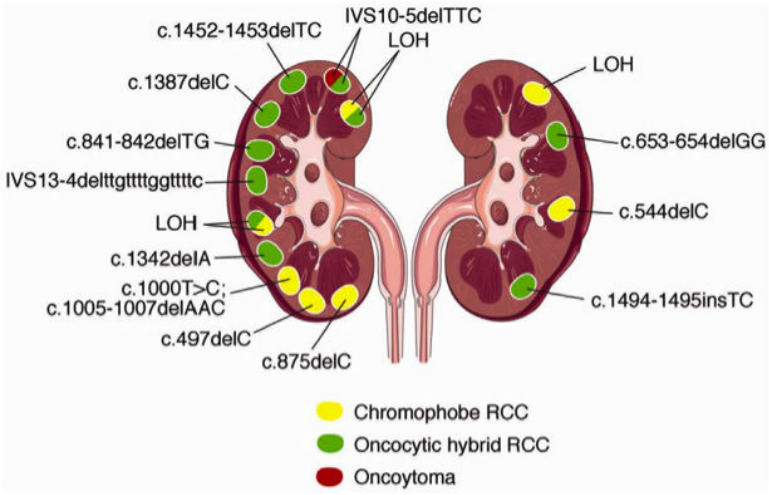

Figure 6. Somatic second hit *FLCN* mutations in multiple renal tumors from a BHD patient with a germline *FLCN* mutation

Somatic inactivation of the remaining wild-type *FLCN* allele by mutation or chromosomal loss drives renal tumorigenesis in BHD syndrome. Multiple tumors in a BHD patient's kidneys have different *FLCN* second hit mutations. Used with permission from Vocke *et al.*[52]

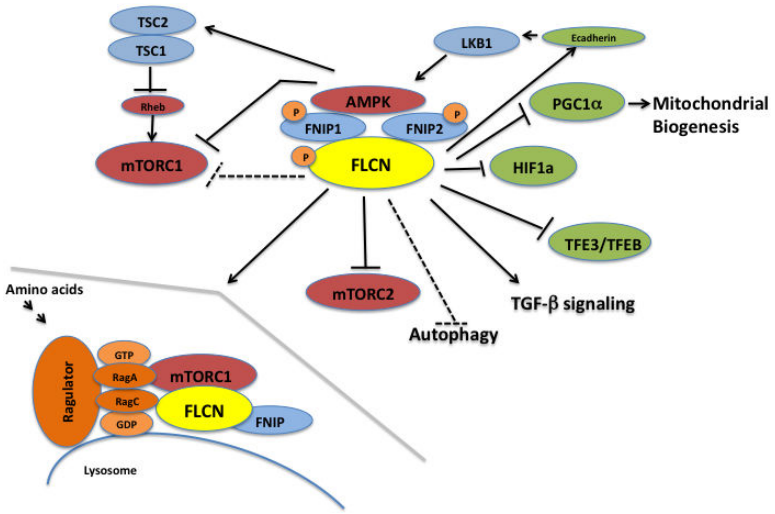

Figure 7. FLCN-associated pathways that may contribute to renal tumorigenesis under *FLCN* deficiency

Arrows indicate activation, T indicates inhibition; where both are indicated the data are conflicting or unclear.

Table 1

Metastatic renal carcinoma in BHD syndrome

FLCN Mutation	Age at Dx (y)	RCC Histology (# Tumors)	Outcome (Dead/Alive/Therapy)	Reference
p.His429Profs*27	35	Undifferentiated (1)	Nx @ 35 y D.@ 62 y, unk. cause	Benusiglio *et al.*[30]
p.Met222Aspfs*26	42	Hybrid (multiple)	3 yrs sys.therapy A., 8 yr post dx	Benusiglio *et al.*[30]
p.Val400Ileu	46	Chromophobe (1)	Sys. therapy A., 5 yr post surg.	Benusiglio *et al.*[30]
p.Lys508Arg	56	Chromophobe (1)	Nx, sys. therapy A., 7 yr post surg	Benusiglio *et al.*[30]
NA	NA	Clear cell (multiple)	Bilateral Nx D.<12mo post surg.	Pavlovich *et al.*[33]
NA	NA	Clear cell/Chr/Pap (1)	Nx, sys. therapy D. <20mo post surg	Pavlovich *et al.*[33]
p.His429Profs*27	39	Pap/clear cell (1)	Nx im. therapy D., 12 mo post surg	Houweling *et al.*[29]
p.Ala204X	56	Clear cell/Chr/Sarc (1)	Nx sys. therapy D., 12 mo post surg	Houweling *et al.*[29]
p.Val107Hisfs*26	51	Clear cell/Chr (1)	Nx sys. & rad. therapy D., 12 mo post surg	Houweling *et al.*[29]
p.Val107Hisfs*26	52	Clear cell (1)	Radiation therapy D. <12 mo post dx	Houweling *et al.*[29]
p.Val107Hisfs*26	43	Clear cell/Chr (1)	Nx, Mx D., 14 yr post surg	Houweling *et al.*[29]
p.His429Profs*27	55	Clear cell/Pap(1)	Nx, im. therapy D., 27mo post surg	Murakami *et al.*[112]
p.His429Profs*27	20	Clear cell (1)	Nx, parotid mets(?)/Mx A. 40 y post dx	Khoo *et al.*[31]
p.Trp511X	61	Papillary (1)	Nx A., currently	Furuya *et al.*[100]
p.His429Profs*27	56	Papillary (1)	Nx, sys. therapy D., 78 mo post surg	Furuya *et al.*[100]

NA, not available; Nx nephrectomy; Mx, metastatic surgery; Dx diagnosis; D, dead; A, alive; Pap, papillary; Sarc, sarcomatoid; Chr, chromophobe; sys., systemic; im., immuno; rad., radiation; surg, surgery; mo, months