HHS Public Access

Author manuscript

Expert Opin Orphan Drugs. Author manuscript; available in PMC 2015 November 16.

Published in final edited form as:

Expert Opin Orphan Drugs. 2015 ; 3(1): 15–29. doi:10.1517/21678707.2014.987124.

Clinical Features, Genetics and Potential Therapeutic Approaches for Birt-Hogg-Dubé Syndrome

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Abstract

Introduction—Birt-Hogg-Dubé (BHD) syndrome is an autosomal dominant disorder that predisposes to fibrofolliculomas, pulmonary cysts, spontaneous pneumothorax and renal neoplasia. BHD is characterized by germline mutations in tumor suppressor *FLCN*. Inactivation of the remaining *FLCN* allele in kidney cells drives tumorigenesis. Novel FLCN-interacting proteins, FNIP1 and FNIP2, were identified. Studies with *FLCN*-deficient *in vitro* and *in vivo* models support a role for FLCN in modulating AKT-mTOR signaling. Emerging evidence suggests that FLCN may interact in a number of pathways/processes. Identification of FLCN's major functional roles will provide the basis for developing targeted therapies for BHD patients.

Areas covered—This review covers BHD diagnostic criteria, clinical manifestations and genetics, as well as molecular consequences of *FLCN* inactivation. Recommended surveillance practices, patient management, and potential therapeutic options are discussed.

Expert opinion—In the decade since *FLCN* was identified as causative for BHD, we have gained a greater understanding of the clinical spectrum and genetics of this cancer syndrome. Recent studies have identified interactions between FLCN and a variety of signaling pathways and cellular processes, notably AKT-mTOR. Currently, surgical intervention is the only available therapy for BHD-associated renal tumors. Effective therapies will need to target primary pathways/processes deregulated in *FLCN*-deficient renal tumors and fibrofolliculomas.

Keywords

Birt-Hogg-Dubé syndrome; BHD; inherited renal cancer syndrome; tumor suppressor; FLCN; folliculin; fibrofolliculoma; chromophobe renal cancer; mTOR; rapamycin

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Declaration of interest

The authors declare no conflict of interest and have received no payment in preparation of this manuscript. This work was supported by the Intramural Research Program of the National Institutes of Health (NIH), National Cancer Institute (NCI), Center for Cancer Research. This project has been funded in part with Federal funds from the Frederick National Laboratory for Cancer Research, NIH, under Contract HHSN261200800001E (LSS). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsements by the US Government.

1. Introduction

Birt-Hogg-Dubé syndrome (BHD) is an autosomal dominant disorder in which affected individuals with germline mutations in the *folliculin* gene (*FLCN*) are at risk for developing benign hair follicle tumors (fibrofolliculomas), pulmonary cysts, spontaneous pneumothoraces and renal neoplasia. In 1977, three physicians for whom the disease was named, Arthur Birt, Georgina Hogg and James Dubé, described a large Canadian family with three generations of individuals who presented with tiny, smooth, flesh-colored papules on the face and neck called fibrofolliculomas (1). Trichodisomas (2) and acrochordons were also reported in the Canadian family and together with fibrofolliculomas represent the classic cutaneous features of BHD syndrome. In 1975 Hornstein and Knickenberg described perifollicular fibromas in a father and two children (3) that histologically and clinically resemble fibrofolliculomas, leading to the hypothesis that fibrofolliculomas and perifollicular fibromas may represent the same lesions. (4). Early reports followed by expanded studies documented the presence of lung cysts in BHD patients and a predisposition to develop spontaneous pneumothoraces (5–7). A single case of bilateral, multifocal chromophobe renal carcinoma in a BHD patient was reported by Roth and colleagues in 1993 (8) raising the possibility of an association between BHD syndrome and renal neoplasia. This was confirmed when cosegregation of fibrofolliculomas with papillary renal tumors and renal oncocytomas was identified in 7 members of 3 familial renal cancer families seen at the National Institutes of Health (7).

Over a decade has passed since the cloning of the gene responsible for BHD syndrome. Studies aimed at uncovering the function of the FLCN protein are underway in many laboratories and have identified a number of pathways in which FLCN may play a role. In this report we summarize the clinical presentation and genetics of BHD syndrome, discuss recent findings implicating FLCN in a number of cellular processes and/or pathways, present recommendations for clinical management of BHD patients, and discuss the potential for developing targeted therapies for BHD-associated renal cancer and cutaneous fibrofolliculomas.

2. Clinical Manifestations of BHD

2.1 Fibrofolliculomas, trichodiscomas and acrochordons

The most frequently observed clinical manifestation of BHD syndrome is the cutaneous fibrofolliculoma. The dome-shaped, pale yellow to white papules, 2–4 mm in diameter, generally occur on the face including ears and eyelids, neck and upper torso and usually develop after puberty (Figure 1A). These lesions are not painful or pruritic and can occur singly or coalesce in a plaque of more than 100 lesions. Fibrofolliculomas develop in 82–92% of BHD-affected individuals over 25 years of age (9–12). Histologically they consist of epithelial strands extending out from a central aberrant hair follicle into specialized fibrous tissue (Figure 1B). They are often accompanied by trichodiscomas, which may be a different stage of development of the same process that produces fibrofolliculomas (13–15), and acrochordons (common skin tags).

2.2 Pulmonary cysts and spontaneous pneumothorax

Lung manifestations that occur with high penetrance in BHD syndrome include multiple bilateral pulmonary cysts seen on high-resolution chest computed tomography scans (HRCT; Figure 1E) in 70–84% of affected members of BHD families (9, 10,12). The cysts are well circumscribed, irregularly shaped, lined by a smooth thin wall that does not enhance, vary in size and generally are located in the basilar and mediastinal regions of the lungs (16,17). They can form large bullae on the lung surface as seen by thoracoscopy (Figure 1D) and can present as the only manifestation in a BHD patient (18–21). Pulmonary function tests in BHD patients have generally shown normal lung function, mild nonspecific patterns or mild airway obstruction (16,22).

Affected members of a BHD family have a 50-fold greater risk (when adjusted for age) for developing spontaneous pneumothorax than their unaffected siblings (23), and the presence of lung cysts was found to be significantly associated with history of pneumothorax (17). About 30% of BHD affected individuals will develop spontaneous pneumothorax, most frequently before the age of 40 years (median age of onset, 38 years) (17) and as young as 7 years of age (9, 10, 12, 23, 24). The majority of patients report more than one episode of pneumothorax and some experience recurrent pneumothoraces requiring medical intervention.

2.3 Renal neoplasia

In a risk assessment study of a large BHD cohort, affected members of BHD families had a 7-fold increased risk for developing renal neoplasia compared to their unaffected siblings (23). Renal tumors develop at a median age of diagnosis of 48–52 years in 12–34% of BHD affected individuals (Figures 1C, 1D), although studies reporting the higher frequencies may reflect ascertainment bias (9, 10, 25). On the other hand, some families do not present with renal tumors, which underscores the phenotypic heterogeneity of BHD syndrome (12). Unlike other inherited renal cancer syndromes that present with a single histologic tumor subtype (i.e., von Hippel-Lindau disease, clear cell renal cancer; hereditary papillary renal carcinoma, papillary type I renal cancer), BHD patients develop bilateral multifocal renal tumors with a wide spectrum of histologies. Most frequently BHD patients develop hybrid oncocytic tumors with features of chromophobe renal cancer and renal oncocytoma (50%), but also present with chromophobe renal carcinoma (35%), clear cell renal carcinoma (9%) and renal oncocytoma (5%) (26, 27). Multiple tumors with different histologies may develop in a single kidney of a BHD patient. Additionally, tumors with different histologic subtypes may present in multiple affected members from the same BHD family. Microscopic foci of dysplastic cells defined as renal “oncocytosis” have been observed in adjacent “normal” kidney parenchyma in the majority of BHD patients who present with renal tumors independent of histology, suggesting that these oncocytic cells may be precursor lesions to hybrid oncocytic tumors as well as chromophobe and clear cell renal carcinomas (26). Although uncommon, renal tumors that develop in the context of BHD can metastasize (25).

2.4 Other malignancies reported in BHD

In addition to fibrofolliculomas, lung cysts, spontaneous pneumothorax and renal tumors, early case studies reported colon polyps and colorectal carcinoma in BHD patients (3, 5, 28,

29). A risk assessment study of BHD kindreds evaluated by colonoscopy at the National Cancer Institute, however, did not find a statistically significant increase in colon polyps or carcinoma in BHD-affected family members when compared to their unaffected siblings (23). On the other hand, one study of a large European BHD cohort reported that the risk for developing colonic neoplasia was 8% by age 60 and 20% by age 80, which was significantly greater than the risk in the general United Kingdom population of 0.8% by age 60 and 4.9% by age 80 (30). Furthermore, Khoo et al. (31) described a high risk of colorectal neoplasia in a large French BHD family. It is possible that environment, genetic alterations in other genes or specific *FLCN* genotypes may increase the risk for colonic neoplasia in an individual affected with BHD.

Parotid oncocytomas have been identified in a number of BHD patients (9, 10, 32, 33, 34), although it is unclear if they are a true phenotypic manifestation of BHD syndrome. Multiple lipomas (6,7, 9), oral papules (7, 12), and thyroid nodules (12) and cancer (10) have also been reported in association with BHD.

2.5 Clinical presentation and diagnostic criteria for BHD syndrome

BHD syndrome displays phenotypic heterogeneity in which individuals affected with BHD may develop cutaneous lesions only, cutaneous lesions and pulmonary manifestations, cutaneous lesions and renal tumors, or all three manifestations. Within a BHD family, affected members may each develop a different spectrum of manifestations. BHD-affected individuals in which lung cysts and history of spontaneous pneumothorax are the only manifestation (18–20), and BHD-affected individuals in which chromophobe renal tumors are the only manifestation (9) have been reported.

Because of phenotypic heterogeneity, diagnostic criteria suspicious for BHD syndrome should include the following: 1) 2 or more cutaneous papules clinically compatible with fibrofolliculoma/trichodiscoma with a minimum of at least one histologically proven fibrofolliculoma developing generally after puberty, 2) bilateral, multifocal basilar lung cysts with or without spontaneous pneumothorax that develops prior to 40 years of age, especially with a family history of these lung manifestations, 3) bilateral, multifocal chromophobe or hybrid oncocytic tumors especially with a family history of renal tumors, or 4) any combination of cutaneous, lung or renal manifestations known to be associated with BHD in an individual or his family (Table 1). The definitive diagnosis of BHD syndrome is a mutation-positive *FLCN* genetic test. At-risk BHD family members are recommended to undergo screening for renal tumors by abdominal magnetic resonance imaging with contrast or computed tomography starting at the age of 21 years.

3. Genetics of BHD Syndrome: *FLCN* Gene

3.1 Cloning of the gene responsible for BHD syndrome

The *FLCN* gene was discovered as a consequence of efforts to identify the genetic cause for familial renal oncocytoma (FRO) (35). Individuals from families in which multiple members developed bilateral, multifocal renal oncocytomas were also found to have cutaneous lesions that were histologically proven to be fibrofolliculomas. Genetic linkage analysis in these and additional families recruited on the basis of BHD skin lesions, followed by positional

cloning efforts, identified a novel gene, f*olliculin* (*FLCN*), located on the short arm of chromosome 17 (36–38) that was mutated in the germline of BHD-affected individuals.

3.2 FLCN mutations in BHD syndrome

In the decade or more since the *FLCN* gene was discovered, over 100 unique mutations have been reported in the LOVD *FLCN* mutation database (39, 40)Mutations have been identified in all 11 coding exons of the *FLCN* gene. The majority of *FLCN* mutations in BHD syndrome are insertions/deletions, nonsense or splice-site mutations that result in a shift in the reading frame and/or introduction of a premature termination codon (9, 10, 12, 25). The predicted consequence is loss of function of the FLCN protein. Partial *FLCN* gene deletions have been identified in the germline of BHD patients (25, 41, 42) that also are expected to result in FLCN inactivation. With the advent of multiplex ligation-dependent probe amplification (MLPA) technology for detecting gene deletions, and highly accurate sequencing methods, the *FLCN* mutation detection rate is high, ranging from 81–89% (9, 10, 25).

Notably, a small number of missense mutations have been reported in the LOVD *FLCN* mutation database (40). The functional significance of these mutations is unclear since they simply exchange one amino acid for another; however, Nahorski and colleagues showed that His255Pro and Arg239Cys mutations, but not Lys508Arg or Val400Ile mutations, reduced the stability of the FLCN protein (43). Consistent with these data, *in silico* analysis of these mutations predicted that His255Pro and Arg239Cys, but not the other missense variants, were probably pathogenic (43).

Insertion or deletion of a cytosine in a tract of 8 cytosines in exon 11 (c.1285dupC or delC) is a mutational “hot spot” in the *FLCN* gene that was identified in up to 50% of BHD patients in a number of large cohort studies (9, 10, 11, 30, 37, 38). Where evaluated, absence of a shared haplotype ruled against a common ancestor.

3.3 Genotype-phenotype correlations in BHD syndrome

To date, no clear genotype-phenotype associations have been reported in BHD syndrome. *FLCN* mutations occur throughout the length of the gene in all coding exons. No correlation between location of a mutation within the gene or type of mutation (frameshift, nonsense, missense or deletion) and the presence of one or another of the cutaneous, lung or kidney manifestations has been identified (9, 10, 17), although in a Japanese BHD cohort in which pulmonary manifestations were the presenting feature, mutations were most frequent in the 3′ end of the *FLCN* gene (41). Interestingly, Schmidt and colleagues (9) found that patients with the cytosine deletion in the C8 tract in exon 11 of the *FLCN* gene had fewer renal tumors than those with the cytosine insertion (1/26 vs. 13/56). No differences in frequency of fibrofolliculomas, lung cysts or spontaneous pneumothorax were seen between these two C8 tract mutation groups. They also noted an increased frequency of renal tumors in two unrelated families with a splice-donor mutation in intron 9 [c.1062+2T>G (previously known as IVS9+2T>G]. Toro et al. (17) observed a trend toward experiencing more pneumothoraces in BHD patients with *FLCN* mutations in exons 9 and 12, and a statistically significant association between increased numbers of lung cysts and *FLCN* exon 9

mutations. Finally, Nahorski and colleagues (30) found a significantly higher risk of colorectal neoplasia in a group of c.1285dupC mutation carriers (χ^2=5.78; p=0.016) when compared to a group of c.610delGCinsTA mutation carriers, in contrast to the negative association of colorectal neoplasia with BHD in the risk assessment of Zbar et al. (23). Confirmation of these potential genotype-phenotype associations awaits validation in additional large BHD cohorts.

3.4 FLCN is infrequently mutated in sporadic counterpart renal tumors

Unlike the *VHL* gene, which is mutated in both the familial clear cell renal cancer syndrome VHL disease and the majority of sporadic clear cell renal cancers, *FLCN* mutations occur infrequently in sporadic chromophobe renal tumors and renal oncocytomas. Gad and colleagues found *FLCN* mutations in 5 of 46 chromophobe renal carcinomas of which 3 were most likely germline, and in 1 of 18 renal oncocytomas (44). In another study, Khoo et al. looked at 39 sporadic renal tumors including renal oncocytoma, chromophobe, papillary and clear cell renal carcinoma for *FLCN* mutations, and found only one frameshift mutation in a papillary renal tumor (without matched normal); however, loss of heterozygosity (LOH) on chromosome 17p and partial promoter region methylation was detected in 36% and 28% of the samples, respectively (45). In contrast to that study, da Silva and colleagues reported no epigenetic silencing of *FLCN* in 30 sporadic renal tumors and cell lines and identified only two *FLCN* missense substitutions, one in a clear cell tumor (but without matched normal) and another in a renal tumor cell line (46). Nagy et al. did not find *FLCN* mutations in 16 sporadic renal oncocytomas and chromophobe renal tumors, but did detect LOH on chromosome 17p in the chromophobe tumors (47). Finally, no *FLCN* mutations were found in 66 sporadic chromophobe renal tumors subjected to whole exome sequencing as part of the Cancer Genome Atlas (TCGA) project (48).

3.5 FLCN is a tumor suppressor gene

Conclusive experimental evidence has been presented to support a tumor suppressor function for *FLCN*. The majority of *FLCN* mutations identified in BHD families are predicted to truncate the protein, indicating that loss of FLCN function is responsible for BHD syndrome. The first report of a somatic *FLCN* “second hit” mutation in a chromophobe renal tumor from a BHD patient with a germline *FLCN* mutation was described by Khoo et al. (31). Subsequently, Vocke et al. (49) performed a large study of 77 renal tumors from BHD patients with germline *FLCN* mutations in which they investigated loss of the wild-type copy of the *FLCN* gene. Sequence alterations, most of which were predicted to truncate the FLCN protein, were identified in 53% of the samples and LOH on chromosome 17p was detected in 17% of the tumors tested (Figure 2A). Moreover, immunocompromised mice developed tumors when injected with a *FLCN*-null renal tumor cell line established from a BHD patient tumor (50,51) or with other *FLCN*-deficient cell lines (52), but tumorigenic potential was lost when the cell lines were restored with wild-type *FLCN* (Figure 2B; 51), further confirming that FLCN functions as a tumor suppressor.

Notably, although both copies of *FLCN* must be inactivated for renal tumor development, van Steensel et al. sequenced DNA from microdissected fibrofolliculoma tissue and found retention of the wild type *FLCN* sequence (53). Similarly Koga et al. showed FLCN-positive

immunostaining in pneumocytes lining BHD-associated lung cysts (54), suggesting that haploinsufficiency is sufficient for the formation of the BHD cutaneous and pulmonary lesions.

4. Molecular Consequences of FLCN Inactivation in BHD

4.1 FLCN and its binding partners FNIP1 and FNIP2 interact with the AMPK-mTOR pathway

No clues to FLCN function were provided by its protein sequence. Although it is highly conserved across species, FLCN is a novel protein with no significant homology to known proteins and without any classic domains to suggest its function (38). Co-immunoprecipitation experiments in cells overexpressing FLCN identified a novel FLCN interacting protein, FNIP1, that associates with FLCN through its C-terminus (55) and also interacts with 5′-AMP-activated protein kinase (AMPK), a kinase-competent complex that functions to monitor and maintain energy homeostasis in cells by negatively regulating mechanistic target of rapamycin (mTOR), the master controller of protein synthesis and cell growth (56). Baba et al. showed that FLCN exists in multiple phosphorylated forms, which are dephosphorylated by different mechanisms (inhibition of mTOR signaling and inhibition of AMPK activity), suggesting the existence of phosphorylated FLCN residues that function to either inhibit or activate FLCN function, respectively (55). FLCN phosphorylation sites at serine 62 and serine 302 were identified (57, 58), which appear to be differently regulated by the mTORC1-dependent pathway. FNIP1 expression enhances FLCN phosphorylation and both proteins can serve as direct or indirect substrates of AMPK (55, 57).

Bioinformatics searches uncovered a second novel FLCN-interacting protein FNIP2 [also known as FNIPL (59) or MAPO1(60)] with 49% homology to FNIP1 and similar characteristics (interacts through FLCN C-terminus and binds AMPK) (61). Hasumi et al. showed that FLCN, FNIP1 and FNIP2 could exist in homo-or hetero-multimeric complexes suggesting FNIP1 and FNIP2 may function independently or coordinately with FLCN. Differences in *FNIP1* and *FNIP2* mRNA expression from tissue to tissue suggest potential redundancy in some tissues but functional specificity of FNIP1 or FNIP2 in other tissues (61). Lim et al. identified a role for the MAPO1(FNIP2)/FLCN complex in controlling induction of apoptosis in response to O^6-methylguanine through activation of AMPK. O^6-methylguanine is produced in cells as a consequence of exposure to alkylating agents and can contribute to mutation and cancer (60). Based on mouse models of *Fnip1* inactivation by two independent groups, homozygous inactivation of *Fnip1* was not embryonic lethal and kidneys in these mice were apparently normal, further supporting a potential Fnip1/Fnip2 redundancy during embryogenesis and major organ development including kidney (62, 63). Interestingly, both *Fnip1*−/− mouse models developed defects in immune cell function and development, specifically a block in B cell development with enhanced apoptosis of B cell precursors, suggesting a role for Fnip1 in these developmental pathways.

4.2 Functional studies in Flcn conditional knockout mouse models suggest a role for Flcn in modulating the mTOR pathway that may be context-dependent

Several lines of evidence from *Flcn*-deficient mouse models suggest that Flcn may modulate the AKT-mTOR pathway. Conditional inactivation of *Flcn* specifically in mouse kidneys

resulted in the development of enlarged multicystic kidneys with hyperplastic cells protruding into the cystic lumen (64), and cystic renal cell carcinomas (65) that resulted in morbidity by 21 days of age due to renal failure. *Flcn*-deficient kidneys displayed activated mTOR pathway downstream effectors. Rapamycin treatment extended the life span and reduced the cystic kidney phenotype confirming that the AKT-mTOR pathway was activated in the *Flcn* knockout kidneys. In support of these data, *Flcn* heterozygous knockout mice developed solid renal tumors at median age of 23 months that displayed loss of the remaining *Flcn* allele and histologies similar to human BHD tumors. These tumors demonstrated activated phospho-AKT, phospho-mTORC1 and phospho-mTORC2 (66). Human BHD tumors also showed activated AKT-mTORC1 and AKT-mTORC2 pathways supporting a role for FLCN in suppressing AKT-mTORC1-mTORC2 signaling in human kidney. In another study, N-ethyl-N-nitrosourea (ENU) mutagenesis of *Flcn* heterozygous-null mice shortened the tumor latency with oncocytic cysts and occasional renal tumors developing at 3–5 months of age (67). In contrast to the previous data in *in vivo* mouse models, these lesions showed reduced phospho-S6 activity (a read out of mTOR activity) that is in agreement with their *in vitro FLCN*-knockdown cell line results and yeast homolog *bhd* mutant data (68). A third group reported mTOR activation (elevated phospho-S6) in large renal cysts that developed in a *Flcn* heterozygous mouse model by 6 months of age, but conversely, they saw reduced phospho-S6 in small renal cysts within the same *Flcn* knockout *in vivo* model (52). Based on these conflicting results in different *in vitro* and *in vivo* systems, it has been hypothesized that activation or inhibition of the mTOR pathway under *FLCN* deficiency is complex, most likely context dependent, and may involve additional genetic events in other pathways (Figure 3).

4.3 FLCN may play a tumor suppressor role in a number of cellular pathways in addition to the AKT-mTOR pathway

Since the discovery of *FLCN* as the gene responsible for BHD syndrome, there has been an intense research effort in multiple laboratories to elucidate the mechanism by which FLCN controls cell growth and to identify the biological pathways in which FLCN interacts. A number of recent reports have presented data that support a role for FLCN in regulating growth through signaling pathways other than the AKT-mTOR axis that are often dysregulated in cancer (Figure 3).

4.3.1 TGFβ/BMP signaling—Deregulation of TGFβ signaling has been demonstrated in a number of cancers including pancreatic, gastrointestinal and colorectal cancers. Using gene expression microarrays, Hong and colleagues (51) evaluated genes that were differentially expressed in *FLCN*-deficient, *FLCN*-mutant, and *FLCN*-restored renal tumor cell lines and found that genes known to signal through and activate TGFβ signaling including *TGFB2*, *INHBA*, *THBS1* and *SMAD3*, were downregulated in *FLCN*-deficient and *FLCN*-mutant but not *FLCN*-restored cells. These findings were confirmed in BHD-associated renal tumors relative to normal kidney from the same patients. *GREM1*, an antagonist of BMP that signals through SMADs was upregulated in *FLCN*-mutant and -deficient but not in *FLCN*-restored renal tumor cells. Cash and colleagues investigated *FLCN*-null mouse embryonic stem cell lines and found defects in cell-intrinsic apoptosis caused by decreased pro-apoptotic Bim transcription. They confirmed these results in BHD renal tumors, and showed

that *FLCN* deficiency was associated with loss of TGFβ–dependent chromatin modifications at target gene promoters leading to altered transcription of its targets including Bim (69). These studies suggest a functional role for FLCN in activating the TGFβ signaling pathway.

4.3.2 PGC-1α-driven mitochondrial biogenesis—In an effort to identify FLCN-interactive pathways, Klomp and colleagues performed gene expression microarray analyses to look for genes differentially expressed in BHD renal tumors compared with sporadic chromophobe renal tumors and renal oncocytomas. They identified a distinct gene expression pattern in BHD tumors not seen in the sporadic counterpart tumors, which included high expression of mitochondrial genes involved in the deregulation of the peroxisome proliferator-activated receptor-γ-coactivator 1α(PPARGC1A/PGC-1α)-transcriptional factor A, mitochondrial (TFAM) axis (70). In support of these findings, Hasumi et al. found that *Flcn*-deficient muscle from a muscle-targeted conditional *Flcn* knockout mouse model underwent a metabolic shift toward oxidative phosphorylation with increased expression of genes involved in mitochondrial biogenesis that was PGC-1α-dependent (71). Genetic inactivation of *Ppargc1a/Pgc-1α* in the *Flcn*-deficient muscle partially rescued the phenotype. PPARGC1A-dependent activation of mitochondrial biogenesis was also seen in a *FLCN*-null renal tumor cell line from a BHD patient. Ppargc1a deregulation leading to increased mitochondrial metabolism was recapitulated in heart tissue from a cardiac-specific *Flcn* knockout mouse model that developed cardiac hypertrophy (72). These independent studies support a role for FLCN in regulating mitochondrial biogenesis through PPARGC1A/PGC-1α.

4.3.3 TFE3/TFEB transcriptional regulation—Hong et al. was the first to demonstrate that FLCN controlled the cellular localization of the basic-helix-loop-helix transcription factor TFE3, a member of the microphthalmia transcription (MiT) family of transcription factors that include transcription factor EB (TFEB) and microphthalmia transcription factor (MiTF). *FLCN* inactivation in human and mouse renal tumors, human renal cell lines and mouse embryo fibroblasts was correlated with nuclear localization of TFE3 associated with decreased TFE3 phosphorylation, and increased TFE3 transcriptional activity as measured by increased mRNA and protein expression of the TFE3 target gene *GPNMB* (73).

Petit et al. has demonstrated a role for FLCN in regulating lysosome function through control of nuclear localization of the MiT family member TFEB (74). They previously showed that nuclear localization of TFEB (and other MiT family members) was controlled by mTORC1-dependent phosphorylation of TFEB at serine 211 triggering its interaction with 14-3-3 proteins, which prevented nuclear accumulation when the lysosome was functioning efficiently (75). In their subsequent study, they demonstrated that FLCN, facilitated by its protein partner FNIP1, localized to the lysosome under starvation conditions and that, in response to amino acid stimulation, the FLCN/FNIP1 complex was required for recruitment of mTOR to the lysosome where it is activated (74) (see below). They further showed that mTOR-dependent TFEB phosphorylation at serine 211 was inhibited in *FLCN* knockdown cells.

Further support for FLCN control of TFE3 transcriptional activity has come from a study by Betschinger et al. who performed a functional screen of mouse embryonic stem cells (ESC)

to identify genes required for exit from ground-state pluripotency to cell lineage differentiation. They discovered that Flcn in complex with both binding partners Fnip1 and Fnip2 drives differentiation of ESC by sequestering Tfe3 in the cytoplasm and thereby reducing transcriptional activation of the Tfe3 target, *Esrrb*, an orphan nuclear receptor and core pluripotency factor (76).

4.3.4 GAP/GEF toward Rag GTPases for amino acid-dependent activation of mTOR—Amino acid sensing by mTOR at the lysosome surface is a complex process involving Rag GTPases that function as heterodimers made up of one from each redundant pair: RagA/B and RagC/D. These Rags are part of a multimeric protein complex that includes Ragulator, GATOR1, GATOR2 and vacuolar (v)-ATPase. The bound nucleotide state of the Rags determines whether they will recruit mTOR to the lysozyme surface. RagA/B binds GDP during amino acid starvation and is exchanged for GTP by the guanine nucleotide exchange factor (GEF) activity of the Ragulator complex when amino acids are restored (reviewed in reference 77). The functional significance of and positive/negative regulators for RagC/D were unknown until recently. Tsun and colleagues have proposed a role for FLCN along with its partner FNIP2 (and to a lesser extent FNIP1) as a RagC/D GTPase activating protein (GAP) resulting in GDP loading of RagC/D to facilitate recruitment of mTOR to the lysosome for amino acid-dependent activation (78). Petit et al. also presented evidence that FLCN with interacting partner FNIP1 was required for amino acid-stimulated recruitment of mTORC1 to lysosomes by the Rags; however, their data support FLCN binding to and serving as a GEF for Rag A (not Rag C) through its GTPase domain (74). Interestingly, the crystal structure of C-terminal FLCN was recently solved at 2 Å resolution and found to be distantly related to differentially expressed in normal cells and neoplasia (DENN) domain proteins, a family of Rab-guanine exchange factors (GEFs) (79). In fact, Nookala et al. showed that FLCN had GEF activity against Rab35 *in vitro.* Taken together, these studies support a functional role for the FLCN/FNIP1/2 complex as a regulator of Rags to facilitate amino acid-dependent recruitment of mTORC1 to lysosomes.

4.3.5 Regulation of cell-cell adhesions, cell polarity and Rho A signaling—Two reports have described results from yeast two hybrid screens that identified an interaction between the FLCN protein and p0071 (plakophilin-4), an armadillo repeat containing protein that localizes to the cytoplasm and to adherens junctions, which are important for maintenance of cell architecture in epithelial tissues (80, 81). Loss of FLCN resulted in increased cell-cell adhesions and loss of cell polarity suggesting that FLCN in complex with p0071 is a negative regulator of cell-cell adhesion. Rho A signaling, a function of p0071, was deregulated in *FLCN*- deficient cells although these studies conflict on whether FLCN loss leads to up- or down-regulated Rho A activity. Khabibullin et al. observed increased cell-cell adhesions in *FLCN*-deficient human lung cell lines *in vitro* (82) supporting the previous studies reported by this group (80).

4.3.6 Regulation of the E-cadherin-LKB1-AMPK axis—The mechanism of BHD lung pathogenesis is currently under study. It is well established that E-cadherin regulates localization of LKB1 to epithelial cell junctions, where it controls maturation of apical junctions in bronchial epithelial cells through phosphorylation and activation of AMPK.

FLCN interacts with AMPK through its interacting proteins FNIP1 and FNIP2 (55,61) suggesting a role for FLCN in AMPK regulation in lung. Lung-targeted *Flcn* inactivation in a mouse model developed by Goncharova and colleagues (83) resulted in increased pulmonary alveoli, increased alveolar epithelial cell apoptosis and impaired lung function. They reported loss of E-cadherin membrane localization and downregulation of LKB1, leading to impaired AMPK activation in *Flcn*-deficient lung. Supporting data were obtained in *FLCN*-deficient mouse lung and human renal cancer cells. This work supports a role for FLCN in regulating the E-cadherin-LKB1-AMPK axis and presents a novel model to explain lung cyst formation in BHD, which might provide the basis for new therapeutic approaches for treating BHD cystic lung disease.

4.3.7 Additional pathways in which FLCN interacts—Recent reports have presented new evidence that FLCN may interact in pathways that modulate unique cellular processes. They include ciliogenesis (84) and autophagy. Possik and colleagues report a role for FLCN in the negative regulation of autophagy through an AMPK-dependent mechanism using a *Caenorhabditis elegans* model and mouse embryonic fibroblasts (85). Conversely, Dunlop et al. demonstrated positive regulation of autophagy by FLCN through its interaction with the autophagosome maturation effector GABARAP (GABA(A) receptor-associated protein) in complex with FNIP1/2, modulated by ULK1 (unc-51 like autophagy activating kinase 1)-dependent phosphorylation of FLCN, which they established in a human kidney cell line model (86). As more and more functions for FLCN are uncovered by new research efforts, it appears that FLCN may play a broader, more fundamental role in the regulation of cellular homeostasis.

5. Clinical Management of BHD Syndrome

5.1 Fibrofolliculomas

While the classic cutaneous lesions of BHD, fibrofolliculomas, are not painful or pruritic, patients may seek treatment for removal of the lesions for cosmetic reasons. Surgical intervention employing hyfrecation (electrocoagulation) is the only option currently available, which may be followed by curettage if necessary (87). Resurfacing with ablative lasers (erbium:YAG or CO2) has also been used as a treatment option for BHD although regrowth occurred after 6 months (88). While the pathogenesis of fibrofolliculomas is unknown, Vernooij et al. suggested that these BHD lesions, similar to “mantleomas”, may be derived from sebaceous glands whose growth has been shown to respond to retinoic acid derivatives, presenting an as yet untested therapeutic option (89).

5.2 Pulmonary manifestations

Once the diagnosis of BHD syndrome has been made, computed tomography (CT) imaging of the chest is recommended to identify the presence of pulmonary cysts and/or evidence of pneumothoraces. Periodic chest imaging is not routinely recommended in asymptomatic patients without history of pneumothoraces. BHD patients who develop pneumothoraces are treated by a variety of approaches depending upon the severity of their symptoms including simple observation, or tube thoracostomy to evacuate air from the pleural space and reinflate the compressed lung, either alone or with chemical pleurodesis. When lung bullae or blebs

are present or if pneumothoraces are recurrent, surgical intervention (i.e., thoracotomy) with mechanical pleurodesis and resection of bullae may be recommended (17, 90). Patients should inform health care providers of their history of pneumothorax to avoid problems during medical procedures that require anesthesia (91). The World Recreational Scuba Training Council recommends that history of spontaneous pneumothorax should be a contraindication to scuba diving even if the individual has undergone surgical procedures such as pleurodesis (92).

5.3 Renal tumors

It is recommended that at-risk BHD family members undergo baseline surveillance by abdominal magnetic resonance imaging (MRI) with intravenous contrast or computed tomography (CT). The use of ultrasonography is not recommended for routine kidney screening; however, ultrasonography may be helpful for surgical planning (93). Screening is recommended starting at 21 years of age at a frequency of every 36 months in patients with no renal mass lesions. MRI is often recommended to reduce radiation exposure. If renal tumors are detected, annual or more frequent imaging is recommended, depending on the size, location and growth rates of the tumors. Surgical intervention is recommended when the largest renal tumor reaches 3 cm in diameter (27, 93).

Nephron-sparing surgical management (i.e., partial nephrectomy with or without robotic assistance) is recommended for preserving maximum renal function, as at-risk BHD patients may develop multiple bilateral tumors during their lifetime and may require multiple surgeries. During a surgical procedure, removal of all detectable renal lesions is undertaken and can be facilitated by intraoperative renal ultrasound to detect small endophytic tumors (27). Regional lymph nodes may be removed if tumors are greater than 4 cm or if a pre-operative biopsy indicates a high grade clear cell histology. In healthy patients able to undergo surgery, ablative procedures (i.e., cryotherapy or radiofrequency ablation) are not recommended because of the frequency of multifocal tumors in BHD that are more completely removed by surgical procedures. Ablative therapy can also potentially interfere with success of subsequent surgeries and complicate the interpretation of imaging (93). The majority of BHD patients have excellent prognosis when managed by nephron-sparing surgery of tumors that reach 3 cm because BHD-associated renal tumors tend to be indolent. However, metastasis of BHD renal tumors does occur leading to patient mortality (27, 94, 95), so proper management of BHD tumors is crucial.

6. Potential Targeted Therapies for BHD Syndrome

Currently there are no approved therapeutic options for BHD-associated renal tumors other than surgical intervention. While some studies have shown that loss of FLCN function leads to mTOR activation, mTOR inactivation under *FLCN* deficiency has also been reported. Rapamycin treatment has been shown to partially reverse the cystic kidney phenotype in the kidney-targeted *Flcn* knockout mouse models (64, 65). Nakamura, et al. reported the use of everolimus as a second line systemic treatment of a BHD patient with metastatic papillary renal cancer. (94).

Rapalogs have also been tested against fibrofolliculomas. A double-blind randomized controlled trial of topical rapamycin (0.1% vs placebo) was performed in 19 BHD patients over a 6 month period (96). No clear change in the appearance, size or number of fibrofolliculomas was seen between rapamycin and placebo treatment. Side effects including burning, itching and dryness were reported in patients treated with rapamycin. The development of an effective therapeutic agent to treat the cutaneous lesions will require clarification of the dominant deregulated pathway driving development of BHD manifestations during FLCN deficiency.

Several *in vitro* studies have suggested potential therapeutic approaches to treat BHD-associated kidney cancer other than targeting AKT-mTOR signaling. A recent report by Preston and colleagues demonstrated that *FLCN*-null renal tumor cells have increased hypoxia-inducible factor (HIF) transcriptional activity and expression of HIF target genes, which activate glycolytic metabolism and a greater dependency upon glucose (so called "Warburg effect") (97). Results from a subsequent study by this group support a mechanism by which FLCN loss activates its binding partner AMPK resulting in increased PGC1-α, upregulated mitochondrial biogenesis, and increased ROS production leading to elevated HIF transcriptional activity, thereby driving Warburg metabolic reprogramming (98). Preston et al. treated *FLCN*-deficient tumor cells with 2-deoxyglucose, which blocked glycolysis and inhibited growth of *FLCN*-null cells (97), suggesting a therapeutic approach for treatment of BHD patients that targets the glycolytic pathway (Table 2).

In a study to identify candidate anticancer drugs that inhibit growth of NCI 60 cancer cell lines with low, but not high, *FLCN* expression, Lu and colleagues found that mithramycin produced a selective growth inhibition of *FLCN*-null renal cancer cells when compared with *FLCN*-restored cells, which was potentiated by low dose rapamycin. Their findings support further evaluation of this agent as a potential therapeutic option for BHD (Table 2; 99). Subsequently, the same group performed a phosphatase siRNA library screen for synthetic lethal targets in isogenic *FLCN*-null cell line pairs, and identified Slingshot 2 (SSH2) as a candidate synthetic lethal target for *FLCN*-null cells, which leaves open the possibility of targeting SSH2 phosphatase activity with small molecules as a strategy for targeted therapy in BHD (100). Clinical trials will be necessary to determine the efficacy of novel agents for BHD-associated kidney cancer.

7. Conclusions

Germline mutations in a novel tumor suppressor gene, *FLCN*, are responsible for Birt-Hogg-Dubé syndrome, which predisposes affected individuals to fibrofolliculomas, lung cysts, spontaneous pneumothorax and kidney cancer that is most frequently chromophobe or hybrid oncocytic histology. The majority of *FLCN* mutations are predicted to truncate the FLCN protein, and loss or mutational inactivation of the remaining *FLCN* allele is found in BHD-associated renal tumors, underscoring a classic tumor suppressor role for FLCN. The initial studies to elucidate FLCN function identified FLCN interacting partners FNIP1 and FNIP2 that in turn interact with 5′AMPK, and results of research using *in vitro* and *in vivo* *FLCN*-deficient models suggested that FLCN may modulate AKT-mTOR signaling in a context-dependent manner. However, numerous reports published over the past few years

have supported a potential role for FLCN in a wide variety of cellular processes including TGFβ signaling, TFE3/B transcriptional control, PGC-1α-dependent mitochondrial biogenesis, vesicular trafficking, cell-cell adhesion, cell polarity, ciliogenesis, autophagy, and HIFα-driven Warburg metabolic reprogramming. The major challenge of BHD researchers and clinicians treating BHD patients in the future will be to determine which deregulated process/pathway is the primary event leading to BHD renal tumorigenesis and identify molecular targets for design of effective therapeutic agents to treat BHD-associated kidney cancer and other BHD manifestations.

8. Expert Opinion

Over the decade or more since the causative gene for Birt-Hogg-Dubé syndrome was identified, our knowledge regarding the clinical spectrum of BHD has expanded and we now have a much more detailed understanding of the genetics of this rare autosomal dominant disorder. The evidence is irrefutable that *FLCN* is a tumor suppressor gene and that loss of *FLCN* expression leads to BHD-associated renal tumorigenesis in keeping with the two-hit Knudson model of tumor suppression.(101) However, studies to elucidate the functional consequences of FLCN inactivation by comparing *FLCN*-deficient and *FLCN*-restored *in vitro* and *in vivo* models have uncovered a surprising diversity of potential pathways in which FLCN appears to play a role and it is not yet clear which single pathway or interconnected signaling network, when deregulated, is responsible for the cutaneous, pulmonary and renal manifestations associated with BHD. Future aims for BHD research should focus on determining which major signaling axis is deregulated by FLCN loss in order to identify molecular targets and design therapeutic agents for treatment of BHD-associated manifestations.

The earliest FLCN functional studies pointed to a role in modulating the mTOR pathway based on the interaction between FLCN, its binding partners FNIP1 and FNIP2, and AMPK, well-known negative regulator of mTOR. *In vivo* data showing activation of mTOR and its downstream effectors in *Flcn*-deficient mouse kidneys (64–66) further supported this idea. Partial rescue of the enlarged polycystic kidney phenotype in this *in vivo* model by mTOR inhibitor rapamycin was encouraging and suggested that targeting the mTOR pathway might be an effective therapeutic approach for treatment of BHD-associated renal tumors and cutaneous fibrofolliculomas. However, topical application of rapamycin in a Netherlands clinical trial failed to have any effect on treatment of fibrofolliculomas (96). Additionally, subsequent studies using several different heterozygous *Flcn*-knockout mouse models (52, 67) and a *bhd* mutant yeast strain (68), reported inhibition of mTOR signaling under *Flcn* deficiency. The observation of reduced phospho-S6 (an mTOR readout) in solid tumors, but elevated phospho-S6 in renal cysts in one of the heterozygous *Flcn*-knockout mouse models has resulted in the proposed idea that FLCN loss may modulate mTOR signaling in a context- and/or cell type-dependent manner (52).

Another dichotomy in studies of FLCN function has recently arisen from data reported in two publications that connect FLCN with regulation of autophagy. Using *FLCN*-deficient human kidney cells, one group presented evidence that FLCN, in complex with its interacting partners FNIP1 and/or FNIP2, acts as a positive regulator of autophagy through

FLCN/FNIP1/FNIP2 interaction with GABARAP, which plays a role in autophagosome maturation (86). This was mediated by site-specific FLCN phosphorylation that was dependent upon ULK1, the key activator of the autophagic cascade. In direct contrast, a *flcn-1* mutant of C. elegans was shown to have activated autophagy that conferred resistance to energy-depleting stresses, which was driven by constitutive activation of AMPK resulting from the *flcn-1* mutation (85). This observation was confirmed in *Flcn*-deficient MEFs supporting the notion that FLCN is a negative regulator of autophagy in these *in vivo* systems. It is difficult to resolve these opposing roles for FLCN in such an important nutrient-sensing process as autophagy. One might postulate that, as was concluded from studies to elucidate how FLCN modulates mTOR, the role of FLCN in facilitating or inhibiting autophagy may depend upon the cell type or environmental context.

One promising approach for the discovery of new drugs to treat BHD has utilized a synthetic-lethal screen that identified mithramycin as a potential therapeutic agent, which was cytotoxic in F*LCN*-deficient, but not *FLCN*-expressing cell lines (99). Future synthetic-lethal screens using an expanded drug library to maximize information gained from this kind of approach may prove highly informative for identifying potential new therapeutic agents. Yeast two-hybrid screens are also useful tools for the identification of protein-protein interactions as a means to elucidate the function of novel proteins. Using this approach, two independent laboratories succeeded in uncovering an interaction between FLCN and plakophilin-4 (p0071) that has suggested a role for FLCN in cell-cell adhesion, cell polarity and Rho A signaling (80, 81). Targeting Rho A activity in a *FLCN*-deficient cell line with Y-27632, an inhibitor of ROCK, the downstream effector of Rho, was effective against the migratory phenotype of these cells (81), and may offer a novel therapeutic approach to treatment of metastatic BHD renal tumors with deregulated Rho A signaling. Further exploration of FLCN interactors using the yeast two-hybrid system should be encouraged to uncover more details of FLCN function in cellular homeostasis.

As previously stated, the development of effective therapeutic drugs to target FLCN-deficient renal and hair follicle tumors awaits the elucidation of the primary deregulated pathway in BHD affected tissues. Currently surgical intervention is the only available therapy for BHD-associated renal tumors. Based upon our experience with renal tumors that develop in the setting of BHD, many BHD-associated renal tumors are relatively indolent. Our approach is to manage these patients with active surveillance until the largest tumor reaches the 3 cm threshold, at which time surgical intervention is often recommended (93).

These are exciting times for researchers who conduct FLCN functional studies and clinicians who manage BHD patients given the explosion of BHD-related research that has been published in the last several years. We can expect that the primary pathway in which FLCN exerts its tumor suppressive function to control proliferative cell growth will be elucidated, providing the opportunity for development of targeted therapies that will hopefully improve the prognosis and quality of life for patients affected with BHD.

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Article Highlights

- Birt-Hogg-Dubé (BHD) syndrome is an autosomal dominant disorder that predisposes to fibfolliculomas, pulmonary cysts, spontaneous pneumothorax and renal neoplasia.
- The cutaneous fibrofolliculomas and the pulmonary cysts are highly penetrant;, spontaneous pneumothorax occurs in about 30% of BHD patients by the age of 40, and renal tumors with variable histology develop in 15–30% of individuals affected with BHD.
- Mutations in the tumor suppressor gene, *folliculin* (*FLCN*), have been identified in the germline of BHD patients; while the majority of mutations are predicted to prematurely truncate the FLCN protein, missense and partial gene deletions have also been detected.
- FLCN, through its binding partners FNIP1 and FNIP2, interacts with AMPK, cellular energy sensor and negative regulator of mTOR, master controller of protein synthesis and cell growth.
- Functional studies in *FLCN*-deficient models have revealed potential roles for FLCN in regulating mTOR activity, TGFβ signaling, PGC-1α-driven mitochondrial biogenesis, TFE3/TFEB transcriptional control, regulating Rag GTPases for amino acid-dependent activation of mTOR at the lysosome, cell-cell adhesions and cell polarity, the E-cadherin-LKB1-AMPK axis, ciliogenesis and autophagy.
- It is hoped that effective targeted therapeutic approaches will emerge as a result of the ongoing studies focused on understanding FLCN function and the cellular consequences of *FLCN* inactivation.

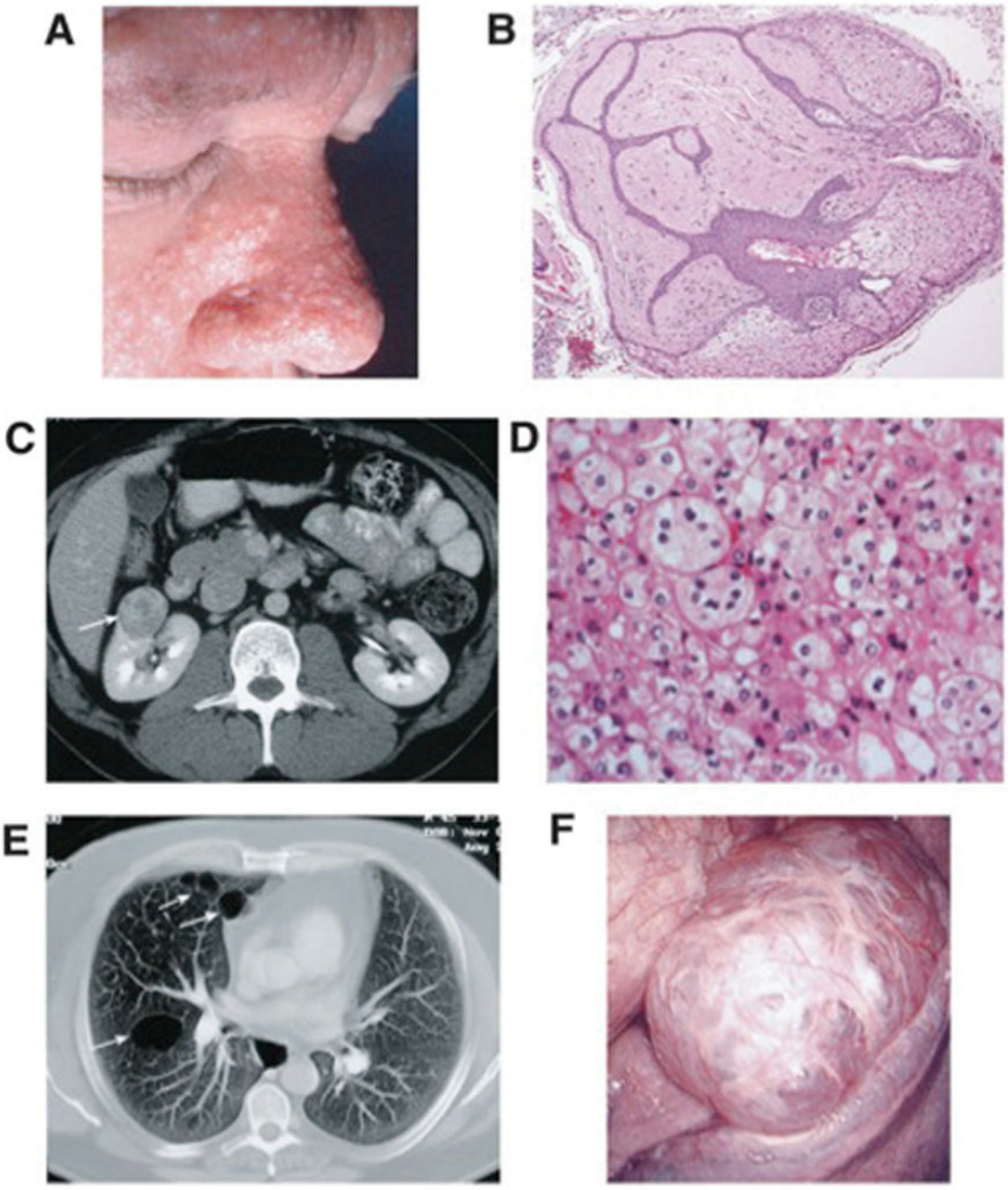

Figure 1.

Clinical manifestations of Birt-Hogg-Dubé (BHD) Syndrome. A) Fibrofolliculomas on a BHD patient present as numerous flesh-colored papules, 2–3 mm in diameter, on the nose and forehead. B) Photomicrograph of a fibrofolliculoma. The elongated, anastomosing epithelial strands and the dense connective tissue stroma are visible. C) Abdominal CT scan of a BHD patient showing a renal tumor; the resected neoplasm was a chromophobe renal carcinoma. D) Photomicrograph of a chromophobe renal carcinoma from a patient with BHD; 200x. E) Thoracic CT showing appearance of lung cysts in a patient with BHD. F)

Appearance of the pleural surface of the left lower lobe from a 46-year-old man with BHD and recurrent spontaneous pneumothorax. Numerous thin-walled bullae on the surface of the lung can be seen. The image was obtained by video-assisted thoracoscopy. Used with permission (23).

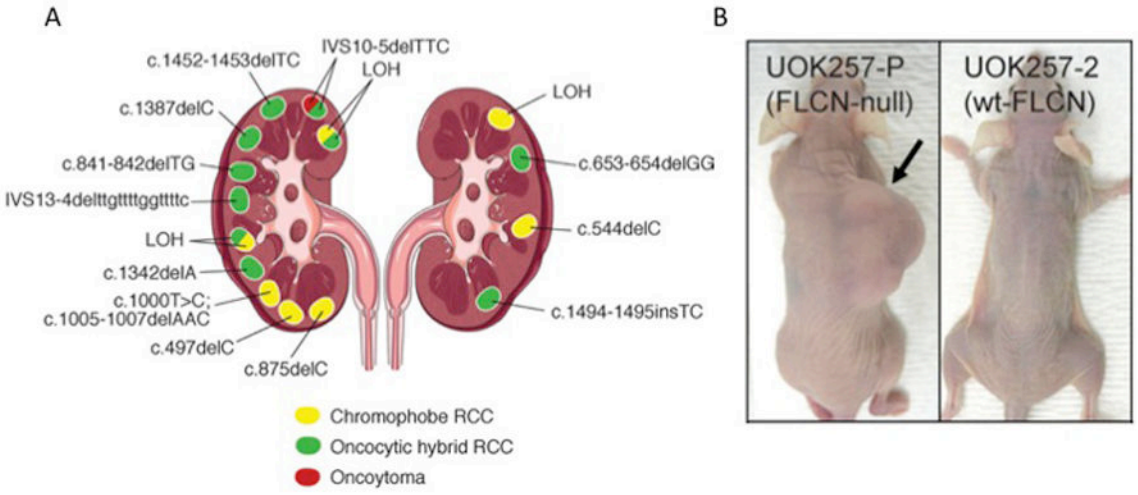

Figure 2.

Homozygous inactivation of *FLCN* drives renal tumorigenesis in BHD patients and xenograft tumors in mice. A) Schematic representation of tumors and mutations found in the kidneys of a BHD patient with a germline *FLCN* mutation. Of 18 tumors examined, 15 tumors (11 from the left kidney and four from the right kidney) exhibited a somatic second hit *FLCN* mutation. Used with permission (49). B) Tumorigenic potential of *FLCN*-null renal cancer cell line (UOK257-P) is suppressed by re-introduction of wild-type *FLCN* (UOK257-2) Arrow indicates xenograft tumor from UOK257-P cells. Used with permission (51).

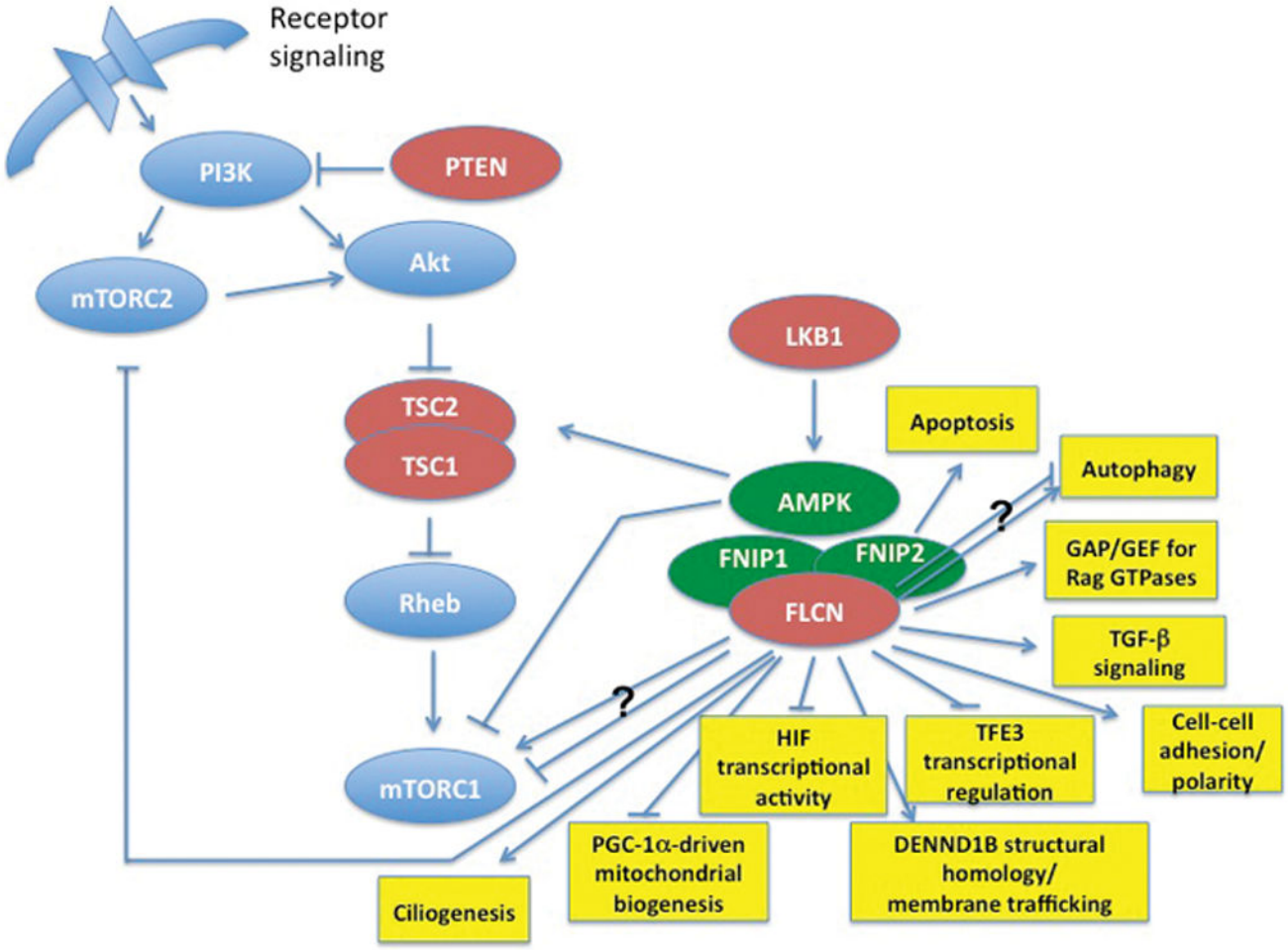

Figure 3.

FLCN-FNIP axis and potential interactions with cellular pathways and processes. FLCN, FNIP1 and FNIP2 interact with AMPK and modulate mTOR signaling. Tumor suppressors are red. FLCN interactors are green. Potential interactions of FLCN/FNIP1/FNIP2 with cellular pathways/processes are in yellow boxes. → indicates activation. ⊣ indicates inhibition. ? indicates evidence for both inhibition and activation. Adapted from reference 102; used with kind permission of Springer Science+Business Media.

Table 1

Diagnostic Criteria for Birt-Hogg-Dubé (BHD) Syndrome

Major criteria (high likelihood of BHD)

- 2 or more cutaneous papules clinically compatible with fibrofolliculoma/trichodiscoma with at least one biopsy proven/ histologically confirmed fibrofolliculoma; adult onset

Minor criteria (suspicious for BHD)

- multiple bilateral basilar lung cysts with or without spontaneous pneumothorax (<40 years of age)especially with family history of these lung manifestations
- bilateral multifocal chromophobe or hybrid oncocytic renal tumors especially with family history of this histologic subtype
- any combination of cutaneous, lung or renal manifestations known to be associated with BHD in an individual or his family

Definitive diagnosis

- positive germline *FLCN* mutation test

Table 2

Potential Therapeutic Approaches for Birt-Hogg-Dubé(BHD) Syndrome

Therapeutic Agent or Class	Target/Pathway	Supporting Evidence	Reference
glycolytic inhibitors	glycolytic metabolism	*FLCN*-deficient *in vitro* model	97
autophagy inhibitors	autophagy	*flcn-1* mutant *C. elegans* and *in vitro FLCN*-deficient models	85, 86
mithramycin	apoptosis	*FLCN*-deficient NCI 60 cell lines	99