Birt-Hogg-Dubé syndrome

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Birt-Hogg-Dubé syndrome

From patient to protein

Marigje Vernooij

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From patient to protein

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Promotores

Prof. dr. M.A.M. van Steensel

Prof. dr. P.M. Steijlen

Copromotores

Dr. M. van Geel

Dr. B.J. Coull

Beoordelingscommissie

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Dr. E.M. Leter

Prof. dr. E.J.M. Speel

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1

General introduction

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General introduction

Birt-Hogg-Dubé syndrome

Birt-Hogg-Dubé (BHD) syndrome or Hornstein-Knickenberg syndrome (MIM 135150) is a rare, autosomal dominantly inherited disorder. Patients can develop fibrofolliculomas¹, lung cysts, spontaneous pneumothorax and kidney tumours.² The syndrome was first described by Hornstein and Knickenberg in 1975 and again by Birt, Hogg and Dubé in 1977.^{3,4} Hornstein and Knickenberg described two siblings with perifollicular fibromatosis cutis and skin tags, as well as colonic polyps of which one showed malignant degeneration. Their father had identical skin lesions and both lung and kidney cysts.³ Birt Hogg and Dubé reported a family with fibrofolliculomas together with trichodiscomas and skin tags. The fibrofolliculomas they described resembled the perifollicular fibromatosis described earlier by Hornstein and Knickenberg, though Birt Hogg and Dubé did not consider them to be the same.⁴ Later histopathologic studies did show that both groups had, in fact, described identical lesions.⁵⁻⁸ A decade after first publication of the syndrome, spontaneous pneumothorax was first noted in a BHD syndrome family and later lung cysts were reported as well.⁹⁻¹¹ A bilateral multifocal chromophobe renal carcinoma was first described in a patient with BHD syndrome in 1993. In 1999, Toro and colleagues reported renal tumours to co-segregate with the skin lesions and lung manifestations.¹⁰

Clinical manifestations

The clinical manifestations vary widely between BHD syndrome families and between patients within one family. Fibrofolliculomas, pneumothoraces, lung cysts and kidney tumours are not necessarily all present in one individual or one family and some patients may not have clinical manifestations at all. The majority of studies on people with BHD syndrome are from Europe and Northern America and most families are therefore Caucasian. Reports of cohorts from Japan show a much higher rate of spontaneous pneumothorax and low prevalence of fibrofolliculomas in BHD syndrome patients.^{12,13} Due to the heterogeneity of clinical manifestations between patient of different ethnicity and familial descent, but also between patients within one family, BHD syndrome may be underreported. Moreover, morbidity can be low, so not all patients seek medical attention and not all clinicians are aware of this rare syndrome.

The fibrofolliculomas in BHD syndrome appear as 2-4mm, pale or skin coloured, dome-shaped papules (Figure 1). They are typically seen around the nose, but can also be present on other locations of the face, ears, neck and upper trunk. Their number increases with age and can reach up to hundreds. The lesions are usually not painful or pruritic. Fibrofolliculomas mainly occur in the third or fourth decade of life and are found in 82-90% of patients with BHD syndrome.¹⁴⁻¹⁷ The trichodiscomas that were described by Birt, Hogg

and Dubé are indeed fibrofolliculomas. Different histologic findings were due to different sectioning techniques.¹⁸ The current best treatment for fibrofolliculomas seems to be hyfrecation (electrocoagulation), if necessary followed by curettage. Alternatively, resurfacing with ablative lasers may be attempted. The effect of both treatments is temporary. The lesions recur in months to years.¹⁹⁻²¹ Preventive therapy is not available at this moment.

Figure 1. Fibrofolliculomas on the nose and cheeks of a patient with BHD syndrome.

Lung cysts are a common feature in BHD syndrome as well. They are present in 70-84% of the patients.¹⁴⁻¹⁶ The cysts are irregularly shaped, well circumscribed and vary in size. They are usually multiple, bilateral and are often located in the basal and mediastinal areas of the lungs.^{22, 23} Lung function is generally normal.²³ Cysts also occur in other organs such as the kidneys, liver and thyroid.^{10, 16}

The lung cysts may cause spontaneous pneumothorax²², which is reported in about 30% of BHD syndrome patients, mostly before the age of 40 years.^{15, 22, 24} Spontaneous pneumothorax has been reported in patients as young as 7 years and as old as 73 years of age.^{25, 26} Compared to their unaffected siblings, BHD syndrome patients have a 50 times greater risk to develop a pneumothorax.² In some patients pneumothorax recurs and may need medical intervention.²⁷

Kidney tumours occur in 16-34% of patients with BHD syndrome.^{14, 15, 24, 28} Patients have a 7-fold greater risk than their unaffected siblings.² The renal tumours develop at a median age of about 50 years, but tumours in 14 years old patients have been reported.^{29, 30} They are often bilateral and multifocal and can have a diverse histology. Over half of the renal tumours are hybrid chromophobe-oncytomas, but also chromophobe renal cell carcinomas, clear cell renal cell carcinomas, oncocytomas and papillary renal cell carcinomas occur.^{29, 31} The tumours usually progress slowly, but they can occasionally metastasise.^{24, 31} Hence, lifelong kidney surveillance is recommended. Computed tomography (CT) is recommended for baseline screening, followed by magnetic resonance imaging (MRI) with

intravenous contrast every 36 months, to prevent high levels of radiation exposure.³²⁻³⁶ When a kidney lesion smaller than 2-3cm is detected, closer monitoring is required. Imaging intervals will be based on tumour size, growth rate and the history of tumours in the patient and its family. Surgical resection is indicated when the tumour is larger than 2-3cm. As patients may require multiple surgeries in their lives^{28, 36, 37} nephron-sparing surgery, which preserves renal function, is the preferred method. The prognosis after nephron sparing surgery is excellent for the majority of patients and most of them only need one intervention during their lifetime.³⁶ Hence, kidney surveillance for timely detection of tumours is important. We evaluated the compliance with surveillance in a cohort of 32 BHD syndrome patients from the Maastricht University Medical Centre. These data, as well as the prevalence of clinical manifestations in this cohort are presented in **chapter 2**.

Colon polyps and colon carcinomas, as described by Hornstein and Knickenberg in their first report, have often been reported in BHD syndrome patients^{6, 7, 11, 38-43}, but a significantly increased risk was not confirmed in a large cohort study.² The first reports describing BHD syndrome also mentioned acrochordons, or skin tags, as specifically associated with BHD syndrome. However, these are very common in the general population and are therefore not likely to be a typical clinical marker for BHD syndrome. Desmoplastic melanoma and lipoma have also been reported in association with BHD syndrome and causal connections were suggested.^{9, 44} However, both melanoma and lipoma are sufficiently common for these associations to have been coincidental.

Compliance with kidney surveillance

Renal malignancies often are asymptomatic before metastasising. Therefore, follow up with regular kidney surveillance is essential to detect renal neoplasms in an early stage. Moreover, detection before tumours reach a size of 2-3cm enables nephron sparing surgery and could improve patients' prognosis. For BHD syndrome no prospective cohort studies are available that investigate the association between surveillance and survival. In other monogenic diseases that predispose to cancer, surveillance is shown to noticeably improve life expectancy.⁴⁵ For von Hippel-Lindau (VHL) syndrome, a disease that involves a risk of up to 30% for renal cell carcinomas⁴⁶, surveillance was positively correlated with increased survival.^{47, 48} Evidently, compliance to surveillance is important to encounter this. Studies in patients with familial colon carcinoma revealed that an older age of the patient is associated with higher compliance. Also having a mutation confirmed is positively associated.⁴⁹ Furthermore, compliance is higher when patients perceive control over the development of a tumour by undergoing periodic surveillance.⁵⁰ Denial, fear for being diagnosed with cancer, lack of knowledge about the imaging method and perceived obstacles to imaging such as physical or emotional discomfort are all negatively associated with compliance. Financial restrictions as reason for noncompliance vary per health care system.^{51, 52}

Cues for action, such as a reminder letter, stimulate compliance.⁵² We have investigated the compliance with kidney surveillance, as well as the reasons for noncompliance, in our cohort of 32 patients with BHD syndrome. The data are presented in **chapter 2**.

The molecular basis of BHD syndrome

BHD syndrome is caused by mutation of the *FLCN* gene (MIM 607273) on chromosome 17p11.2, encoding the folliculin (FLCN) protein; as discovered by Nickerson *et al.* in 2002.⁵³ To date, over 150 unique mutations in *FLCN* have been reported in the Leiden Open Variation Database.⁵⁴ The majority of mutations are nonsense or frameshifts that cause a premature stop codon and result in truncation of the FLCN protein, but also splice site, missense and large genomic deletion mutations are found in BHD syndrome patients. Mutations are found throughout the full length of *FLCN*, but about 50% occur in a hyper mutable tract of 8 cytosines in exon 11, making this a mutation “hot spot”.^{14, 43, 53, 55} Three previously unpublished *FLCN* mutations are reported in **chapter 2**.

FLCN is considered a tumour suppressor gene, since *FLCN* mRNA expression is lost in kidney tumours of BHD syndrome patients⁵⁶ and second hit mutations or loss of the remaining *FLCN* allele found in the majority of renal tumours of the naturally occurring Nihon rat model of BHD syndrome.⁵⁷ In fibrofolliculomas however, the wild type allele of *FLCN* was still present⁵⁸ and *FLCN* mRNA is strongly expressed⁵⁶, indicating that haploinsufficiency of *FLCN* may be enough to cause the growth of the fibrofolliculomas in BHD syndrome patients.

The *FLCN* gene is highly conserved in Animals and Fungi⁵³, indicating that it is ancient and has an important role in biology. In the rat and mouse models of BHD syndrome, homozygous mutation of *FLCN* is embryonically lethal^{57, 59} and also in dogs carrying mutations in canine *FLCN*, homozygous mutation is strongly suggested to be lethal.⁶⁰ Hence, *FLCN* is expected to have a crucial function in embryogenesis. The gene encodes the FLCN protein, which was identified as a novel protein with unknown function. First analysis of its sequence in 2002 did not reveal any functional domains that could indicate what role FLCN plays in the human cell.⁵³ Only in 2012, when the crystal structure of FLCN's C-terminus was presented, FLCN was found to be distantly related to proteins with a DENN (differentially expressed in normal cells and neoplasia) domain. DENN proteins act as guanine exchange factor (GEF) towards Rab GTPases⁶¹, which are proteins that regulate many aspects of membrane traffic.⁶² Recent data suggest that FLCN interacts with several Rabs and towards those can act as a GEF as well as a GAP (GTPase-activating protein).^{63, 64} This function is consistent with a potential role in orchestrating vesicular transport⁶⁵, a notion that is supported by known activity of FLCN's yeast ortholog LST7.⁶⁶

In 2006, the first protein interaction studies on FLCN suggested that the mTOR (mammalian target of rapamycin) complex and AMPK (5' AMP-activated protein kinase) may be involved in regulating FLCN, because phosphorylation of FLCN is diminished upon inhibition of both mTOR complex and AMPK.⁶⁷ AMPK is upstream of the mTOR complex, which is a key regulator of cell growth.⁶⁸ See Figure 2 for an overview of the position of mTOR in different signal transduction pathways.

Since, many publications were dedicated to the role of mTOR in BHD syndrome. The results, however, are conflicting as both upregulation^{67, 70-73} and downregulation⁷³⁻⁷⁶ are seen upon impaired FLCN expression. Altogether, FLCN might affect the mTOR complex, though it is not clear how. Yet, the full function of FLCN remains to be elucidated, as well as how its absence or aberrant forms cause BHD syndrome.

Figure 2. Simplified overview of the position of mTOR complex 1 (mTORC1) in different signal transduction pathways. As reviewed by Laplante and Sabatine⁶⁹, with incorporation of FLCN's role in this pathway, as described in the main text.

Learning from diseases that look like BHD syndrome

Tuberous sclerosis complex

BHD syndrome clinically resembles tuberous sclerosis complex (TSC). Both disorders combine renal cysts and tumours with cystic pulmonary lesions and hair follicle tumours.⁷⁷ The clinical similarities might be reflected in the underlying biology. In TSC, mutations in either of two genes, *TSC1* (encoding hamartin) or *TSC2* (encoding tuberin), result in deregulation of the mTOR complex⁷⁷, which may be deregulated in BHD syndrome as well.

The macrolide antibiotic rapamycin can inhibit mTOR activity and has been shown to reduce facial angiofibromas in TSC.^{78, 79} Rapamycin solution can be applied topically as a local treatment of the skin lesions, that directly targets the molecular pathway involved in the pathogenesis of TSC. Skin lesions are highly accessible and thus potentially are an excellent system for trying to understand if the mTOR pathway is also involved in the pathobiology of BHD syndrome.

Moreover, fibrofolliculomas are often the first symptom in BHD syndrome that patients notice. It is also the most visible one, representing a daily confrontation with their disease. They can be a psychological and social burden to the patient⁸⁰, by giving cosmetic complaints. Patients may therefore seek treatment for the fibrofolliculomas. The currently available treatments all are destructive and have a risk of causing inflammation, scarring, hypo- and hyperpigmentation, worsening the cosmetic outcome. Moreover, they only have a temporary effect since the lesions recur within a few years. Hence there is a need for a more subtle and targeted therapy that cures the existing fibrofolliculomas and/or prevents the growth of new ones.

From the clinical similarities between BHD syndrome and TSC, one would expect increased mTOR activity in the context of BHD syndrome, and thus a potential use for rapamycin in prevention or control of the symptoms. This was investigated in a double-blind placebo controlled randomized split-face trial that investigated this, as presented in **chapter 3**.

Ciliopathies

A feature of BHD syndrome is the formation of cysts in various organs.^{10, 16} Lung cysts are a recognised manifestation in BHD syndrome. While liver cysts occur in about 5% of the population, their frequency in a series of BHD syndrome patients was 43%. About the same percentage of patients had renal cysts and no less than 65% had thyroid nodules or cysts.¹⁶

Although fibrofolliculomas are not commonly considered as such, they are probably cysts as well. Already in 1996 Chung and colleagues published the cystic appearance of fibrofolliculomas.⁹ When we examined them closer, it seems that fibrofolliculomas are not hair follicle tumours, but rather cystic-sebaceous lesions.⁸¹ Many of the typical epithelial strands that make up much of the lesion are continuous with sebaceous glands which consistently show strong FLCN expression (Figure 3). In this respect it is of interest to note that lesions very similar, if not identical, to fibrofolliculomas have been previously described as “mantleomas”.⁸² These mantleomas are benign tumours that emanate from a skirt- or mantle-like continuum of the infundibulum of the hair follicle: the mantle.⁸³ This structure consists of cords formed by undifferentiated cells from which sebaceous glands presumably develop upon appropriate hormonal stimulation. Interestingly, fibrofolliculomas in BHD syndrome preferentially affect areas that are rich in sebaceous glands, such as the nose and perinasal skin. Hence, the fibrofolliculomas could be considered cystic-sebaceous lesions.

Cyst formation is a hallmark of ciliopathies. This is a group of diseases caused by impaired function of the cilium.⁸⁴⁻⁸⁶ There are motile and non-motile cilia. The latter is called the primary cilium and is involved in ciliopathies. Primary cilia are best understood as cellular antennae. They form as membrane extensions growing from the basal body, which is a modified centriole, and they serve primarily as sensory organelles. Interestingly, the hamartin and tuberlin proteins that are mutated in TSC (which resembles BHD syndrome) are involved in cilia formation.⁸⁷ Von Hippel-Lindau disease, a syndromic disorder that, like BHD syndrome, predisposes to cyst formation and renal cell carcinomas⁴⁶, has also been linked to impaired cilia formation.⁸⁸

It is not completely understood how ciliary dysfunction causes cyst formation. However, there are strong indications that the planar cell polarity (PCP) pathway is at least partly controlled by the cilium.⁸⁹ PCP ensures that cells in polarized epithelia divide in the plane of the tissue so that its spatial organization remains intact. Thus, in growing kidney tubules (or lung vesicles), cells should divide in a plane oriented along the tubule's main axis. If this does not happen and division is out of plane, cyst formation can ensue.⁹⁰

If FLCN has a role in cilia, ciliary dysfunction and disrupted PCP might explain the cysts that occur in various organs in BHD syndrome. The fibrofolliculomas could likewise be due to ciliary dysfunction. They may reflect abnormal growth and differentiation of the sebaceous mantle, as it has been demonstrated that lack of epidermal cilia in mice results in epidermal hyperproliferation and sebaceous differentiation of hair follicles.⁹¹

Figure 3. Fibrofolliculomas contain cystic sebaceous structures. (A) H&E stained section of a fibrofolliculoma (composed of 4 images) showing a cyst containing keratin (arrowheads), sebaceous differentiation (arrows) and epithelial strands connecting the cyst to the sebaceous gland; reprinted with permission of Elsevier; Journal of Investigative Dermatology.⁵⁸ (B) H&E stained section of a fibrofolliculoma showing pronounced sebaceous hyperplasia (x10). (C) FLCN is strongly expressed in sebaceous glands. Immunohistochemistry with a custom-made antibody directed against the FLCN N-terminus, visualization with DAKO Envision system (x5). Adapted from Vernooij *et al.*⁸¹ with permission of Springer; Familial Cancer.

Because of the cystic phenotype of BHD syndrome and its resemblance to TSC and VHL syndrome, we hypothesised that BHD syndrome is a ciliopathy. This hypothesis is further explored in **chapter 4**.

Discovery of novel functions for FLCN

When the studies described in this thesis were started, most research on BHD syndrome pathophysiology focused on the mTOR signal transduction pathway. Results were contradictory, and did not satisfactorily explain the clinical manifestations of patients with BHD syndrome. Hence, the actual function of FLCN and the pathogenicity – if any – of the mutant protein are yet to be determined. It would therefore be worth to deploy a completely different approach than those used before and attempt to identify the function of FLCN using an unbiased method. How we employed such a method for FLCN is described in **chapter 5**.

Aims and outline of this thesis

The aims of this thesis are to improve the clinical management of patients with BHD syndrome and to gain more insight in the pathophysiologic mechanism of the syndrome. The first objective is to describe the clinical characteristics and follow up characteristics of our cohort of BHD syndrome patients in order to identify factors that could improve patients' compliance to follow up. This is described in **chapter 2**.

The second objective is to explore a potential targeted treatment for the fibrofolliculomas that present in BHD syndrome patients, as reported in **chapter 3**.

The third objective is to further elucidate the pathophysiology of BHD syndrome. As described in **chapter 4**, we test the hypothesis if BHD syndrome is a ciliopathy, based on the resemblance with other ciliopathies. In **chapter 5** we developed an unbiased approach to identify novel pathways that may be involved in the pathogenesis of BHD syndrome.

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Topical rapamycin as a treatment for fibrofolliculomas in Birt-Hogg-Dubé syndrome: a double-blind placebo-controlled randomized split-face trial

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Gijzen L.M.C.*, Vernooij M.*, Martens H., Oduber C.E.U., Henquet C.J.M., Starink T.M., Prins M.H., Menko F.H., Nelemans P.J., van Steensel M.A.M.

* These authors contributed equally to this work

Abstract

Background

Birt-Hogg-Dubé syndrome (BHD) is a rare autosomal dominant disorder characterised by the occurrence of benign, mostly facial, skin tumours called fibrofolliculomas, multiple lung cysts, spontaneous pneumothorax and an increased renal cancer risk. Current treatments for fibrofolliculomas have high rates of recurrence and carry a risk of complications. It would be desirable to have a treatment that could prevent fibrofolliculomas from growing. Animal models of BHD have previously shown deregulation of mammalian target of rapamycin (mTOR). Topical use of the mTOR inhibitor rapamycin is an effective treatment for the skin tumours (angiofibromas) in tuberous sclerosis complex, which is also characterised by mTOR deregulation. In this study we aimed to determine if topical rapamycin is also an effective treatment for fibrofolliculomas in BHD.

Methods

We performed a double blinded, randomised, facial left-right controlled trial of topical rapamycin 0.1% versus placebo in 19 BHD patients. Trial duration was 6 months. The primary outcome was cosmetic improvement as measured by doctors and patients. Changes in fibrofolliculoma number and size were also measured, as was occurrence of side effects.

Results

No change in cosmetic status of fibrofolliculomas was reported in the majority of cases for the rapamycin treated (79% by doctors, 53% by patients) as well as the placebo treated facial sides (both 74%). No significant differences between rapamycin and placebo treated facial halves were observed ($p=1.000$ for doctors opinion, $p=0.344$ for patients opinion). No significant difference in fibrofolliculoma number or change in size of the fibrofolliculomas was seen after 6 months. Side effects occurred more often after rapamycin treatment (68% of patients) than after placebo (58% of patients; $p=0.625$). A burning sensation, erythema, itching and dryness were most frequently reported.

Conclusions

This study provides no evidence that treatment of fibrofolliculomas with topical rapamycin in BHD results in cosmetic improvement.

Introduction

Birt-Hogg-Dubé syndrome (BHD, MIM #135150) was first described in 1975 by Hornstein and Knickenberg and again in 1977 by Birt, Hogg and Dubé.^{1,2} It is a rare autosomal dominant disorder characterized by the occurrence of benign, mostly facial, hair follicle tumours called fibrofolliculomas (FF)s², multiple lung cysts, spontaneous pneumothorax³, and an increased renal cancer risk.^{4,6} The FFs can be quite disfiguring and are often the reason why patients come to medical attention. FFs occur in around 80% of patients and usually appear after the age of 20 years as dome-shaped, whitish or skin-coloured papules. Typically presenting around the nose, they can also occur on the ears, neck and trunk.² Although they do not grow beyond 3-4 mm in size, their numbers increase with age. Consequently, patients can eventually have hundreds of papules that may cause emotional distress and have a strong impact on their quality of life.

Current treatment options for FFs include destructive approaches such as ablative laser, electrocoagulation, excision and other invasive interventions.⁷⁻¹⁰ These treatments have high recurrence rates (for laser therapy it is known that FFs recur after 2-3 years or even after months⁹) and are not effective in preventing early lesions. Moreover, they have a significant risk of complications such as scarring, inflammation, hypo- and hyperpigmentation. Therefore, there is a medical need for a more targeted therapy that is suitable for chronic use, reduces the number of tumours and/or prevents the growth of new ones. Insights from genetic and cell biological studies have suggested such a potential approach.

BHD syndrome is caused by germline mutations in the *FLCN* gene on chromosome 17p11.2 coding for the protein folliculin (FLCN).^{11,12} FLCN's functions are mostly unknown, although recent data suggest that it might be involved in cellular energy sensing through the mammalian target of rapamycin (mTOR) signalling pathway.¹³ A considerable body of data suggests that in BHD, the mTOR pathway is activated.¹⁴⁻¹⁸ Thus, we theorized that BHD syndrome belongs to a larger family of disorders characterized by mTOR deregulation, such as tuberous sclerosis complex (TSC).^{14,19} In yeast, the homologue of *FLCN* is found to have opposing roles to the genes involved in TSC (*TSC1* and *TSC2*), but all are suggested to regulate common downstream targets.²⁰ In TSC, as in BHD, patients develop facial hair follicle tumours, called angiofibromas. These tumours strongly resemble FFs and have also been reported in BHD syndrome (Figure 1).^{21,22} Angiofibromas in TSC were coincidentally found to respond favourably to the mTOR inhibitor rapamycin. They disappeared after only a few months of oral rapamycin, that was administered after renal transplantation.²³ Oral administration of rapamycin has potential side effects that are not acceptable in the context of BHD. However, there are several studies showing that topical application is safe.²⁴⁻²⁶ Additionally, multiple reports show successful treatment of angiofibromas in TSC

with topical rapamycin.^{24, 26-30} Thus, we hypothesized that rapamycin oral solution might be used for the topical treatment of FFs.

We here show the results of the first double-blind placebo-controlled randomized split-face trial of topical rapamycin for the treatment of FFs in BHD. The objective of this study was to determine whether topical application of rapamycin 1 mg/ml oral solution could lead to overall cosmetic improvement and reduction in size and number of facial FFs in BHD patients. Secondly, we evaluated safety and formula acceptance.

Figure 1. Skin features in TSC and BHD. Photographs taken from a TSC and a BHD patient show angiofibromas and fibrofolliculomas, respectively. Notice the resemblance between the two types of lesion.

Materials and methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information (Protocol S1 & Checklist S1). Before patient recruitment started the study was deposited at ClinicalTrials.gov (NCT00928798; <http://www.clinicaltrials.gov/ct2/show/NCT00928798?term=rapamycin+BHD&rank=1>).

Ethics Statement

This study was approved by the Medical Ethical Committees of the Maastricht University Medical Centre+ and the VU Medical Centre Amsterdam, Licence number NL28245.068.09 and was conducted in accordance with the Helsinki Declaration of 1975. The individuals in Figure 1 and 3 of this manuscript have given written informed consent (as outlined in PLOS consent form) to publish their pictures.

Patients

Patient recruitment, inclusion and follow-up occurred in the Department of Dermatology in the Maastricht University Medical Centre* and at the Department of Clinical Genetics in the VU Medical Centre Amsterdam, both reference centres for patients with BHD. Patients with genetically proven BHD, a minimum age of 18 years, at least 10 facial FFs (of which at least one histologically confirmed) and a general good health were eligible for inclusion. Pregnant or lactating women, patients with proven or suspected skin or other malignancy in the past 5 years and patients who planned facial surgery in the treatment period were excluded. Because of the increased risk of renal cell carcinoma in BHD patients, screening by an abdominal ultrasound or MRI was scheduled for patients who had their last screening more than 1 year ago. Patients with concomitant disease requiring systemic immunosuppressive treatment and patients using any facial topical immunosuppressive treatment or facial topical drugs interfering with rapamycin during the trial period or within 30 days before starting therapy were excluded, as were patients with facial skin lesions other than FFs that might worsen under rapamycin, such as active infections. Next to these protocolled criteria, patients were excluded when they could not attend any of the follow-up visits, because this made monitoring of (side) effects and adverse events impossible. A biopsy of a facial FF was taken if this had not been performed earlier. Written informed consent was obtained from all patients eligible for participation.

Treatment procedures

Computer generated randomisation was performed using random permuted blocks of 2. Randomisation was stratified by participating centres. For each participant, topical treatment with a commercially available rapamycin (sirolimus) oral solution 1 mg/ml was randomized to one facial half and application of a placebo solution to the other facial half. Patients were instructed to topically apply the two formulations twice daily, for 6 months. The rapamycin solution was decanted at 2-8 °C in a nitrogen atmosphere and packaged in amber glass bottles to preserve solution quality. The solvent was used as a placebo and was packaged in identical bottles. Bottles were numbered and marked 'left' or 'right' by the pharmacist, in order to conceal randomisation. Patients and investigator were both blinded for the treatment of each facial side throughout the study.

Efficacy assessment

Patients were clinically assessed at baseline, 3 months and 6 months by a single investigator (LMCG). Evaluation consisted of careful examination of the face, including assessment of facial FF number and size, as well as the change in general cosmetic FF status compared to baseline. At each visit, facial medical photographs were obtained under standardized conditions concerning: studio lighting, subject positions, predetermined camera settings (distance to subject, resolution, aperture settings, exposure time) and background. For

each patient full front, left and right oblique views (45°) and left and right profile views (90°) were obtained. Photographs were evaluated twice by 3 physicians; the investigator and 2 dermatologists. All were blinded for the treatment. They assessed the size of the largest FF in a predefined area. Right and left facial halves were scored separately and 3-month and 6-month photographs were compared to baseline. The change in general cosmetic FF status was evaluated using a 7-point Likert scale (-3= strong worsening; -2= moderate worsening; -1= minimal worsening; 0= no change; 1= minimal improvement; 2= moderate improvement; 3= strong improvement).^{31, 32} The FF number was registered in categories, (0; 1-10; 11-20; 21-30; 31-40; 41-50; >50). Each of the three observers scored the photographs twice for cosmetic status, FF size and FF number. From the six Likert scores or categories obtained per patient, the most frequently assigned score was used for analysis. When multiple scores were obtained in equal amounts, the score indicating the least improvement was used. The change in FF number was obtained by comparing the 6 month results to baseline results. Reduction of FF number was defined as a decrease of at least one category. Patients' impressions concerning cosmetic FF status were assessed as well, also using the 7-point Likert scale. At follow-up visits we assessed and registered side effects.

Statistical analysis

A minimum of 17 patients was needed to detect an increase in preference for rapamycin over placebo by at least 35% (from 50% to 85%), with a power of 90% and a type 1 error (2 sided) of 0.05. A minimum of 35% difference in response was considered to be clinically relevant, as a smaller difference does not justify the routine treatment of BHD patients with rapamycin. The McNemar's chi-square test was used to test for discrepancies in response to rapamycin and placebo treatment concerning cosmetic improvement and reduction in FF number. This test was also used to compare the proportion of side effects occurring between the two facial sides. Differences in FF size were tested for significance using the T-test for paired samples. A p-value smaller than 0.05 was considered to indicate statistical significance in all analyses. The inter-observer variation of scores on cosmetic status, FF size and number was measured using the intraclass correlation coefficient. For data analysis we used SPSS Statistics version 20.0.0.1 (IBM, Armonk, NY, USA) and for calculation of 95% confidence intervals (95%CI) for difference in paired proportions we used R package ExactCldiff version 2.15.1 (<http://cran.r-project.org/web/packages/ExactCldiff/index.html>).

Results

Study population

Between January 2010 and August 2010, 24 patients with BHD were recruited and assessed for eligibility to participate (Figure 2). One patient was excluded due to excessive use of alcohol and three because of frequent travelling abroad, which made follow-up impossible. After screening, but before randomisation, one person had to be excluded due to the diagnosis of a malignancy not related to BHD. A total of 19 individuals were randomised. The distribution of baseline characteristics of the study population is shown in Table 1. One individual was not able to attend the 3-month follow-up visit due to immobility after back surgery, but could be evaluated at six months. This person was excluded from the data analysis of the 3-month results (n=18). After two months of treatment, one patient had to discontinue the treatment because of a tearing eye. This patient was seen at the 3-month follow-up visit, but was considered lost to follow-up at six months. A second patient was lost to follow-up at six months due to travelling. For both, the 3-month data were carried forward in the 6-month efficacy analysis (n=19). An additional analysis of the 6-month results was performed in which all persons that were lost to follow-up at six months were excluded from the analysis (n=17).

Table 1. Baseline characteristics of included patients

Characteristic		Number of patients; n=19
Gender	Male	13
	Female	6
Mean age, years (range)		52 (34-70)
Number of facial fibrofolliculomas	20-50	4
	50-100	4
	>100	11
FLCN mutation	c.319_320delGTinsCAC	1
	c.610_611delGCinsTA	9
	c.655dupC	1
	c.1177-2A>G	1
	c.1285dupC	3
	c.1408_1418del	1
	c.871+3_c.871+4delGainsTCCAGAT	1
	c.880G>T	1
	c.250-?_1740+?del	1
Previous treatment	None	15
	Laser	4
	Surgical	1

Figure 2. Trial outline. * The patient who was lost to follow-up at 3 months was excluded from the analysis of the 3 month results. ** For the patients who were lost to follow-up at 6 months, the 3-month data were carried forward in the analysis of the 6-month results.

Effects of rapamycin treatment

The effect of rapamycin was assessed by measuring changes in cosmetic status, number and size of the FFs.

Cosmetic improvement of the FFs as judged by doctors and patients is presented in figures 3 and 4. There were no significant differences between rapamycin and placebo treated facial sides. According to doctors' opinion, improvement of cosmetic status was seen in 10.5% of rapamycin treated facial halves and in 10.5% of the placebo treated sides. The difference is 0% with a 95%CI of -19% to +19%; $p=1.000$ (Appendix SI). Patients reported cosmetic improvement upon rapamycin treatment more often than upon placebo treatment (47.3% versus 26.3%). The difference is 21% with a 95%CI of -14% to +51%; $p=0.344$ (Appendix SI).

Figure 3. Photographic recording of cosmetic status during rapamycin treatment. Close up of standardised facial photographs taken at baseline, 3 months and 6 months after starting treatment with topical rapamycin or placebo per facial half. Photos of a representative patient are shown, revealing no visible improvement or worsening of the facial fibrofolliculomas.

Both doctors and patients reported no changes in the majority of cases for the rapamycin treated (79% and 53% respectively) as well as the placebo treated facial sides (both 74%). The doctors reported minimal worsening in 16% of the placebo treated versus 11% of the rapamycin treated facial halves, whereas minimal improvement was observed in 11% of both rapamycin and placebo treated facial sides (Figure 4). Patients reported improvement more frequently for the rapamycin treated compared to the placebo treated facial side. Nine patients (47%) reported minimal to strong improvement of the rapamycin treated facial half, versus five (26%) reporting improvement of the placebo treated half (Figure 4). Additional analysis of the 17 patients that were seen at the 6-month follow-up showed similar results for the doctors' opinion on cosmetic improvement of FFs after treatment. Reduction in FF number was observed in 32% of rapamycin treated sides versus 37% of placebo treated sides, with a difference of 5% with a 95%CI of -20% to +31%; $p=1.000$ (Figure 5 and appendix SI)

Figure 4. Changes in cosmetic status of fibrofolliculomas after treatment with rapamycin scored by doctors and patients. BHD patients with facial fibrofolliculomas were treated with topical rapamycin and placebo for six months. The degree of improvement compared to baseline was scored on a 7-point Likert scale by both doctors (A) and patients (B). Doctors and patients reported no change in the majority of cases. Patients reported improvement more frequently for the rapamycin compared to the placebo treated facial side.

Figure 5. Changes in the number of fibrofolliculomas after treatment with rapamycin. BHD patients with facial fibrofolliculomas were treated with topical rapamycin and placebo for six months. The number of fibrofolliculomas in a predefined area of the face was determined and compared to baseline. The reduction in the number of fibrofolliculomas was stronger upon placebo treatment.

After three months of treatment the mean change in FF size was 0.054 mm on the rapamycin treated sides and 0.027 mm on the placebo treated sides, with a mean difference of 0.027 mm with a 95%CI of -0.210 to +0.264 mm; $p=0.184$ (Table 2).

The inter-observer variation expressed as intraclass correlation coefficients (ICC) for cosmetic status, FF number and FF size are shown in Table 3.

Table 2. Between-treatment comparison of mean change in fibrofolliculoma size compared to baseline (in mm) using the T-test for paired samples

	Rapamycin	Placebo	Difference	95% CI	p-value
At 3 months	0.054 (SD 0.34)	0.027 (SD 0.27)	0.027	-0.21 - +0.26	0.814
At 6 months	0.100 (SD 0.29)	0.096 (SD 0.32)	0.004	-0.16 - +0.17	0.961

SD = standard deviation; CI = confidence interval

Table 3. The inter-observer variation of scores used to measure the effect of rapamycin using the intraclass correlation coefficient (ICC)

Outcome measure	Lowest ICC (95%CI)	Highest ICC (95%CI)
Cosmetic status of fibrofolliculomas	0.517 (0.244-0.758)	0.633 (0.390-0.821)
Number of fibrofolliculomas	0.643 (0.324-0.840)	0.878 (0.757-0.947)
Size of fibrofolliculomas	0.374 (0.075-0.660)	0.653 (0.401-0.834)

95%CI = 95% confidence interval

Side effects of topical rapamycin

The majority of patients reported one or more side effects during the six months of treatment for rapamycin (68%) as well as for placebo (58%) (difference 10% with 95%CI -14.3 to 35.0; $p=0.625$). A burning sensation, erythema, dryness and itching were most frequently reported (Figure 6). Other side effects were tearing and prickling eyes, scaling, moderate pain and a prickling sensation of the skin. Most side effects occurred in the first three weeks of treatment and resolved over time, with 71% reported at the 3-month follow-up visit and only 42% at the 6-month visit. No serious adverse events have occurred during treatment.

Figure 6. Local side effects of topical rapamycin treatment. Side effects of both topical rapamycin and placebo treatment were registered at each clinical assessment. Burning, erythema, dryness and itching were the most commonly reported local side effects. All side effects other than dryness and itching were reported more frequently for the rapamycin treated facial half.

Discussion

This is the first randomised controlled trial in which the effectiveness of rapamycin on persistence and growth of FFs in patients with BHD was studied. We provide evidence that topical rapamycin does not improve the cosmetic status of FFs in these patients and does not reduce the amount or size of the FFs.

Previous studies in rodent and *Drosophila* models have suggested a role for increased mTOR activity in the pathogenesis of BHD, prompting this study.^{14-18, 33} However, other mouse models have subsequently shown conflicting data concerning the nature of the link between FLCN and mTOR. In contrast to other studies, Hudon *et al.* and Hartman *et al.* found decreased mTOR activity in mice.^{14-18, 34, 35} In human BHD tumours, the role of mTOR had also been questioned by Claessens *et al.* based on immunohistochemical assessment of BHD patient skin and kidney tumour tissue.³⁶ More recent in vitro studies using FLCN knockdown cells have not been able to confirm a direct link between FLCN and mTOR³⁷ (and unpublished data). The deregulation of mTOR activity found in the tumours of BHD animal models could be due to mTOR's general involvement in cancerous growth rather than a direct effect of FLCN's absence. Of note, most animal models involve knockout of FLCN. FFs do not have loss of FLCN and we have recently provided evidence that BHD-associated kidney tumours do not have complete loss of

FLCN either.³⁸⁻⁴⁰ Thus, the present state of the art suggests that there is no clear-cut relation between FLCN and mTOR. Our trial data support this assertion and we suggest that mTOR does not play an active role in the maintenance or growth of fibrofolliculomas, in contrast to angiofibromas in TSC.

We have considered alternative explanations for the observed lack of efficacy. Firstly, the concentration of rapamycin in the lotion might have been too low. However, the oral rapamycin solution that we used in this study has been shown to be an effective topical treatment for angiofibromas in TSC, without detectable levels of rapamycin in the serum.²⁶ A 1:1 mixture of rapamycin solution 1mg/ml in an emollient has also shown to be effective in TSC.²⁷ This indicates that rapamycin in this oral solution penetrates into the skin and that the concentration of rapamycin we used is sufficient to cause a clinical effect. Propylene glycol is part of the solvent of the oral rapamycin and is known to enhance topical drug delivery.⁴¹⁻⁴⁴ Secondly, patient compliance might have been too low. To measure compliance we planned to weigh the used bottles with rapamycin and placebo solution at every follow-up visit. Because the treated surface varied widely between patients and not all bottles were retrieved, compliance could not be determined in this way. No patient reported to have deviated from the treatment protocol, but we cannot rule out non-compliance. However, as most patients experienced one or more side effects, we are confident that they did indeed adhere to protocol. Thirdly, there was moderate variation between the doctors in scoring the change in cosmetic status and the size of FFs. Small random errors in classification of cosmetic status theoretically may result in obscuring an effect of rapamycin. However, it seems improbable that large, clinically relevant differences would be missed by lack of perfect agreement. Moreover, ICCs up to 0.878 were obtained for observer agreement on FF number. For this outcome measure the results clearly show no advantage of rapamycin treatment compared to placebo treatment.

In this randomised controlled trial both doctors' and patients' opinion on the efficacy of the topical rapamycin treatment of FFs in BHD was measured, giving disparate results. Patients reported improvement in some cases, whereas doctors observed no efficacy of rapamycin treatment. All three doctors were blinded and they evaluated the treatment twice at separate time points. Patients were also blinded at the start of the treatment, but because side effects occurred more often on the rapamycin treated side blinding could have been compromised. Therefore patients might have been biased towards experiencing improvement on the facial half with most side effects. This can explain the difference in doctors' and patients' opinion on improvement of the FFs after treatment.

Most reported side effects were a sign of skin irritation. Although these irritation signs occurred more often on the rapamycin treated side, they were also reported on the pla-

cebo treated side of the face. This suggests that the excipient, which contains ethanol, may have been responsible for some of the side effects.

In conclusion, we show that topical rapamycin as used by us is not an effective treatment for FFs in BHD. It is still possible that a higher concentration or oral administration could be effective. However, very recent work provides evidence that reduced expression of FLCN does not necessarily cause increased mTOR activity. Thus, our present findings do not support further study of rapamycin or similar compounds for the treatment of FF in BHD. Development of an effective topical treatment will have to await full elucidation of FLCN's functions in cellular signalling.

Supporting information

Supporting information is available at *PLOS ONE* online (<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0099071#s5>)

Appendix S1. Cross tabulations for cosmetic outcome and fibrofolliculoma number

Checklist S1. CONSORT checklist

Protocol S1. Trial protocol

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Birt–Hogg–Dubé syndrome is a novel ciliopathy

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Luijten M.N.*, Basten S.G.*, Claessens T.*, Vernooij M., Scott C.L., Janssen R., Easton J.A., Kamps M.A., Vreeburg M., Broers J.L., van Geel M., Menko F.H., Harbottle R.P., Nookala R.K., Tee A.R., Land S.C., Giles R.H., Coull B.J., van Steensel M.A.M.

* These authors contributed equally to the manuscript

Abstract

Birt-Hogg-Dubé (BHD) syndrome is an autosomal dominant disorder where patients are predisposed to kidney cancer, lung and kidney cysts and benign skin tumors. BHD is caused by heterozygous mutations affecting folliculin (FLCN), a conserved protein that is considered a tumor suppressor. Previous research has uncovered multiple roles for FLCN in cellular physiology, yet it remains unclear how these translate to BHD lesions.

Since BHD manifests hallmark characteristics of ciliopathies, we speculated that FLCN might also have a ciliary role. Our data indicate that FLCN localizes to motile and non-motile cilia, centrosomes and the mitotic spindle. Alteration of FLCN levels can cause changes to the onset of ciliogenesis, without abrogating it. In three dimensional culture, abnormal expression of FLCN disrupts polarized growth of kidney cells and deregulates canonical Wnt signalling. Our findings further suggest that BHD-causing FLCN mutants may retain partial functionality. Thus, several BHD symptoms may be due to abnormal levels of FLCN rather than its complete loss and accordingly, we show expression of mutant FLCN in a BHD-associated renal carcinoma. We propose that BHD is a novel ciliopathy, its symptoms at least partly due to abnormal ciliogenesis and canonical Wnt signalling.

Introduction

Birt-Hogg-Dubé (BHD) syndrome (MIM #135150) is a rare autosomal dominant disorder that was first described in 1975 by Hornstein and Knickenberg as a distinct disorder associated with intestinal polyps.¹ Birt, Hogg and Dubé later reported the same disorder, but in association with medullary thyroid carcinoma.² A clear association with kidney cancer, mostly of mixed clear cell/chromophobe histology³, was recognized in 1999⁴ and has been extensively documented since. The prevalence of BHD is estimated at 1/200,000 and the majority of papers published to date put the lifetime risk of developing renal cell carcinoma (RCC) in BHD patients at ~30%.⁵ Our own, more recent data suggest a range of 16–20%.³ A roughly similar risk exists for pneumothorax, possibly due to basal lung cysts that are present to a varying degree in almost all BHD patients. About 80% of BHD patients will develop benign skin lesions called fibrofolliculomas⁵, generally after the age of 35. An emerging aspect of the BHD phenotype is cyst formation in kidney, liver and the pancreas (Figure 1, and Kluger *et al.*).⁶ BHD is caused by mostly truncating mutations in the gene coding for the protein folliculin (FLCN)⁷, whose functions are largely unknown but which is considered a tumour suppressor.^{8,9} FLCN is an ancient and highly conserved protein, with multiple orthologs present in fungi and animals. Previous research suggests that FLCN is a downstream target of both AMP-dependent protein kinase (AMPK) and mammalian Target of Rapamycin complex 1 (mTORC1) signalling.¹⁰ FLCN might also modulate mTORC1, but conflicting data obtained in cells and tissues that lack FLCN show both up- and down-regulation of mTORC1 activity.^{9, 11–13} We recently reported that the absence of FLCN causes aberrant hypoxia-inducible factor 1 transcriptional activity and the Warburg effect, where FLCN-deficient cells favoured aerobic glycolysis over oxidative phosphorylation.¹⁴ Deregulation of transforming growth factor beta (TGFβ) signalling in FLCN-deficient cells has also been reported, although the reports are contradictory on the nature of FLCN's involvement.^{15, 16} FLCN has recently been implicated in control of ribosomal RNA synthesis through an interaction with the protein RPT4¹⁷, a finding that might explain the aberrant transcriptional activity observed in a number of studies.^{14, 15}

The von Hippel-Lindau (VHL) and Tuberous Sclerosis Complex disease syndromes, predisposing to malignant and benign renal tumours, respectively, have previously been linked to impaired cilia function and cyst formation.^{18, 19} Since BHD patients also develop cysts in multiple organs such as kidney, liver and lungs, we hypothesized that FLCN might similarly have a functional role in primary cilia and that ciliary dysfunction could contribute to the BHD phenotype. Cilia are microtubule-based structures that are enveloped by a highly specialized membrane and protrude from the apical cell membrane. Cilia formation is restricted to cells that have exited the cell cycle, allowing the centrosome to differentiate into a basal body that forms the base of the cilium.²⁰ Different ciliary subtypes have been

described, of which primary (non-motile) cilia are the most common and are considered to have primarily sensory functions. Motile cilia are predominantly involved in generating fluid flow.²¹

Figure 1. Birt-Hogg-Dubé syndrome is associated with development of renal cysts. (A) CT scan of a BHD patient. Coronal plane. Arrows indicate cysts in liver and kidney. (B) Paraffin-embedded samples were obtained from a renal carcinoma from a BHD patient with a c.499C>T mutation (encoding pGln167X). Immunohistochemical staining with custom made C terminal FLCN antibody revealed FLCN around kidney tubules and within the tumour. Highlighted area around the kidney cyst is shown in Figure 1C. Magnification 50x. Scale bar is 400µm. (C) Magnification highlighted area Figure 1B. Magnification 400x (D) H&E stain of highlighted area Figure 1B. Magnification 400x (E) H&E stain (composite of 3 images) of a cyst from Nihon Rat kidney tissue. H&E, Magnification 50x Scale bar is 100µm. (F) H&E stain (composite of 4 images) of a tumour from Nihon Rat kidney tissue. Magnification 50x Scale bar is 100µm. (G) H&E stain (composite of 6 images) of a cyst from Nihon Rat kidney tissue. There are clear cysts containing two distinct populations of cells. The first population with cuboidal morphology (arrow), and the second with an eosinophilic cytoplasm that protruded to into the cyst lumen (arrow-head). Magnification 50x. Scale bar is 100µm.

Dysfunction of (primary) cilia is a universal cause of the ciliopathies, which are characterized by pathophysiology associated with changes in the morphology and/or number of cilia. The large group of disorders classified as ciliopathies is particularly (but not exclusively) associated with cyst formation in several organs, including kidneys.^{22, 23} In recent years, it has become apparent that cilia act as a master switch essential to maintain normal kidney morphology. The mechanisms involved are far from resolved, but many links have

been made between ciliary signalling and planar cell polarity (PCP), the mechanism that defines the cellular orientation in tissue morphology.²⁴ Defective PCP can cause renal tubule elongation defects, which are believed to underlie cystic growth initiation.²⁵ Cysts may represent a potential pre-tumorigenic stage²⁶ and ciliary dysfunction is increasingly implicated in the pathogenesis of several cancer types, including renal carcinoma²⁷, melanoma²⁸, pancreatic ductal adenocarcinoma²⁹ and ovarian cancer.^{30, 31}

We set out to test our hypothesis by examining FLCN's subcellular localization in various cellular models and its influence on ciliary number, morphology and function. Our data suggest that BHD syndrome can be considered a novel ciliopathy, and that FLCN has a role in regulating cellular morphology and PCP, possibly through interaction with Wnt signalling. We also provide evidence that BHD patient derived FLCN mutants retain functionality, which has important implications for understanding and treating BHD manifestations. If therapeutic or preventive approaches to BHD-associated pathology are to be developed, FLCN's functional role(s) must ultimately be elucidated.

Results

BHD syndrome is associated with development of renal cysts

BHD patients develop cysts in multiple internal organs such as kidney and liver (Figure 1A). Using a custom-made antibody directed against the C-terminus of FLCN, we stained kidney tumour material derived from a BHD patient with a c.499C>T mutation (that encodes for a truncated FLCN mutant, pGln167X) (Figure 1B). Hence, this antibody will only detect the wild-type protein, not this mutant form. The kidney sample contains both unaffected kidney (Figure 1B, upper section) and clearly demarcated renal tumour material with mixed chromophobe/clear cell histology (Figure 1B, lower section). We observed strong expression within the normal kidney and clear staining for FLCN around the kidney tubules (Figure 1B and C and Supplementary Material, Figure S1). We also noted a cystic area devoid of cells (Figure 1B–D), which represents a small kidney cyst. Previously, we determined that the remaining *FLCN* allele in this tumour contains a second hit of a 15 base pair deletion, predicted to result in the deletion of exon 6.³² Within the tumour mass, we still observe staining for FLCN (Figure 1B, lower section), suggesting the presence of a mutant form of the protein in this particular tumour. Next we examined kidney material obtained from the *Nihon* rat BHD animal model.³³ The *Nihon* rat has a naturally occurring mutation in *Flcn* that is predicted to result in a severely truncated Flcn protein. Upon examination of the kidney, multiple cysts (Figure 1E) as well as cancerous lesions were evident (Figure 1F). Of note, the cysts contained two populations of cells — one population of clear cells with a cuboidal morphology that lined the cyst (Figure 1G,

Supplementary Figure S1. Additional IHC staining for Paraffin-embedded BHD renal carcinoma. Paraffin-embedded samples were obtained from a renal carcinoma from a BHD patient with a c.499C>T mutation (encoding pGln167X). (A) Immunohistochemical staining (composite of 6 images) with custom made N terminal FLCN antibody revealed FLCN around kidney tubules and within the tumour. Magnification 50x. Scale bar is 100µm. (B) Immunohistochemical staining (composite of 10 images) with FLCN-AP antibody revealed FLCN around kidney tubules and within the tumour. Magnification 50x. Scale bar is 100µm. (C) Control immunohistochemical staining (composite of 6 images) with secondary antibody only. Magnification 50x. Scale bar is 100µm.

see arrow) and another population with eosinophilic cytoplasm that grew into the cyst lumen (Figure 1G, see arrowhead).

Thus, both humans and rats with BHD syndrome develop kidney cysts, highlighting that BHD might be a member of a larger group of disorders called ciliopathies, of which cysts in various organs, particularly the kidneys, are hallmark lesions. Ciliopathies are associated with cystic disease linked to changes in the number and/or shape of cilia.²³ As ciliopathy proteins often localize to the cilium (although this is not always a prerequisite)³⁴, we hypothesized that FLCN might localize to cilia and proceeded to examine whether FLCN is a ciliary protein.

FLCN localizes to a number of microtubule-based structures

We determined the localization of endogenous FLCN in several different cell types that form primary cilia. To rule out the possibility of a non-specific staining, we utilized several different FLCN antibodies. In all cell lines and with all antibodies used, we observed a clear and specific staining of anti-FLCN antibodies to primary cilia (Figure 2A and Supplementary Material, Figure S2). To further rule out the possibility of a non-specific antibody stain, we stably expressed EGFP-tagged FLCN (hereafter referred to as EGFP-FLCN WT) in type II Madin-Darby Canine Kidney (MDCK) cells. In these cells, we observed EGFP-tagged FLCN localizing to cilia (Supplementary Material, Figure S4A). In line with previous data, we observed FLCN localization throughout the cytoplasm and in the nucleus.^{10, 35} Interestingly, we noted that anti-FLCN antibody also localized to 1 or 2 discrete foci within the cell. We subsequently stained with the centrosome marker gamma-tubulin, and found co-localization, indicating that FLCN is a centrosomal protein (Figure 2B). We confirmed these results in the stable cell line, where EGFP-FLCN WT (in addition to cilia) localizes at 1 or 2 discrete foci, indicating that after centrosome duplication FLCN localizes to both the centrosomes (Figure 2C). Next we considered the possibility that FLCN might be a universal constituent of both primary and motile cilia and their respective basal bodies. In primary human nasal epithelial (HNE) cells that contain beating motile cilia, we observed localization of FLCN to the cilia (Figure 2D) and at the base plate, which is the basal body equivalent for motile cilia (Figure 2E). In *Drosophila melanogaster*, *BHD*^{-/-} males exhibit a fertility defect.³⁶ We therefore asked whether FLCN is present in sperm flagellae or their associated structures. The sperm flagellum is structurally identical to motile cilia, although its beating pattern is different.³⁷ Some ciliopathies are associated with sperm motility defects.³⁸ As shown in Figure 2F, in human spermatozoa we observed clear staining of FLCN to the centriole (Figure 2F, arrow) and annulus (Figure 2F, arrowhead). The latter structure is equivalent to the ciliary diffusion barrier for non-motile cilia.³⁷ Faint flagellar staining can also be observed, but due to the low level of staining it is not clear whether this is an artefact or genuine localization of FLCN in the flagellum.

Figure 2. FLCN is a constitutive ciliary, basal body and centrosomal protein.

Figure 2. Continued. In this figure all endogenous FLCN stains were obtained using the FLCN-AP antibody. (A) FLCN localises to primary cilia. HK-2 cells serum starved for 48 hours were fixed and stained for endogenous FLCN (green) and acetylated alpha tubulin (red). Nuclei were stained with DAPI (blue). Cilium in the white box is shown in orthogonal view below. Scale bar is 10µm. (B) Endogenous FLCN localises to the centrosome. IMCD3 cells were fixed and stained for gamma-tubulin (red) and endogenous FLCN (green). Nuclei are stained with

DAPI (blue). Scale bar is 10µm. (C) EGFP-FLCN WT localises to the centrosome. MDCK cells stably expressing EGFP-tagged FLCN WT were fixed and counterstained with anti-gamma-tubulin (red) to mark the centrosomes (indicated by arrows). EGFP-FLCN WT localises at centrosome before (Ci scale bar is 20µm) and after centrosome duplication (Cii scale bar is 10µm). Nuclei are stained with DAPI (blue). (D) FLCN localises to motile cilia. (i) Single stain. Primary Human Nasal Epithelial (HNE) cells were fixed and stained for endogenous FLCN (green). Middle panel is differential interference contrast (DIC) image for visualisation of cilia (indicated by the arrow). Scale bar is 20µm. (ii) Double stain. HNE cells were fixed and stained for endogenous FLCN (red) and acetylated alpha tubulin (green). Scale bar is 10µm. (E) FLCN localises to the basal body. HNE cells were fixed and stained for endogenous FLCN (red) and pericentrin (green) to mark the base plate. Scale bar is 20µm. (F) In human sperm endogenous FLCN localises to the centriole and annulus. Human sperm cells were fixed and stained for endogenous FLCN (red). Note the staining at the centriole (arrowhead) and annulus (arrow). Scale bar is 10µm. (G) FLCN localises to the mitotic spindle. HK-2 cells overexpressing EGFP-FLCN WT were fixed and counterstained with acetylated alpha tubulin (red) to mark the mitotic spindles (indicated by arrows) and DAPI (blue). Scale bar is 20µm.

Finally, several known ciliary proteins such as IFT88, LKB1 and VHL³⁹⁻⁴¹ are known to localize to the mitotic spindle. To see if this is the case for FLCN, we examined mitotic cells expressing EGFP-FLCN WT and indeed, we observed the EGFP-tagged FLCN protein localizing to the mitotic spindle (Figure 2G).

Disease-associated mutations of FLCN do not affect subcellular localization

Having observed full-length FLCN localizing to cilia, the basal body and the centrosome, we reasoned that disease-associated mutations might affect FLCN's subcellular localization. Therefore, we expressed EGFP-tagged versions of several disease causing FLCN mutations in the immortalized proximal kidney tubule cell line HK-2.⁴² The tagged versions of missense mutations p.His255Arg (H255R) and p.Lys508Arg (K508R) could be visualized at the centrosome (Supplementary Material, Figure S3 and Supplementary Material, Table S1). Next, we examined EGFP-tagged patient mutations that result in C-terminal deletions, p.Tyr463Stop (Y463X), and the most common mutation, p.His429ProfsX27. Again, both of these truncating mutants localize to the centrosomes. These observations suggest that BHD-associated truncating FLCN mutations do not prevent centrosomal localization. Unfortunately, due to consistently low expression levels and a deleterious effect on ciliation, localization of the H255R, Y463X and p.His429ProfsX27 mutant FLCN proteins to cilia and the mitotic spindle could not be determined. However, we successfully visualized EGFP-FLCN K508R in the cilia (Supplementary Material, Figure S4A) and at the mitotic spindle (Supplementary Material, Figure S4B). Thus, the localization of the K508R mutant seems comparable with that of the wild-type protein.

Supplementary Figure S2. FLCN is detected at the primary cilia of various cell types using various anti-bodies. Normal human dermal fibroblasts (NHDF), retinal pigmented epithelial cells (RPE), UOK257-2 and MDCK cells were allowed to ciliate, fixed and stained for acetylated alpha tubulin to mark the ciliary axoneme (red) and for endogenous FLCN (green) using indicated FLCN antibodies. Nuclei were stained with DAPI (blue). Cilia in white boxes are shown in orthogonal views below. Scale bar is 10 μ m.

Supplementary Figure S3. Disease-associated mutations of FLCN do not affect FLCN localisation to the centrosome. HK-2 cells were transiently transfected with the indicated EGFP tagged disease-causing mutations. 24 hours post transfection cells were fixed and counterstained with gamma-tubulin (red) to mark the centrosome (indicated by white arrows). Nuclei are stained with DAPI (blue). Scale bar is 20µm.

The BHD-derived renal cancer cell line UOK257 shows abnormal ciliation

As loss of cilia is a frequent occurrence in kidney cancer²⁷, we were interested whether BHD-derived renal cancer cells can form cilia. UOK257 is a BHD-derived kidney tumour cell line carrying the most common germline FLCN mutation, c.1285dupC⁴³, resulting in the FLCN truncation p.His429ProfsX27. Whereas BHD patients are heterozygotes, and still have one wild-type allele for the *FLCN* gene, the UOK257 cell line used in this study has been reported

to have lost the wild-type allele and consequently only possesses the mutant copy.⁴³ The presumed lack of functional FLCN might impair ciliogenesis. To test this idea, we compared the ability of UOK257 and UOK257-2 (UOK257 cells with re-expression of FLCN)¹⁰ to ciliate. Neither cell line ciliated upon serum starvation, however, cilia were evident in both cell lines when left at full confluence for several days (Figure 3A). Interestingly, we noted that after 6 days, confluent FLCN-rescued UOK257-2 cells exhibited fewer cilia than UOK257 cells. When we increased this time to 10 days, this difference between the cell lines was no longer evident (Figure 3A), suggesting that overexpression of FLCN may impede formation of primary cilia. The length of the cilia observed in these UOK257 cells ($6.3 \pm 2.5 \mu\text{m}$ $n=74$) was not significantly different to that observed in our control HK-2 cell line ($7.0 \mu\text{m} \pm 3.2 \mu\text{m}$ $n=147$).

Supplementary Figure S4. The disease-associated mutation K508R of FLCN does not affect FLCN localization to the cilia or mitotic spindle. (A) Orthogonal projections of MDCK cells stably overexpressing EGFP, EGFP-FLCN WT or EGFP-FLCN K508R maintained at confluence for 5 days, fixed and stained for acetylated alpha tubulin to mark the ciliary axoneme (red). Nuclei were stained with DAPI (blue). (B) MDCK cells overexpressing EGFP-FLCN K508R were fixed and counterstained with acetylated alpha tubulin (red) to mark the mitotic spindles (indicated by arrows) and DAPI (blue). Scale bar is $10\mu\text{m}$.

Supplementary Table S1. Localisation of EGFP tagged constructs to the centrosome

construct	Centrosomal localisation
EGFP	No
EGFP-FLCN Wild Type	Yes
EGFP-FLCN His255Arg (H255R)	Yes
EGFP-FLCN Lys508Arg (K508R)	Yes
EGFP-FLCN Tyr463 Stop (Y463X)	Yes
EGFP-FLCN His429 ProfsX27	Yes

Given the apparent differences we observed in ciliation between the UOK cell lines, we then sought to confirm their identity by sequencing the *FLCN* locus. Upon genomic sequencing of all of the *FLCN* exons using intronic primers, the only *FLCN* mutation detected was His429ProfsX27. However, it was also evident that a low level of the wild-type allele was present in the cells (Figure 3B). This finding was subsequently confirmed by both immunofluorescence and western blotting experiments (see Supplementary Material, Figure S5). Thus, we conclude that UOK257 cells are not *FLCN* null, as originally reported, but rather still express full-length, wild-type *FLCN*.

Knockdown of *FLCN* in human kidney cells delays ciliogenesis

Considering that UOK257 cells express a lower level of full length *FLCN* than seen in HK-2 cells (Supplementary Material, Figure S5C), we wondered if the ciliary phenotype observed in these cells resulted from reduced levels of wild-type *FLCN*. Therefore, we generated stable knockdowns of *FLCN* in HK-2 cells (see Supplementary Material, Figure S6). Subsequently, we selected *FLCN* knockdown HK-2 clone A5 (hereafter referred to as *FLCN* KD, estimated 50% knockdown) and the scrambled control HK-2 clone G10 (hereafter referred to as NT; Figure 4A). Knockdown of *FLCN* did not alter cell morphology or growth rate (data not shown). Cells were plated at low density and subsequently serum-starved for 48, 72, 96 and 120 hours to initiate ciliogenesis. As seen in Figure 4B, at 48 and 72 hours, the *FLCN* KD cell line showed a significant reduction in number of ciliated cells compared to the NT control cells. Knockdown or knockout of other ciliary proteins can also cause changes in the length of cilia.²⁰ Cilia in the HK-2 *FLCN* KD cells showed a slight but significant reduction in length versus the NT control cell line after 48 hours of serum starvation. This was not evident after 72 hours of serum starvation (Figure 4C). At the later time points of 96 and 120 hours, all cell lines exhibited more cilia, and no significant difference in the number of ciliated cells between *FLCN* KD and NT control was evident (Figure 4C). Collectively, these data suggest that ciliogenesis is delayed rather than inhibited upon *FLCN* knockdown.

Figure 3. The BHD-derived renal cancer cell line UOK257 shows abnormal ciliation. (A) UOK257 and UOK257-2 cells were maintained at confluence for indicated amount of days, fixed and stained with acetylated alpha tubulin to mark the ciliary axoneme and pericentrin to mark the ciliary base. Nuclei were stained with DAPI and the percentage of ciliated cells was counted using ImageJ. After 6 days of confluence UOK257-2 had a significantly reduced number of cilia compared to UOK257 ($p=0.023$). After 10 days of confluence both cell lines have a similar number of cilia ($p=0.422$). Error bars represent standard error of the mean. A minimum of 365 cells were counted per cell line per time point. * is $p<0.05$. (B) Sequencing of genomic DNA isolated from HK-2 and UOK257 cells showing presence of the wild-type allele (starting sequence indicated by the arrow).

Supplementary Figure S5. UOK257 cells express wild-type FLCN.

Supplementary Figure S5. Continued. (A) UOK257 and UOK257-2 cells were fixed and stained for endogenous FLCN (green) with indicated antibodies. Scale bar is 20µm. (B) MDCK cells transiently transfected with EGFP-FLCN WT or the disease-causing variant EGFP-FLCN p.His429ProfsX27 were fixed and stained with antibodies (red) against full-length FLCN (top panel) and the C-terminus of FLCN (bottom panel). Nuclei were stained with DAPI (blue). Scale bar is 20µm. (C) Western blot on 50 µg of whole cell lysate of indicated cell types probed with custom made N-terminal FLCN antibody. Gamma tubulin was used as a loading control.

Figure 4. Knockdown and exogenous expression of FLCN affects ciliogenesis in kidney cells. (A) Western blot of knockdown and control cells. Western blot on 30 μ g of whole cell lysate showing stable knockdown of FLCN using shRNA in HK-2 clone FLCN KD compared to wild-type HK-2 and HK-2 with scrambled control shRNA clone NT, (also see supplementary Figure S6). Membrane was probed with FLCN-AP antibody. Actin was used as a loading control. (B) Measurement of ciliogenesis in FLCN knockdown versus non target control. HK-2 FLCN KD and NT cells were serum starved for the indicated amount of hours, fixed and stained with acetylated alpha tubulin to mark the ciliary axoneme and pericentrin to mark the ciliary base. Nuclei were stained with DAPI and the percentage of ciliated cells and cilium length was measured using ImageJ.

After 48 ($p<0.001$) and 72 ($p<0.001$) hours of serum starvation, HK-2 FLCN KD cells showed a statistically significant reduction in the percentage of ciliated cells compared to NT cells. After 96 ($p=0.915$) and 120 ($p=0.067$) hours of serum starvation there was no difference in the percentage of ciliated cells between the two cell lines. Error bars represent standard error of the mean. Average of 350 cells counted per cell line per time point. * is $p<0.05$. (C) Measurement of cilia length in FLCN knockdown versus non-target control. Average cilium length of HK-2 FLCN KD cells ($n=40$) was significantly decreased compared to cilia of NT cells ($n=124$) after 48 hours of serum starvation ($p=0.001$). After 72 hours of serum starvation no difference in cilia length was observed between HK-2 FLCN KD ($n=24$) and NT cells ($n=55$) ($p=0.55$). Error bars represent standard error of the mean. * is $p<0.05$. (D) Western blot of FLCN expressing cell lines. Western blot probed with EGFP antibody showing MDCK cells with stable expression of EGFP alone, EGFP-FLCN WT and the disease-causing FLCN missense variant EGFP-FLCN K508R. Actin was used as a loading control. Actin is not detected in the EGFP only MDCK cells, as a result of 10-fold dilution of this sample due to very high EGFP expression in this cell line. (E) Growth curve in FLCN expressing cell lines. At a starting density of 10^5 cells per well, untransfected MDCK cells and MDCK cells with stable overexpression of EGFP, EGFP-FLCN WT or EGFP-FLCN K508R were plated. Cell number was determined at each time point. Each data point is the average of six independent counts. (F) Measurement of ciliogenesis in FLCN expressing cell lines. Untransfected MDCK (MDCK) and MDCK cells stably overexpressing EGFP, EGFP-FLCN WT (WT) or EGFP-FLCN K508R (K508R) were maintained at confluence for 5 days, fixed and stained with acetylated alpha tubulin to mark the ciliary axoneme and with DAPI to stain nuclei. Cells expressing EGFP-FLCN WT showed a statistically significant reduction in the percentage of ciliated cells compared to cells expressing EGFP alone ($p<0.001$) or EGFP-FLCN K508R ($p<0.001$), as counted using ImageJ. Error bars represent standard error of the mean. * is $p<0.05$.

Exogenous expression of FLCN affects proliferation and ciliogenesis

Having noted that exogenous expression of FLCN in UOK257-2 cells seemed to reduce the number of cilia observed we then decided to examine the effect of exogenous FLCN expression on ciliation in more detail. To do this, we generated cell lines expressing EGFP alone or EGFP-FLCNWT from an expression construct. We also attempted to make stable cell lines expressing the different EGFP-tagged patient mutations described above. However, only the FLCN missense variant EGFP-FLCN K508R resulted in a usable stable cell line (Figure 4D). We noted that the cell lines expressing exogenous EGFP-FLCN seem to grow more slowly than the cells expressing EGFP alone and we therefore measured the doubling time of these cells. As shown in Figure 4E and Supplementary Material, Table S2, expression of EGFP-FLCN WT and EGFP-FLCN K508R significantly increased the cellular doubling time, when compared with the control cell lines. This finding contrasts with previously published data, suggesting that a missense mutant (H255R) does not affect cell growth.⁸ Next, we tested the ability of these different cells to ciliate (Figure 4F).

To initiate formation of cilia, all cells were cultured to 5 days post-confluence. Cells expressing EGFP-FLCN WT ($52.5\% \pm 4.77$ $n=705$) exhibited a statistically significant reduction in the number of cilia relative to the EGFP alone control (EGFP $70\% \pm 0.12$ $n=825$). Interestingly, despite the apparently normal localization and similar growth defect to that observed for wild-type FLCN, EGFP-FLCN K508R ($66.5\% \pm 2.03$, $n=670$) did not change the percentage of ciliated cells when compared with untransfected or cells expressing EGFP alone (Figure 4F).

Supplementary Figure S6. Knockdown of FLCN using shRNA in HK-2 cells. (A) Schematic representation of human *FLCN* locus. The 5'UTR is marked in blue, the coding exons in red and the 3' UTR in green. The sequence of *FLCN* shRNA5968 corresponds to bp 775 - 795 of the 3'UTR. (GCTGCTTTCAACATTTACGTTCTCGAGAACG-TAAATGTTGAAAGCAGC). The sequence of *FLCN* shRNA5970 corresponds to bp 1677 -1697 of exon 14 of the coding sequence. (GACCTACAAGTCACACCTCATCTCGAGATGAGGTGTGACTTGTAGGTC). (B) Western blot on 20 µg of whole cell lysate from HK-2 cells stably transfected with shRNA FLCN shRNA5968, shRNA FLCN shRNA5970, both FLCN shRNA's (shRNA5968 and shRNA5970) or the non-target shRNA control. The membrane was probed with FLCN-AP antibody and actin was used as a loading control. FLCN knockdown HK-2 clone A5 and FLCN knockdown non-target control HK-2 clone G10 were selected.

Supplementary Table S2. Average Doubling time of MDCK

Cell Line	Average doubling time (hours)	SEM (hours)
MDCK	13.7	1.8
MDCK EGFP	14.3	1.2
MDCK EGFP-FLCN	17.3	1.1
MDCK EGFP-FLCN K508R	16.3	0.8

Figure 5. Knockdown or expression of exogenous FLCN impairs kidney cell spheroid growth in three-dimensional culture. (A) Knockdown of FLCN impairs kidney cell spheroid growth in three-dimensional culture (i) IMCD3 cells were treated with non-targeting RNAi (SiCON) or FLCN RNAi (SiFlcn) for 48 hours. The cells were subsequently split into 2 equal parts, with the first half cultured for three days in matrigel and the remainder in 2D culture for quantification of knockdown shown in (ii). Cultures were fixed and stained. Tight-junction marker ZO-1 (orange) localises to the apical side, β -catenin (green) is enriched at lateral cell-cell contacts and acetylated alpha tubulin stains cilia (red) that are present protruding into the lumen. Nuclei are stained with DAPI. Representative spheroids are shown. SiCON cells form a typical spheroid that is evenly rounded and has a clear lumen. Cells treated with SiFlcn form spheroids with impaired ciliogenesis, reduced overall and luminal sizes. Arrows indicate cilia containing β -catenin. The RNA was extracted from cells at the same time point as the 3D spheroids shown

Figure 5. Continued. in (i) were fixed. (iii) Quantification of the number of cilia observed in non-target siCON or the *siFlcn* spheroids. Spheroids with reduced *Flcn* levels exhibit significantly decreased numbers of cilia compared to the siCON control ($p > 0.0001$). (B) Knockdown of FLCN in IMCD3 decreases spheroid and lumen size in three-dimensional culture. IMCD3 cells with siRNA mediated knockdown of *Flcn* form spheroids that consist of the same number of cells (i) but are significantly smaller in size ($p = 0.0058$) (ii) and have a smaller lumen (ii, $p = 0.0003$) compared to control spheroids. (C) Knockdown of FLCN impairs spindle orientation in three-dimensional culture. (i) Representative image of misoriented, dividing cell and a cell that ended up outside the normal plane (white arrows) in *siFlcn* spheroid versus control. Cells are stained with DAPI. Scale bar 20 μm . (ii) Quantification of cell division orientation in IMCD3 spheroids treated with *SiFlcn*, which show a significant increase in the number of misorientated spindles per spheroid compared to control spheroids ($p < 0.0001$). (D) Expression of wild type but not K508R

FLCN impairs kidney cell spheroid growth in three-dimensional culture. MDCK cells stably overexpressing EGFP, EGFP-FLCN WT or EGFP-FLCN K508R were grown in collagen for 11 days. Cultures were fixed and stained with DAPI (blue). Note the lack of lumen formation in the cells overexpressing EGFP-FLCN WT in the 2D and 3D projection. Scale bar is 20µm. (E) Quantification of spheroid parameters after FLCN expression in IMCD3. (i) IMCD3 cells with stable expression of EGFP-FLCN WT form spheroids that are significantly smaller in size ($p<0.0001$) and have a smaller lumen ($p<0.0001$) compared to control spheroids expressing EGFP only. (ii) Expression of EGFP-FLCN K508R results in normal-sized spheroids ($p=0.2381$) with a slight reduction in lumen size ($p<0.0001$) compared to control spheroids expressing EGFP only.

Knockdown and exogenous expression of FLCN impairs kidney cell spheroid growth in three-dimensional culture

Having established that exogenous expression and knockdown of FLCN both adversely affect cilia number in monolayer culture, we then assessed the effect of modulating FLCN expression in a 3D culture system. Due to the complex signalling required for proper spatial orientation, a mild ciliopathy phenotype in monolayer culture can translate into a more severe one in 3D cultures.³⁴ MDCK and IMCD3 cell lines cultured in 3D are both established models for renal morphogenesis, mimicking normal renal tubules by forming spheroids or tubules composed of polarized cells exhibiting a lumen lining a ciliated apical membrane. We considered that BHD cyst formation could be related to delayed ciliogenesis resulting from reduced levels of wild-type FLCN. Therefore, we assessed the effects of FLCN knockdown in detail in the 3D system. Spheroid formation in cells treated with siRNA against *Flcn* (*SiFlcn*) was severely impaired when compared with non-target siRNA (*SiCON*) (Figure 5Ai). The level of knockdown achieved was ~60% (Figure 5Aii) and in line with previous data, fewer cilia were observed in *Flcn* knockdowns compared with non-target controls (Figure 5Aiii).

The number of cells composing the median section of a spheroid did not differ significantly in the *SiFlcn*-treated cells versus control knockdown (Figure 5Bi). We then determined the overall and lumen size of these spheroids. As shown in Figure 5Bii, while *SiFlcn* only resulted in a small decrease in the overall size of the spheroid, it did cause a marked and significant decrease in luminal size when compared with the control spheroids. In addition to the effects of *Flcn* knockdown on cilia frequency and lumen size, we observed an increase in improper orientation of cell divisions. In normal spheroids, cell division occurs in plane with the apical membrane, directed towards the lateral membranes (Figure 5Ci). By scoring the number of cells that are either actively dividing in the wrong orientation, or have ended up outside the normal plane, we found that *Flcn* knockdown causes a strong increase in abnormally orientated cell divisions (Figure 5Cii). Having observed that knockdown of *Flcn* affects spheroid formation in 3D cultures, we then assessed the effect of exogenous expression of EGFP-FLCN WT in 3D spheroids. Consistent with the previously observed growth defect in cells expressing EGFP-FLCN WT, the number of cells composing an EGFP-FLCN WT spheroid was reduced when compared with the control cell line (Supplementary Material, Figure S7A). We then compared the overall and lumen size of

the EGFP-FLCN WT spheroids to that of the EGFP alone control cell line (Figure 5Ei). Interestingly, spheroid formation in cell types expressing EGFP-FLCN WT was severely impaired, with smaller spheroids exhibiting a reduction (or absence) in the lumen size. Thus, both knockdown and exogenous expression of FLCN perturb lumen formation, with the latter giving a more severe phenotype.

Supplementary Figure S7. Spheroid formation in IMCD3 cells. (A) Quantification of EGFP-FLCN WT spheroids versus the control reveals a significant reduction in the number of cells composing a spheroid ($p < 0.0001$). (B) Quantification of EGFP-FLCN K508R spheroids versus the control reveals a significant reduction in the number of cells composing a spheroid ($p = 0.0005$). (C) Lack of localisation of β -catenin to cilia in *siFlcn* spheroids. IMCD3 cells were treated with FLCN RNAi (*siFlcn*) and cultured for three days in matrigel. Cultures were fixed and stained. Tight-junction marker ZO-1 (orange) localises to the apical side, β -catenin (green) is enriched at lateral cell-cell contacts and acetylated alpha tubulin stains cilia (red) that are present protruding into the lumen. Nuclei are stained with DAPI. Arrows indicate position of cilia. To rule out the possibility of low level of β -catenin in cilia a long exposure time was used. Scale bar is 10 μ m.

BHD-associated FLCN missense mutation is partially dysfunctional with respect to spheroid formation

Next, we tested the ability of the K508R mutant to influence spheroid formation, as this mutation does not seem to affect ciliogenesis. In contrast to EGFP-FLCN WT, EGFP-FLCN K508R spheroids appear similar to normal spheroids (Figure 5D), where a clear lumen can be observed. The size of the spheroid and lumen was determined and while the overall size was similar, the lumen size was found to slightly deviate from EGFP controls, suggesting that K508R may have a mild effect on lumenogenesis. In line with the previous growth

rate data, the number of cells composing an EGFP-FLCN K508R spheroid was reduced when compared with the control cell line (Supplementary Material, Figure S7B).

These observations further suggest that the spheroid formation defect observed upon FLCN knockdown or exogenous expression could be linked to ciliary dysfunction.

Flcn knockdown results in β -catenin delocalization and activates canonical Wnt signalling

In the spheroid assays, we used β -catenin as a standard marker for the basolateral membranes. When we performed these assays, we noted that β -catenin can often be observed in the cilium (Figure 5Ai, indicated by arrow). Intriguingly, in the few cilia present in the spheroids treated with *SiFlcn*, we rarely observed this localization of β -catenin (Supplementary Material, Figure S7C). The sequestration of β -catenin to cilia has been reported to dampen canonical Wntless signalling⁴⁴ and abnormal Wnt/ β -catenin signalling has previously been implicated in renal cystogenesis.⁴⁵ Therefore, we hypothesized that the observed defects in spheroid formation upon knockdown of *Flcn* might be a consequence of altered Wnt signalling. Thus, we examined canonical Wnt activity by looking at β -catenin phosphorylation and *Axin2*/*CyclinD1* mRNA levels. Upon *SiFlcn* treatment, no reduction in the total amount of β -catenin was evident when compared with control RNAi (Figure 6A). However, an antibody against the active, unphosphorylated form of β -catenin showed clear activation of β -catenin upon *Flcn* knockdown. Furthermore, quantitative RT-PCR of downstream target genes *Axin2* and *CyclinD1* shows up-regulation of these two genes upon *Flcn* knockdown, further supporting activation of the canonical Wnt pathway (Figure 6B).

Figure 6. FLCN knockdown causes activation of canonical WNT signalling. (A) Knockdown of FLCN results in activation of β -catenin. Western blot on 30 μ g of whole cell lysate from treated IMCD3 cells in 2D culture. Cells were harvested at 60 hours post transfection. Cells were serum starved for 18 hours prior to harvesting. IMCD3 express equal amounts of total β -catenin (middle panel) upon treatment with non-targeting SiCON or SiFLcn or treatment with 20 mM KCl or LiCl for 16 hours. Using an antibody to detect the unphosphorylated, active β -catenin, cells treated with LiCl (an activator of β -catenin through inhibition of GSK3 β) showed increased active β -catenin as did SiFLcn treated cells (upper panel). The β -actin (lower panel) was used as a loading control. (B) Activation of downstream targets of the WNT pathway upon FLCN knockdown. The activation of the WNT pathway was further confirmed using quantitative RT-PCR of downstream target genes *Axin2* and *CyclinD1*. Upon *Flcn* knockdown, mRNA levels of both genes exhibited up-regulation.

Discussion

BHD patients show cyst formation in lungs, kidney and liver.⁶ Cyst formation in multiple organ systems is a hallmark characteristic of ciliopathies²³, which led us to hypothesize that ciliary dysfunction could contribute to the BHD phenotype. Our results suggest that this is indeed the case and provide a possible explanation for key aspects of the BHD phenotype. In a kidney sample taken from a BHD patient during surgery for renal cancer, we observed a cyst and a tumour in close proximity. Interestingly, we found similar arrangements in Nihon rat kidneys (data not shown). It is well established that in this model, cancer is preceded by the appearance of cuboidal cells with a clear cytoplasm lining dilated collecting ducts, followed by cells with acidophilic cytoplasm.³³ The cancers that appear around 8 weeks consist of both clear and acidophilic cells. Large cysts such as the one shown in Figure 1E and G have not been reported as a microscopic finding, but macroscopic ones have.³³ Of note, the cysts that we observed in the rat are lined with clear cuboidal cells; acidophilic cells grow into the cyst lumen (Figure 1G). The tumours contain both cell types, with the clear cells consistently located towards the periphery. We note that human BHD RCC show the same arrangement of cells.³ We suggest from these observations that in humans with BHD, as in the Nihon rat, tumours develop from cysts. Hence, understanding cyst formation can help elucidate carcinogenesis in BHD.

FLCN is a dose-dependent regulator of cilia formation

Ciliogenesis is a process requiring complex regulation that is not yet fully understood. Entrance into the G0 phase of the cell cycle and establishment of cell polarity are considered to be the first prerequisites for ciliation.⁴⁶ We observe that levels of FLCN affect the timing of ciliation. We also show that endogenously expressed FLCN, and tagged versions of FLCN, very specifically localize to tubulin-based structures such as motile and non-motile cilia, centrosomes and the mitotic spindle in a variety of cell types. HK-2 cells with reduced endogenous FLCN expression show reduced ciliation and a subtle but significant decrease in ciliary length at early starvation time points, which are subsequently overcome. These findings indicate that cells with less FLCN have a delay in ciliogenesis, compared with normal cells. IMCD3 spheroids recapitulate this reduction in ciliation upon knockdown of *Flcn*. Furthermore, the expression of exogenous wild-type FLCN also resulted in reduced ciliation, suggesting that tight regulation of FLCN levels is required for normal ciliogenesis.

We have not observed any obvious defects in the size or shape of matured cilia, indicating that once ciliogenesis has been initiated, FLCN's role is permissive. In line with other renal cell types examined (MDCK and HK-2), under conditions of confluence, UOK257 cells do have the ability to form cilia. However, despite the apparently normal appearance of these cilia in UOK257 cells, the actual percentage of cells exhibiting cilia is low relative to other cell lines examined. Similarly, ciliation is delayed in these UOK257 cells. The low level of and delay in ciliation observed may be a consequence of a reduced level of full-length FLCN expression in these cells. The abnormal ciliation in UOK257-2 is consistent with our observations in other cell types and suggests that FLCN expression levels are critically important for correct timing of ciliogenesis.

FLCN regulates canonical Wnt signalling and spindle orientation in spheroids

We previously described the IMCD3 spheroid culture system as a suitable method to assess ciliopathy genes and their effects on renal morphology.³⁴ The siRNA-mediated knockdown of FLCN in IMCD3 cells demonstrates a role for the protein in establishing cellular orientation, as the spheroids formed by these knockdown cells exhibit an increased number of cells outside of the normal plane of division. This mitotic spindle misalignment may reflect disturbed PCP (controlled by non-canonical Wnt signalling), abnormal cortical microtubule function or mislocalized polarity proteins. Cilia have a role in the process of polarization by dampening the activity of the canonical Wnt pathway via sequestration of β -catenin.⁴⁴ Accordingly, FLCN knockdown seems to reduce ciliary β -catenin retention and is associated with an increase in the level of unphosphorylated β -catenin. The lack of phosphorylation of β -catenin results in the stabilization of the protein and an increase in canonical Wnt activity.⁴⁷ As a consequence, non-canonical PCP might be inhibited⁴⁸, resulting in spindle misalignment. In view of the increased Wnt activity, it is of considerable

interest that recent observations clearly show an increased incidence of kidney cancer in people who chronically use lithium chloride for psychiatric conditions.⁴⁹ Lithium activates canonical Wnt signalling through repression of GSK3 β activity.⁵⁰ The spectrum of renal tumours associated with lithium use is remarkably similar to that seen in BHD. Thus, we propose that carcinogenesis in BHD may be partly due to deregulated canonical Wnt signalling.

An alternative explanation for the spindle orientation defect could be that FLCN does not exert a direct effect on PCP signalling, but rather is actively involved in spindle orientation. The observation that EGFP-FLCN WT localizes to the centrosome and mitotic spindle is compatible with this idea. Other ciliary proteins have been shown to affect spindle positioning. For instance, the classic ciliary transport protein IFT88 localizes to the centrosome in mitosis, where it plays a fundamental role in microtubule transport.³⁹ FLCN might exert a similar function in controlling essential processes at the spindle pole, defects of which result in misorientation of the mitotic spindle. Finally, a recent report describes that LKB1 regulates spindle orientation through AMPK, possibly through microtubule destabilization⁴⁰, and we have previously demonstrated an increase in AMPK phosphorylation in UOK257 cells.¹⁴

Very recently, Nookala *et al.*⁵¹ provided evidence that the C-terminal half of FLCN may act *in vitro* as a guanine nucleotide exchange factor for Rab35, a poorly characterized GTPase involved in endocytotic membrane recycling and in the final steps of cytokinesis.⁵² How this function relates to the pervasive cellular abnormalities caused by FLCN's absence and the BHD phenotype is not yet clear. However, both ciliogenesis and mitotic spindle positioning require vesicular transport, which exhibit significant overlap⁵³ and we show that these processes are affected in BHD. Thus, FLCN may have a function in intracellular transport events involved in ciliogenesis.

FLCN might exhibit a partial genotype-phenotype correlation

To date, no clear correlation has been found between mutations in *FLCN* and the severity of BHD symptoms observed in patients. However, our findings with FLCN K508R may indicate that there might be a milder form of BHD associated with specific missense mutations. The growth defect caused by K508R and its cellular localization are indistinguishable from the wild-type protein. However, in contrast to wild-type FLCN, expression of K508R does not seem to adversely affect ciliogenesis and only mildly affects the spheroid forming abilities of the cell. These results are suggestive of at least some (partial) functionality of K508R mutant. This is in line with the published clinical information.⁵⁴ The patient, in whom the mutation was found, presented with bilateral multifocal benign renal oncocytomas and was only diagnosed with BHD upon sequencing of the oncocytomas. At the time of

publication, they did not manifest any of the other known BHD symptoms (W.M. Linehan, personal communication, 2013). It is tempting to speculate that, if kidney cancer in BHD arises from cysts, mutations that do not affect ciliogenesis will not be associated with malignancy. Another missense mutation (p.Arg239Cys) has been reported in a patient presenting with kidney cancer.⁵⁵ If our reasoning is correct, this particular mutant should affect ciliogenesis.

Mutant FLCN alleles might contribute to the phenotype

From our observations, we think that the clinical manifestations of BHD may not result from simple loss of one FLCN allele. Rather, the mutant versions of FLCN could also contribute to the BHD phenotype, perhaps in a dominant negative manner. Relative to the UOK257 cell lines, the restoration and exogenous expression of FLCN in UOK257-2 cells does not increase the low percentage of ciliated cells upon confluence. Mutant versions of FLCN that were tested localized to the centrosome and (at least for K508R) negatively impact cellular proliferation. Expression of truncated or wild-type FLCN seems to impede ciliogenesis (with the notable exception of K508R).

Complete loss of FLCN has been shown to be lethal^{9,13} and we would therefore not expect to encounter it in patients or in any tissue. Accordingly, the majority of BHD-associated kidney tumours have somatic second hits affecting FLCN, rather than loss of the wild-type allele.⁵⁶ We also show that truncated FLCN is still expressed in a renal cancer from a BHD patient (Figure 1 and (32). Finally, people with the Smith-Magenis syndrome (MIM182290), a congenital disorder caused by heterozygous deletion of chromosome 17p11.2 (including the region containing the *FLCN* locus), do not develop symptoms consistent with BHD.⁵⁷ In particular, patients do not exhibit an elevated risk of developing kidney cancer.

Based on these observations, we propose that a ‘just right’ hypothesis, as for the APC tumour suppressor⁵⁸, also holds true for FLCN.

In conclusion, we provide evidence that BHD syndrome is a novel ciliopathy. The causative protein, FLCN, localizes to motile and non-motile cilia, centrosomes and the mitotic spindle. It is involved in the initiation of ciliogenesis — once ciliation is underway, FLCN's role seems to be permissive. Knockdown of FLCN affects PCP, which might be due to the ciliation defect. Additional roles for FLCN in establishing cellular polarity seem likely and we have evidence for abnormal canonical Wnt signalling in the context of FLCN knockdown. Wnt signalling is potentially oncogenic and might thus present a new mechanism for carcinogenesis in BHD. Our data further suggest that the correct level of FLCN expression is critically important for its optimal function. Delayed ciliogenesis is likely to affect the ability of cells to orientate themselves in newly generated polarized tissues. The observations in Nihon rat kidney suggest

that cyst formation may be a prerequisite to cancer development. Hence, FLCN mutations that do not affect ciliogenesis, such as K508R, might not cause cancer and it would be of considerable interest to test this hypothesis using knock-in mouse models.

The classification of BHD as a ciliopathy should help better understand its pathogenesis, as the study of BHD can now benefit from the intense research effort on ciliopathies.

Materials and methods

DNA constructs and antibodies

Human *FLCN* was cloned into pEGFP C1 (Clontech, Mountain View, CA, USA) using *EcoRI*. EGFP-tagged FLCN mutant constructs were made by site-directed mutagenesis using the Quikchange II Site Directed Mutagenesis kit according to the manufacturer's instructions (Agilent, Santa Clara, CA, USA) with the following primers (Supplementary Material, Table S3). EGFP-tagged FLCN WT and mutants were cloned into the pQCXIP vector (Clontech) using *AgeI* and *EcoRI*.

Supplementary Table S3.Primer sequences used for site-directed mutagenesis

Construct	Primer Sequence
pEGFP-FLCN H255R	GGCAAAGGAGGTGCGCAGGCACGC
pEGFP-FLCN H255R	GCGTGCCTGCGCACCTCCTTTGCC
pEGFP-FLCN H429	AGGAGAGCACGTGGGGGGGGGATCT
pEGFP-FLCN H429	AGATCCCCCCCCACGTGCTCTCT
pEGFP-FLCN Y463X	GGTCACCACAACTCCTACTTGCTGAGAGACTG
pEGFP-FLCN Y463X	CAGTCTCTCAGCAAGTAGGAGTTTGTGGTGACC
pEGFP-FLCN K508R	CCACTCCTCCCTGAGGCAGACGAG
pEGFP-FLCN K508R	CTCGTCTGCCTCAGGGAGGAGTGG

Human-specific FLCN shRNA plasmid pLKO.1-puroshRNA5968 and the Non Target shRNA plasmid pLKO.1- puro-NonTarget (Sigma) were a kind gift from Prof. A. Pause (McGill University, Canada). Murine-specific Flcn siRNA oligos were purchased from Dharmacon (Lafayette, CO, USA).

Primary antibodies used for immunofluorescence microscopy are anti-FLCN-AP (rabbit polyclonal, a kind gift from Prof. A. Pause, 1:200), anti-FLCN (rabbit monoclonal, 3697 Cell Signalling, Danvers, MA, USA 1:200), anti-FLCN (rabbit polyclonal¹², a kind gift from Dr T. Cash 1:100), anti-pericentrin (rabbit polyclonal, ab4448 Abcam, Cambridge, UK, 1:1000), anti-gamma-tubulin (mouse monoclonal, T3559 Sigma 1:1000), anti-acetylated-alpha-tu-

bulin (mouse monoclonal, ab24610 Abcam 1:200), anti-acetylated- α -tubulin (mouse, T7451 Sigma, 1:20 000), anti- β -catenin (rabbit, AHO0462 Invitrogen, Paisley, UK, 1:500) and anti-ZO-1 (rat, R40.76 Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:500).

Secondary antibodies used were goat-anti-mouse Texas red (1010-07 Southern Biotech, Birmingham, AL, USA, 1:80), goat-anti-rabbit FITC (4050-02 Southern Biotech, 1:80), goat-anti-mouse Cy5 (1034-15, Southern Biotech, 1:80) and anti-Rabbit-488, anti-mouse-568 and anti-Rat-647 (all from Molecular Probes, Invitrogen).

Primary antibodies used for western blot were raised against EGFP (mouse, 11814460001 Roche, Basel, Switzerland 1:2000), actin (mouse monoclonal, A5441 Sigma 1:5000), β -tubulin (ab6046, Abcam, 1:500), γ -tubulin (mouse monoclonal, T3559 Sigma 1:1000), total β -catenin (Ab6302, Abcam, 1:400) and unphosphorylated β -catenin (ABC; clone 8E7, Millipore, 1:400).

Secondary antibodies used include HRP-conjugated goat-anti-mouse and goat-anti-rabbit (Jackson Immuno Research Laboratories, West Grove, PA, USA, 1:10 000).

Custom-made rabbit antibodies against the N-terminus and C-terminus of FLCN were generated and affinity purified by Eurogentec (Seraing, Belgium). The N-terminal antibody was raised against a peptide consisting of amino acids 29–41 (LPQGDGNE₂₉SPGQCE) and the C-terminal antibody was raised Human Molecular Genetics, 2013 11 Downloaded from <http://hmg.oxfordjournals.org/> at Maastricht University on July 30, 2013 against a peptide consisting of amino acids 522–536 (TKVD SRPKEDTQKLL) of human FLCN (NP_659434).

Cell lines and cell culture

Normal Human Dermal Fibroblasts (NHDF) were purchased from Lonza (Blackley, UK). MDCK cells type II were a gift from P. Zimmermann (Department of Human Genetics, University of Leuven, Belgium). HK-2 cells were provided by Prof. Dr. M. van Engeland (Department of Pathology, Maastricht University Medical Centre). UOK257 and UOK257-2 cell lines were kindly supplied by Dr L. Schmidt (NIH, USA). All cells were cultured in tissue culture grade plastics, in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/l D-glucose and L-glutamine (Lonza) supplemented with 10% FCS (Lonza) and 50 μ g/ml gentamycin (Lonza) at 37 °C in 5% CO₂ (humidified atmosphere). The culture medium was changed every 2 to 3 days. At confluence, cells were split using trypsin-EDTA (Lonza). Primary ciliated HNE cells were obtained from consenting donors with no known history of BHD under local ethical approval from East of Scotland Research Ethics Service (EoSRES) REC1 (12/ES/ 0081). A 2 mm diameter cytology brush was inserted into each nostril and cellular material was recovered by abrasion of the nasal turbinate. This was washed with

several changes of serum free Medium 199 containing 1 mM Dithiothreitol (Sigma) and Primocin antibiotic/antimycotic (Invivogen, Toulouse, France). Cells were either processed immediately for immunofluorescence or maintained in culture for up to 1 nweek by placing into Bronchial Epithelial Growth Medium (Lonza), supplemented with SingleQuot Kit Supplements and appropriate growth factors. Human sperm was obtained from volunteer donors (healthy men with no known fertility problems randomly selected from the general public) who were recruited in accordance with the HFEA Code of Practice (version 8) under ethical approval (08/S1402/6) from the EoSRES REC1. Capacitated sperm cells were prepared using a swim-up assay⁵⁹ and were then immediately processed for immunofluorescence by spreading 10 ml sperm solution onto Polysine slides (VWR, Leicestershire, UK). Slides were air-dried prior to fixation in Methanol:Acetone (1vol:1vol) for 20 min at -20 °C and staining was performed as described for 2D cultures.

Immunocytochemistry of 2D cultures

For fluorescent microscopy, cells were cultured on glass cover slips. Cilium formation was induced by culturing the cells for at least 4 days after reaching confluence or by serum starving the cells for at least 48 hours in DMEM without FCS, depending upon the cell type. When ready for staining, cells were fixed using either ice-cold 100% methanol or 4% formaldehyde at room temperature and permeabilized using 0.2% Triton X-100, depending upon the primary antibody. Cells were incubated overnight at 48 °C in primary antibody appropriately diluted in 3% BSA in TBS block buffer followed by 1 hour incubation at room temperature in secondary antibody appropriately diluted in block buffer. DNA was stained with DAPI. Fluorescent samples were visualized using a Leica TCS SPE confocal laser scanning fluorescence microscope (Leica DMRBE, Mannheim, Germany). Images were further processed using ImageJ (NIH) software to determine the percentage of ciliated cells (n=3, average 200 cells per group per experiment) and cilia length (n=3, average 40 cilia per group per experiment).

Immunohistochemistry

Sections of 4 µm were processed using standard methodology; antigen retrieval was performed after blocking in H₂O₂, using 10 mM citrate buffer pH 6.0. For FLCN stain of BHD kidney sections, the custom-made C-terminal, N-terminal antibodies, or FLCN-AP antibody was diluted in 3% BSA in PBS. To visualize we used the Dako REAL EnVision Detection System Peroxidase/ DAB + Rabbit (Dako Heverlee, Belgium). The slides were counterstained with haematoxylin and embedded in Entellan.

Transfection and generation of stable cell lines

Transient transfections were performed using FuGENE HD Transfection Reagent (Roche) according to the manufacturer's instructions. To generate EGFP expressing stable cell

lines, cells were transfected with appropriate plasmid DNA using FuGENE HD. Twenty-four hours post transfection cells were selected using 3 µg/ml puromycin (Invivogen). Clonal cell populations were picked using cloning cylinders and analysed for the expression of the protein of interest by western blot. To generate *FLCN* knockdown and non-target cell lines, HK-2 cells were transfected with appropriate plasmid DNA using FuGENE HD. Twenty-four hours after transfection selection of 1 µg/ml puromycin (Invivogen) was added. Clonal cell populations were picked using cloning cylinders and subsequently analysed by western blot for FLCN expression.

Growth curve

MDCK cells were seeded into six-well plates at a starting density of 10^5 cells per well. The cell number was determined at 48 hours intervals using a haemocytometer. After counting cells were reseeded into a fresh six-well plate at an appropriate sub-confluent density and allowed to grow for another 48 hours.

3D spheroid growth

MDCK cells were resuspended in a collagen matrix and seeded on a glass cover slip. The mixture was allowed to polymerize for 30 min at 37 °C, and fresh medium was put on top to cover the matrix. After 11 days spheroids had formed. IMCD3 cells were cultured and reverse transfected with 50 nM ON-TARGETPlus siRNA murine-specific oligos (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's recommendations. Twenty-four hours later, cells were collected and resuspended, mixed 1:1 with growth factor-depleted Matrigel (BD Bioscience, San Jose, CA, USA) and seeded in a glass bottom Lab-tek II chamber (Nunc Thermo Scientific). The mixture was allowed to polymerize for 30 min at 37 °C, and fresh medium was put on top to cover the matrix. After 3 days spheroids had formed.

Immunocytochemistry of 3D cultures

After spheroid formation, the medium was removed and the gels containing the spheroids were washed with warm PBS 12 Human Molecular Genetics, 2013 Downloaded from <http://hmg.oxfordjournals.org/> at Maastricht University on July 30, 2013 supplemented with calcium and magnesium and fixed in fresh 4% PFA for 30 min at room temperature. After washing with PBS, the cells were permeabilized for 20 min in gelatin dissolved in warm PBS (7 mg/ml) with 0.5% Triton X-100 added. Primary antibodies were diluted in the permeabilization buffer and the gels were incubated at 4 °C overnight. After washing the spheroids three times for 30 min in permeabilization buffer, secondary antibodies were diluted in permeabilization buffer and the gels were incubated overnight at 4 °C. The next day, the gels were washed three times in permeabilization buffer immersed in Fluoromount-G (Cell Lab, Beckman Coulter, Brea, CA, USA). Images were taken with a Zeiss

LSM510 inverted confocal microscope. Using confocal laser scanning microscopy, several parameters of these spheroids can be determined, including the number of cells forming the median section of a spheroid. Additionally, the total spheroid size (basal membrane) and the luminal size (apical membrane) can be determined by measuring the distance of three lines spanning the maximal distance at a roughly 120° angle of one another. Also the number of cilia present parallel to the transverse section (over a 6–8 µm Z-section depending on spheroid size) can be counted.

Statistical analyses

The percentage of ciliated cells (n=3, average 200 cells per group per experiment) was analysed using a two-tailed Pearson’s Chi-square test for categorical data (significant p<0.05). Cilium length (number of experiments = 3, average 40 cilia per group per experiment) and all spheroid parameters were analysed using non-parametric two-tailed Mann–Whitney tests (significant p=0.05). For SiCON, Si*Flcn* and EGFP-FLCN K508R cells 30 spheroids were analysed, for EGFP cells 36 spheroids were analysed and for EGFP-FLCN WT 43 spheroids were analysed. Distances are averages of three measurements at a 120° angle of each other in µm.

RNA isolation and quantitative RT-PCR

RNA was isolated using the RNA isolation kit from Qiagen (Hilden, Germany), according to the manufacturer’s recommendations. Probes *Flcn* mm00840973_m1 and *Rpl19* Mm01606 037_g1 form RNA analysis were purchased from Applied Biosystems. Real-time PCR was performed in triplicate using the one-step RT-PCR kit and the 7500 system from Applied Biosystems. Normalized Ct values were used to calculate relative expression levels per condition and to determine the standard deviation (p=0.05; n=3). Real-time PCR for *Axin2* and *CyclinD1* was performed as previously described⁶⁰ using the primers listed in Supplementary Material, Table S4.

Supplementary Table S4. Primer sequences used for real-time PCR of Axin2 and CyclinD1

Cyclin D1	gtgctgcgaagtggaaacc
Cyclin D1	atccaggtggcgacgatct
Axin2	agccaaagcgatctacaaaagg
Axin2	ggtaggcatttctccatcac

Sequencing

1 × 10⁶ UOK257 and HK-2 cells were harvested using trypsin- EDTA. Genomic DNA was isolated using the Genomic DNA purification kit from Gentra Systems (Minneapolis, MN, USA) according to the manufacturer’s recommendations. Subsequently, the BHD locus was sequenced using primers directed to intronic sequences adjacent to the *FLCN* exons.

Western blotting

Cells were harvested using trypsin-EDTA and whole cell lysate (WCL) was obtained using NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 250 mM Tris pH 7.3) supplemented with protease and phosphatase inhibitors (Roche). Protein concentration was determined using the Bradford protein assay (Sigma). An appropriate volume of Laemmli sample buffer was added to equal amounts of total protein followed by boiling for 5 min. Samples were subjected to SDS-PAGE electrophoresis before transfer to an Amersham Hybond™-PVDF membrane (GE Healthcare Life Sciences, Buckinghamshire, UK). After overnight incubation at 4 °C in primary antibody appropriately diluted in 0.5% BSA in TBS block buffer, membranes were incubated with secondary antibody appropriately diluted in block buffer. Signal was detected using enhanced chemiluminescence (ECL) system (Thermo Scientific, Waltham, MA, USA).

Supplementary material

Supplementary material is available at *Human Molecular Genetics* online.

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Conflict of Interest statement. None declared.

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5

Identifying binding partners of folliculin
by tandem affinity purification
Establishing the method

Preface

This chapter describes the laboratory work conducted during my PhD studies. I established a method to identify protein interactions of folliculin, the protein that is mutated in Birt-Hogg-Dubé syndrome. This method has been extensively used for further studies, but will not be published other than in this thesis.

The project was started in December 2009 and predominantly performed in the laboratory of Dermatology of the Maastricht University Medical Centre + (MUMC+), under supervision of Prof. Dr. M.A.M. van Steensel, Dr. B.J. Coull and Dr. M. van Geel. Work was partially performed during an invited stay at the department of Biochemistry of the University of Cambridge, in collaboration with Dr. R.K. Nookala.

Co-workers R.S. Bladergroen, T. Claessens, J.A. Easton, R. Janssen, M.N.H. Luijten and M.A.F. Kamps are acknowledged for their support in the MUMC+ laboratory of Dermatology.

Introduction

Birt-Hogg-Dubé (BHD) syndrome is caused by mutations in the *FLCN* gene, encoding the protein folliculin (FLCN).¹ FLCN's function is yet to be fully elucidated. It is also not known how mutations in *FLCN* cause the symptoms of BHD syndrome, such as fibrofolliculomas in the head and neck region, lung cysts, pneumothorax and both benign and malignant renal tumours.²⁻⁴ To gain more insight into FLCN's function, we decided to study its interactions with other proteins.

Folliculin and studying its interactions

A protein's interaction with other molecules is important for its function in the cell, regarding cell structure and cellular processes such as transcription and translation, metabolism and cell signalling/communication between cells.⁵ Because most cells are in constant motion, the interactions are time and space dependent.⁶ Since proteins are involved in the majority of activities of the cell, abnormal interactions can cause disease.⁷ Understanding the interactions is a key principle in molecular biology research and gives insight in the function of proteins.^{5, 8}

At the time we started this project, two proteins were known to interact with FLCN: folliculin-interacting protein 1 (FNIP1)⁹ and folliculin-interacting protein 2 (FNIP2; also called FNIP1 or MAPO1).¹⁰⁻¹² FNIP1 and FNIP2 were identified as novel proteins with unknown function. Therefore, the interaction of FLCN with FNIP1 and FNIP2 did not give any insight in the function of FLCN at that moment. However, FNIP1 and FNIP2 were found to interact with AMPK (5' AMP-activated protein kinase), in a complex together with FLCN. In the absence of FNIP1 and FNIP2, FLCN does not form a complex with AMPK.^{9, 10} AMPK itself is involved in the energy sensing of the cell and inhibits the mTOR (mammalian target of rapamycin) complex. The role of mTOR signalling in BHD syndrome has been studied by several groups, though the results are conflicting (as reviewed by Smith and Linehan¹³). It is not yet clear if and how FLCN's interaction with AMPK contributes to the manifestations of BHD syndrome.

The focus of this project is to identify more binding partners of FLCN and to elucidate its cellular function. FLCN is likely to play a role in the same cellular processes that its interacting proteins are involved in, because proteins that have related functions preferentially interact with each other.¹⁴ The identification of such a protein-protein interaction (PPI) could therefore give new insights in the function of FLCN itself. Since examining the known PPIs of FLCN has not yet clarified its function, we aim to gain more insights by establishing an unbiased approach for identifying proteins that interact with FLCN. In this chapter, we will first describe the different methods that are available for identifying PPIs, then we will discuss which method we chose and why. Finally, we will show how it was established for FLCN.

Methods for identifying protein-protein interactions of FLCN

There are different methods to identify PPIs. We evaluated the advantages and limitations of these techniques to decide which method to use for the identification of binding partners for FLCN. Commonly used methods for PPI identification are: protein-fragment complementation assays, protein complex purification followed by mass spectrometry, protein microarrays, phage display and in silico analysis.^{5, 7, 15} Each method will be briefly described below. An overview is given in Table 1, together with their fulfilment of two major selection criteria. The first criterion is the source of proteins that PPI's are identified from, since a pre-selected subset of proteins could limit the PPI's that can be detected. The second criterion is if PPI's are formed under natural conditions of the cell, because cellular processes, such as phosphorylation, may be necessary to form PPI's. Finally, we will discuss which method we selected, and why.

Protein-fragment complementation assays

A commonly used type of a protein-fragment complementation assay is yeast two-hybrid screening (Y2H), based on the complementation of a transcription factor with a reporter gene when two proteins bind. Yeast strains are made to express a protein of interest, called bait, with a specific DNA binding domain attached to it. The bait will therefore bind the DNA in front of an endogenous yeast reporter gene. Each yeast strain is also made to express a possible binding partner, called prey, with a transcription factor attached to it. Binding of the bait and prey leads to transcriptional activity of the reporter gene, which is measured for each bait-prey combination as indicator of their binding. The properties of Y2H are further reviewed by Westermarck *et al.*⁷ Next to Y2H, protein-fragment complementation assays can be performed in other organisms, such as bacteria, or in mammalian cells.¹⁶

Protein complex purification followed by mass spectrometry

In protein complex purification followed by mass spectrometry, the protein of interest is isolated from the cell, together with any proteins that are bound to it. Subsequently, the components of this complex are identified by mass spectrometry. Over time, multiple techniques have been developed to isolate protein complexes for mass spectrometry. These are briefly described below. Because non-specific binding of proteins can occur in all purification methods, PPIs identified in this manner should always be validated in subsequent experiments. Carneiro *et al.* reviewed mass spectrometry and different purification techniques.⁵

Immunoprecipitation

In immunoprecipitation, the protein of interest is purified from cells by binding to an antibody that is fixed to a solid matrix. When the protein, together with its binding partners,

is subsequently released from the matrix, it can be used for mass spectrometry. The antibody can target the endogenous protein or, alternatively, it can target a tag that is attached to the protein of interest by gene-fusion.

Affinity purification

Affinity purification is based on chimerically tagging the protein of interest with a tag that has a specific affinity to a certain matrix. As examples, a tag consisting of histidine residues has high affinity for nickel, whereas a biotin tag will bind to streptavidin. The tagged protein of interest is isolated from cells by its direct binding to the solid matrix.

Tandem affinity purification

A two-step purification method can be used to improve specificity. For this, a tandem affinity purification (TAP) tag can be used. The protein of interest is linked with 2 combined tags, which can be used for sequential immunoprecipitation and/or affinity purification.

Affinity chromatography

Affinity chromatography also uses an affinity tag fused to the protein of interest. However, the protein is not produced and processed in human cells, but in bacteria and is subsequently bound to a column. Next, lysed cells are passed through the column, where binding partners can bind the protein of interest. These bound proteins are subsequently released from the column and can be analysed by mass spectrometry.

Proximity-dependent biotin identification

An alternative approach is to purify proteins based on proximity to the protein of interest, using proximity-dependent biotin identification (BioID). The protein of interest is fused to a biotin ligase and expressed in cells. The ligase will biotinylate any protein in its proximity. Affinity purification with a streptavidin matrix, which has a very high affinity for biotin, will then purify all biotinylated proteins, which are subsequently subjected to mass spectrometry for identification.

Protein microarrays

Another method to identify PPIs is the protein microarray. This method uses a chip containing a large number of different proteins, to which the protein of interest is added. When the protein of interest is fluorescently labelled, its binding to one of the proteins on the chip can be visualised by a laser scanner.

Phage display

The phage display method uses genetically recombined bacterial viruses (phages) that express a library of prey proteins on their viral coat. These phages are added to the protein

of interest (bait), which is fixed to a matrix. Phages that express a binding partner of the protein of interest will bind the bait. Successively, the bound phages can be amplified in bacteria to enrich the sample for phages expressing binding partners. Finally, the binding partners are identified by sequencing the recombined part of the phage DNA.

In silico analysis

Ever more data on PPIs is available in digital databases for computational analysis. Binding partners can be predicted when the protein of interest is compared to known proteins in a database, for example if they have similar active domains or a similar structure. PPIs can also be predicted based on the knowledge about orthologs in other species. Evidently, prior knowledge of the protein of interest is crucial for *in silico* analysis. Furthermore, all *in silico* predicted PPIs need to be experimentally validated.

Table 1. Overview of commonly used PPI identification methods and their ability to use all cellular proteins and native conditions for PPI formation.

Identification method	Source of proteins to identify PPIs from	Enables PPI under natural conditions?
Protein-fragment complementation assays	Protein library	Not fully
Protein complex purification followed by mass spectrometry	Whole cell	<i>Depends on purification method:</i>
		Immunoprecipitation Yes
		Affinity purification Yes
		Tandem affinity purification Yes
		Affinity chromatography No
		Biotin identification Yes
Protein microarrays	Protein library	No
Phage display	Protein library	No
<i>In silico</i> analysis	All cellular proteins known in database	No

Selecting a method

FLCN's function has not yet been elucidated. At the time we started this project, FLCN was known to be involved in mTOR signalling. However, results were conflicting (as reviewed¹³) and mTOR was known to be involved in many different cellular signal transduction pathways (as reviewed¹⁷). Hence, FLCN could play a role in many different pathways. Next to its role in mTOR signalling, FLCN could have mTOR-independent functions in signal transduction or cell structure as well. We therefore wanted to use an unbiased approach for identifying new PPIs, not limiting our search for novel binding partners to a library of pre-selected proteins (table 1). Hence, we opted for protein complex purification followed by mass spectrometry. Since we aim to identify a significant binding partner, rather than

identifying all binding partners of FLCN, protein purification by TAP suits our purpose best, as purifying the protein complex twice will highly increase specificity and reduce the presence of non-specifically bound proteins.¹⁸ Using TAP tagged FLCN expressed in human cells, FLCN can form PPI's under natural cellular conditions, where i.e. posttranslational modifications can take place. Moreover, cells grown under different conditions can be easily compared, to study the influence of these conditions on the formation of PPIs. Besides creating opportunities for future research, an additional advantage of using TAP tagged FLCN is its optional use for single purifications in subsequent validation experiments (i.e. co-immunoprecipitation) and in future studies. To our knowledge, this is the first time that an attempt was made to identify FLCN-interacting proteins by TAP.

Methods

A TAP tag for FLCN was designed, created, recombined with FLCN and expressed in cells. Subsequently, the FLCN protein complex was tandem purified and its components were analysed by mass spectrometry. Each of these steps will be described below. Cloning, sequencing, transfection, cell culture, immunofluorescence sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as previously described.¹⁹ An overview of the primers used for Sanger sequencing is given in appendix 2 of this chapter.

Designing a tandem affinity purification tag for FLCN

Tags

Many different tags are available for TAP tagging. An overview of the most commonly used tags for TAP is given in Table 2, together with their characteristics, advantages and limitations.²⁰

For the purification of the FLCN complex, we wanted to use a small size TAP tag in order to limit the probability of the tag interfering with FLCN and its interactions. We decided to use a histidine (His) tag in combination with a haemagglutinin (HA) tag. This tag combination has previously been successfully used for protein purification.^{23, 24} A 10His-HA TAP tag was designed. The histidine tag was chosen because we could work with a Fast Protein Liquid Chromatography (FPLC) machine that uses nickel columns as affinity matrix. Since hexa-histidine (6His) is known for often pulling down contaminating proteins²⁵, we used a deca-histidine (10His) instead. 10His has a stronger affinity to nickel^{26, 27} and therefore allows more stringent purification conditions in order to remove non-specifically bound proteins. The HA tag was selected because we have an anti-HA antibody producing cell line, significantly reducing the costs of using this tag. Moreover, our laboratory has

Table 2. Commonly used tags for tandem affinity purification.²⁰⁻²²

Tag name	Size in number of amino acids	Affinity matrix	Elution method (other than denaturing)
Calmodulin binding peptide (CBP)	26	Calmodulin	EGTA
Z-domain	58	IgG	IgG binding domain mimics.
6His	6	Ni ²⁺ , Co ²⁺	Imidazole, L-histidine peptide
FLAG	8	Antibody	FLAG peptide
HA	9	Antibody	HA peptide
Myc	10	Antibody	Myc peptide
Strep II	8	StrepTactin, streptavidin	Desthiobiotin
Biotinylation tag	15	Avidin	Biotin
S-peptide	15	S-protein	None
Streptavidin binding peptide (SBP)	38	Streptavidin	Biotin

experience with the use of HA tags and the HA-antibody. HA is a small non-eukaryotic peptide and is therefore unlikely to interfere with FLCN's function and protein interaction. Importantly, it has previously been successfully used to identify a large network of protein interactions.²⁸ Finally, the HA tag is suitable for immunofluorescence. This enables localisation experiments and fluorescence-activated cell sorting, convenient for future research opportunities.

Additional features of the TAP tag

A recognition site for tobacco etch virus (TEV) protease was designed flanking both tags to create an alternative method for releasing FLCN from the tag, in the event that elution would not suffice. *In silico* analysis predicted that TEV would not cleave either FLCN or its known binding partners, FNIP1 and FNIP2. We also designed a glycine linker between our TAP tag and FLCN, as between the 10His and HA tag, to enhance flexibility and therefore limit the chance of our TAP tag interfering with FLCN's function and PPI formation. The final TAP tag we designed is 10His-linker-HA-TEV-linker (Figure 1A). From here on, it will be called 'TAPtag'.

Position of the TAPtag to FLCN

We chose to tag the N-terminus of FLCN, because the C-terminus is known to interact with other proteins.^{9, 10} Furthermore, nearly all known germ-line FLCN mutations causing BHD syndrome result in a premature stop-codon which causes truncation of the C-terminus of FLCN.^{1, 29-32} This implies that the C-terminus is important for the physiological functioning

Advantages	Limitations
	Large size; Low specificity in mammalian cells; Decreased sensitivity; Cannot be combined with purification using Ni ²⁺ resin; EGTA elution may affect cation-dependent PPIs and may interfere with subsequent functional analysis
	Large size
Small size. Cheap matrix	Non-specific binding to Ni ²⁺ by histidine containing proteins
Small size	Costs of antibody
Small size	Costs of antibody
Small size	Costs of antibody
Small size. Low costs	
Strong binding to matrix	Co-transfection of <i>BirA</i> gene is required
Highly soluble	Elution requires harsh conditions
	Large size

of FLCN. Hence, we do not want our TAPtag to interfere with this terminus. Moreover, if in future research BHD syndrome causal mutant forms of TAP tagged FLCN have to be made, the C-terminus needs to be eliminated. Therefore, tagging FLCN on the N-terminus is most suitable.

Recombinant FLCN

The *FLCN* cDNA was available from the pCAGGS-FLAG-FLCN construct which was a kind gift from prof. O. Hino (Juntendo University School of Medicine, Tokyo, Japan).¹¹ The FLAG tag in front of FLCN was maintained. Removing the tag would be technically challenging and we considered that a third tag would create more opportunities for future experiments. The FLAG tag is a newly designed peptide.³³ It has a unique sequence, not originating from any known protein. Hence, PPI formation by the FLAG tag itself is unlikely.³⁴ TAPtagging FLAG-FLCN generated a protein designated TAPtag-FLAG-FLCN.

A control for TAP tagged FLCN

A control was designed to differentiate between proteins that specifically bind FLCN and non-specifically co-purified proteins. The TAPtag on its own is not suited as a control, as it is small (4.1 kDa) and therefore does not require the same cellular processing as bigger proteins such as TAPtag-Flag-FLCNwt (73.4 kDa) do. Moreover, it is too small to visualise by Western blotting. Therefore, EGFP (enhanced green fluorescent protein) was added to the TAPtag. Green fluorescent protein is known to have low non-specific binding in

mammalian cells.³⁵ Besides, it has fluorescent properties which enables easy visualisation of expression and localisation in the cell. This control-construct does not contain a FLAG tag. However, non-specific binding of FLAG to mammalian proteins is not expected.³⁴ The control we designed was designated TAPtag-EGFP (33.7 kDa).

cDNA features for recombination

TAPtag cDNA was designed for genetic recombination with FLCN and EGFP. A start codon was placed in front of the 10His tag to enable translation. Recognition sites for DNA restriction enzymes were designed at either end of our TAPtag, to enable cloning by genetic recombination into eukaryotic expression vectors. Recognition sites for *Bgl*II and *Sma*I (also recognised by *Xma*I) will be used for cloning into the pEGFP-N1 vector (*Clontech*). *Acc*65I, *Sbf*I and *Mlu*I are additionally included for future flexibility in cloning. To keep the reading frame intact after cloning the TAPtag cDNA into the desired vectors, two single nucleotides were inserted between the *Sbf*I and *Mlu*I and between the *Mlu*I and *Sma*I/*Xma*I recognition sites. A 5' to 3' DNA fragment encoding part of the TAPtag and a partly overlapping 3' to 5' DNA fragment were designed to eventually create the TAPtag. Both fragments contain 84 nucleotides of which 32 bases overlap (Figure 1B).

Figure 1. Components of the TAPtag on (A) protein level and (B) cDNA level. The arrows underline the nucleotide sequence of the single stranded DNA fragments, 5'-3' (continuous arrow) and 3'-5' (dotted arrow), that were used to generate the TAPtag cDNA.

Creating the TAPtag cDNA constructs

The TAPtag and FLAG-FLCN were made as shown in the flowcharts of Figure 2. Figure 2A shows how the TAPtag was created by annealing and subsequently complementing the two single stranded DNA fragments shown in Figure 1B, that were ordered from *MWG Eurofins Genomics*. Its sequence was verified by Sanger sequencing. The flowchart of Figure

2B shows how FLAG-FLCN was obtained from the pCAGGS-FLAG-FLCN construct and that additional restriction sites were added to enable further and future cloning of FLAG-FLCN into expression vectors. Sanger sequencing confirmed the correct sequence of the FLAG-FLCN construct. It also revealed one clone harbouring a c.1285dupC mutation in *FLCN*. This c.1285dupC mutation is also found in BHD syndrome patients. It occurs in a hypermutable tract of 8 cytosines in exon 11, in which about 50% of all patient mutations are located.^{1, 29, 31, 36} Moreover, in 16-23% of sporadic colon carcinomas with microsatellite instability, mutations in this 8 cytosine track of *FLCN* are seen.^{37, 38} The hypermutability of this track could explain the occurrence of the spontaneous mutation during the genetic recombination of *FLCN*. The mutation results in a frameshift and premature truncation of the protein, p.His429Profs*27. Truncation of FLCN changes its structure. This may not only lead to loss of crucial PPIs, but also to the formation of new ones. The mutated FLAG-FLCN construct can be used to study whether (loss of) a specific PPI may be involved in the pathogenesis of BHD syndrome. The mutant variant will be called FLCN1285dupC and the wild type FLCNwt.

TAPtag FLCN and EGFP fusion constructs

The TAPtag was cloned in-frame to FLCN and EGFP in a eukaryotic expression vector. The flowchart in Figure 3 shows step by step the construction of pTAPtag-EGFP, pTAPtag-FLAG-FLCNwt and pTAPtag-FLAG-FLCN1285dupC. The sequences of the inserts of these vectors were confirmed by Sanger sequencing. These three inserts will be collectively called 'the TAPtag constructs'.

TAPtag constructs expression in human cells

To generate a model for studying the PPI formation of FLCN, we aimed to express the TAPtag constructs in UOK 257 cells, which are epithelial cells derived from a renal cell carcinoma of a patient with BHD syndrome.^{9, 39} It harbours a c.1285dupC mutant copy of *FLCN* and has lost wild type *FLCN*.³⁹ We have previously seen a low level of the wild type *FLCN* allele when Sanger sequencing UOK 257 cells, but its relevance is unknown.¹⁹ No full length FLCN protein was detected in these cells.^{40, 41} The plasmids pTAPtag-EGFP, pTAPtag-FLCNwt and pTAPtag-FLCN1285dupC were transfected into UOK 257 cells and grown under selection with G418. Fluorescence microscopy of pTAPtag-EGFP transfected UOK 257 cells showed that they exhibited low fluorescence, indicating low expression of the construct. Immunofluorescence using an anti-FLAG antibody (mouse monoclonal, F1804 *Sigma* 1:500) showed no expression of TAPtag-FLAG-FLCNwt and TAPtag-FLAG-FLCN1285dupC (data not shown). We concluded that the transfection of UOK 257 cells was not efficient enough to create cell lines expressing the TAPtag constructs. As an alternative, we decided to use retinal pigment epithelium (RPE) cells (a kind gift from Dr. R. Giles, UMC Utrecht, Utrecht, The Netherlands), because these cells are more susceptible to transfection. RPE

cells were transfected with pTAPtag-EGFP, pTAPtag-FLCNwt and pTAPtag-FLCN1285dupC and grown under selection with G418. Evaluation of fluorescence showed expression of TAPtag-EGFP in only 2% of the cells. Immunofluorescent staining using anti-FLAG antibody showed expression of TAPtag-FLAG-FLCNwt in part of the cells, but no expression of TAPtag-FLAG-FLCN1285dupC (data not shown). Based on the experiments with UOK 257 and RPE cells, we concluded that our method of transfection was not efficient enough to create stable cell lines expressing the TAPtag constructs.

Figure 2. Schematic overview of the method used to create (A) TAPtag cDNA, and (B) FLAG-FLCNwt and FLAG-FLCN1285dupC cDNA.

* See Figure 1B for sequences

** Sequence of forward primer T7: TAATACGACTCACTATAGG

Figure 3. Schematic overview of the method used to create the expression vectors pTAPtag-EGFP, pTAPtag-FLAG-FLCNwt and pTAPtag-FLAG-FLCN1285dupC.

G = G418 resistance cassette.

TAPtag constructs in a retroviral expression vector

Retroviral transduction of a gene into cells is more efficient than conventional transfection. We therefore cloned the TAPtag constructs into the retroviral expression vector pQCXIP (Clontech), generating three retroviral expression plasmids called pQCXIP-TAPtag-EGFP, pQCXIP-TAPtag-FLAG-FLCNwt and pQCXIP-TAPtag-FLAG-FLCN1285dupC, as shown in Figure 4. Sanger sequencing confirmed the correct inserts.

Figure 4. Schematic overview of the method used to create the retroviral expression vectors pQCXIP-TAPtag-EGFP, pQCXIP-TAPtag-FLAG-FLCNwt and pQCXIP-TAPtag-FLAG-FLCN1285dupC.

G = G418 resistance cassette; P = puromycin resistance cassette.

* Restriction with *Bam*HI and *Bgl*II results in compatible overhangs.

** Restriction with *Eco*RI and *Mfe*I results in compatible overhangs.

Creating stable cell lines and confirming expression

We generated stable cell lines expressing the TAPtag constructs by retroviral transduction of human kidney-2 (HK-2) cells⁴² and UOK 257 cells with virions from Phoenix-Ampho cells (*ThermoFischer Scientific*) that were transfected with pQCXIP-TAPtag-EGFP, pQCXIP-TAPtag-FLAG-FLCNwt, or pQCXIP-TAPtag-FLAG-FLCN1285dupC construct. The Phoenix-Ampho cells excrete virions that have packaged the transfected DNA. The virions introduce the DNA into the HK-2 and UOK 257 cells by infection. Next, we grew the cells under selection with puromycin, in order to create polyclonal stable cell lines expressing the TAPtag constructs. The UOK 257 and HK-2 cells transduced with pQCXIP-TAPtag-EGFP and pQCXIP-TAPtag-FLAG-FLCNwt did grow properly under selection. On the other hand, the UOK 257 cells transduced with pQCXIP-TAPtag-FLAG-FLCN1285dupC did not survive. The HK-2 cells transduced with this construct grew very slowly. Western blotting was used to assess the expression of the TAPtag constructs in the surviving cell lines. Figure 5 shows that TAP-

tag-FLAG-FLCNwt (73.4 kDa) and TAPtag-EGFP (33.7 kDa) are expressed in both UOK 257 and HK-2 cells. TAPtag-FLAG-FLCN1285dupC could not be detected by Western blotting in the slow growing HK-2 cell line (data not shown) and therefore this cell line was discarded.

Figure 5. TAPtag-FLAG-FLCNwt and TAPtag-EGFP are expressed in HK-2 cells and UOK 257 cells. Western blot of whole cell lysate of HK-2 cell lines and UOK 257 cell lines grown under puromycin selection after transduction with pTAPtag-FLAG-FLCN-wt or pTAPtag-EGFP, probed with anti-HA (mouse monoclonal, 2367 *Cell Signalling* 1:1000). In the first and third lane, the band just below 80kDa represents TAPtag-FLAG-FLCNwt in HK-2 and UOK 257 cells respectively. The faint band at about 46 kDa in the first lane is expected to be a degradation product of TAPtag-FLAG-FLCNwt or a protein the antibody non-specifically binds to. In the second and forth lane, the band below 30 kDa represents TAPtag-EGFP in both HK-2 and UOK 257 cells. This band presents slightly lower than expected, possibly because some of the secondary or tertiary structure of the protein remained during sample preparation for sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Tandem affinity purification of folliculin

TAP was performed by nickel affinity purification (Ni-AP) and subsequent immunoprecipitation with anti-HA antibody (HA-IP). HK-2 cells expressing the TAPtag constructs were used for all optimisation experiments (see appendix 1 of this chapter) and first purifications, because of their higher growth rate. Ni-AP was performed first, since non-denaturing conditions with imidazole are preferred for elution, whereas denaturing conditions that disrupt the purified protein complex can be used for HA-IP. For a schematic overview of the purification steps, see Figure 6.

Figure 6. Schematic representation of tandem affinity purification. (A+B upper left part) Whole cell lysate, containing all proteins that are released from the cells, is used as input for nickel affinity purification (Ni-AP). It is loaded onto a column with nickel beads, where the deca-histidine of the TAPtagged proteins (purple dots) will bind. When the lysate is passed through the column, most other proteins (triangles and rectangles) will leave unbound, but some may non-specifically bind the nickel column. Washing the column will remove part of them. (A upper right part) In conventional Ni-AP, the bound proteins are eluted with elution buffer containing a standard concentration of imidazole. The elution step is repeated multiple times, resulting in elution 1, 2, etc. Most of the bound protein is released in the first elutions and the least or none in the last elution. After elution, the nickel beads are left. A Western blot will show in which of these samples the TAPtagged protein is present. It is expected in the input and in decreasing amounts in the elutions. (B upper right part) In Ni-AP by Fast Protein Liquid Chromatography (FPLC Ni-AP), the bound proteins are eluted in multiple fractions with increasing concentration of imidazole. Non-specifically bound proteins are more likely to have a weaker binding to nickel than TAPtagged proteins do. They are therefore eluted in the first elution fractions. The more tightly bound TAPtagged proteins will require higher imidazole concentrations to be released and they are thus present in later elution fractions. During FPLC Ni-AP, the UV absorption of the sample that leaves the column is continuously measured. When proteins are present in the sample, the UV absorption peak will rise. During elution, the first peak indicates the non-specifically bound proteins that are released and a second, higher, peak indicates that the release of specifically bound proteins. A Western blot will confirm in which samples the TAPtagged protein is present. It is expected in the input and in a continuous subset of elution fractions. (A+B lower part) The second purification step is a HA immunoprecipitation. The elution fractions of the Ni-AP that contain the TAPtagged protein will be used as input. The input is loaded onto an agarose column that is coated with anti-HA antibody. The TAPtagged protein will bind the antibody. Most of the proteins that non-specifically bound the nickel in the previous purification, will not bind the antibody coated agarose and hence will flow through the column. Finally, the TAPtagged protein, together with its binding partners, will be eluted off the agarose column. The elution step is repeated multiple times, resulting in elution 1, 2, etc.

Nickel affinity purification

TAPtag-FLAG-FLCNwt was successfully purified by conventional Ni-AP with nickel beads (17371221 *GE Healthcare*) and using 80 mM imidazole in the binding- and wash buffer, after lysing the cells with a triton X-100 (polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) based buffer (Figure 7). FPLC Ni-AP, enabling more specific purification, was performed by loading cells lysed with octyl glucoside (n-octyl- β -D-Glucopyranoside) lysis buffer on a His column (11-0004-58 *GE Healthcare*) and using a 5 – 600 mM imidazole gradient for elution in 30 fractions of 1ml (Figure 8).

Figure 7. TAPtag-FLAG-FLCNwt and its EGFP control are purified by conventional Ni-AP. Western blot of conventional Ni-AP in HK-2 cells expressing TAPtag-EGFP or TAPtag-FLAG-FLCNwt, probed with anti-HA. Both constructs are present in the respective inputs and enhanced in the elutions after successful purification.

Figure 8. TAPtag-FLAG-FLCNwt and its EGFP control are purified by FPLC Ni-AP. Western blot of FPLC Ni-AP in HK-2 cells expressing TAPtag-EGFP or TAPtag-FLAG-FLCNwt, probed with anti-HA or anti-EGFP (rabbit polyclonal CAB4211 *Thermo Fischer Scientific* 1:5000). Both constructs are present in the inputs. TAPtag-FLAG-FLCNwt is eluted in fractions 8 to 15. A small fraction was left unbound and none was washed of the column prior to eluting. TAPtag-EGFP is eluted in fractions 8 to 17. None was left unbound or washed off the column.

FLCN is known to be phosphorylated on serine 62 by AMPK.⁴³ Serine 62 phosphorylation either enhances the formation of the FLCN-AMPK complex or stabilises it. Binding of FLCN to its known binding partners FNIP1 and FNIP2 increases the phosphorylation of serine

62.⁴³ All of this strongly suggests that phosphorylation of FLCN on serine S62 plays a role in its interaction with other proteins. We confirmed phosphorylation of TAPtag-FLAG-FLCNwt on this serine (Figure 9A), as well as its purification by Ni-AP (Figure 9B).

The known binding partners of FLCN, FNIP1 and FNIP2, were successfully co-purified with TAPtag-FLAG-FLCNwt, this confirms the FLCN protein complex stays intact during the Ni-AP, which is essential for identifying new binding partners of FLCN (Figure 10).

Figure 9. TAPtag-FLAG-FLCNwt is phosphorylated on serine 62 and this modified form of TAPtag-FLAG-FLCNwt is purified by Ni-AP. (A) Western blot of HK-2 cells expressing TAPtag-FLAG-FLCNwt or TAPtag-EGFP, probed with anti-pFLCN(S62) (rabbit polyclonal, generated and affinity purified by Eurogentec, raised against amino acid 56 – 68 of human FLCN with serine 62 phosphorylated, 1:1000). pFLCN(S62) is present in the cells that overexpress TAPtag-FLAG-FLCNwt. In HK-2 cells expressing TAPtag-EGFP the endogenous level of pFLCN(S62) is too low to be detected by this anti-pFLCN(S62) antibody (generated by Eurogentec). (B) Western blot of a conventional Ni-AP in HK2-cells expressing TAPtag-EGFP or TAPtag-FLAG-FLCNwt, probed with anti-pFLCN(S62). pFLCN(S62) is purified from the HK-2 cells expressing TAPtag-FLAG-FLCNwt.

HA immunoprecipitation

HA-IP was performed with anti-HA-agarose (mouse monoclonal, A2095 *Sigma*) on the elutions of a Ni-AP that contain TAPtag-FLAG-FLCNwt or TAPtag-EGFP, using mild conditions with low salt concentration in all buffers and continuously handling samples at 4 °C. A buffer with 9 M urea was used for elution. Proteins in the FPLC Ni-AP elutions were visualised by SDS-PAGE and subsequent Western blotting using anti-6His antibody (mouse monoclonal, 51-9000012 *BD Pharmingen* 1:2000). Elution fractions containing TAPtag-FLAG-FLCNwt or TAPtag-EGFP were pooled prior to HA-IP.

Successive nickel affinity purification and HA immunoprecipitation

Tandem purification of TAPtag-FLAG-FLCNwt was performed by FPLC Ni-AP and subsequent HA-IP (Figure 11). Multiple proteins were effectively co-purified, as shown in Figure 12.

Figure 10. FNIP1 and 2 are co-purified with TAPtagged FLCN by Ni-AP. Western blot of a conventional Ni-AP in HK-2 cells expressing TAPtag-FLAG-FLCNwt or TAPtag-EGFP, probed with anti-FNIP1 (mouse monoclonal ab134969 *Abcam* 1:1000), anti-FNIP2 (rabbit polyclonal, generated and affinity purified by *Eurogentec*, raised against amino acid 117-131 of human FNIP2, 1:1000) and anti-HA. FNIP1 (upper panel) and FNIP2 (middle panel) are present in the elution of TAPtag-FLAG-FLCNwt and not in the elution of the TAPtag-EGFP negative control. The anti-FNIP2 antibody was not capable of detecting FNIP2 in the inputs (middle panel). However, FNIP2 is detectable when it is enhanced in the sample by co-purifying it with FLCN, which increases its concentration. Anti-HA was used as a control for the Ni-AP (lower panel).

Figure 11. TAPtagged FLCN and its EGFP control are tandem purified by FPLC Ni-AP and subsequent HA-IP. Western blot of HA-IP performed on pooled elution fractions from FPLC Ni-AP in HK-2 cells that contain TAPtag-FLAG-FLCNwt or TAPtag-EGFP, probed with anti-6His. A small portion of TAPtag-FLAG-FLCNwt did not bind the anti-HA agarose. None was released in the washing step. TAPtag-FLAG-FLCNwt is mainly present in the first three elutions of the HA-IP and part of it is captured on the agarose after elution. An overload of TAPtag-EGFP was present in the HA-IP, a portion is left unbound and a portion is washed off the agarose. Consequently TAPtag-EGFP is present in all elutions and some is left on the anti-HA agarose after elution.

Identifying co-purified proteins by mass spectrometry

The tandem purified FLCN protein complex was reduced to its individual, unfolded components by boiling in sample buffer with β -mercaptoethanol. The proteins were visualised by SDS PAGE and silver staining (k0681, *Fermentas*, using the maximum sensitivity protocol). Two individual protein bands, that seemed to be specific for FLCN (Figure 12), were obtained and subjected to mass spectrometry at the Cambridge Centre of Proteomics at

Cambridge University in Cambridge, United Kingdom. After releasing the proteins from the gel, they were digested, reduced and alkylated to generate unfolded peptides for detection. Proteins possibly present in the sample were predicted after comparing the detected peptides to a database of human proteins.

Figure 12. Protein bands co-purified with TAPtagged FLCN. Silver staining of proteins in a SDS-PAGE gel containing the tandem affinity purified TAPtag-FLAG-FLCNwt complex or TAPtag-EGFP complex, as purified from HK-2 cells by FPLC Ni-AP and subsequent HA-IP. The two outer lanes contain marker proteins with a known weight in kDa, as indicated on the sides. The second lane contains TAPtag-FLAG-FLCNwt and all proteins that were co-purified. As a control, the third lane contains TAPtag-EGFP and its co-purified proteins. The band just above 64 kDa in the second lane (*) represents TAPtag-FLAG-FLCNwt. The band between 28 kDa and 39 kDa in the third lane (•) represents TAPtag-EGFP. Bands present in both samples most likely contain non-specifically co-purified proteins. In the TAPtag-FLAG-FLCNwt sample, a clear, distinct band is present above 191 kDa (arrow 1). No band at this height can be detected in the sample of TAPtag-EGFP, indicating that the band was specifically co-purified with TAPtag-FLAG-FLCNwt. Another distinct and intense band is present below 51 kDa (arrow 2). No band with this intensity is detected for TAPtag-EGFP.

Selecting proteins that could be true binding partners of FLCN

Mass spectrometry identified 71 proteins for band 1 and 104 proteins for band 2 (bands are indicated in Figure 12). Proteins with the highest probability of being specifically co-purified with FLCN were selected. First, all proteins present in both samples were discarded, because these most likely are contaminants of the gel. Subsequently, all proteins from skin and saliva were rejected, since these are common contaminants in laboratory experiments. Finally, all proteins with a size that does not correspond with the height of the band they were identified from, were excluded. One protein remained for band 1 (Table 3). The eleven proteins remaining for band 2 were further confined by selecting the five proteins with the highest protein score (Table 3). This score is an indicator for the probability of the identification not being a random event.

Table 3. Proteins with the highest probability of being specifically co-purified with TAPtagged FLCN, as identified by mass spectrometry after tandem affinity purification of FLCN.

Protein name	kDa	Protein score	No. of identified peptides
Protein identified in band 1 (>191 kDa)			
Nucleoprotein TPR	268	33	1
Protein identified in band 2 (>39 <51 kDa)			
Elongation factor 1-alpha 1	50	215	7
Elongation factor 1-alpha 2	51	194	6
Immunoglobulin heavy chain variable region	53	157	5
Immunoglobulin heavy chain variable region	53	148	4
Tubulin alpha-1A/B/C	38/50	104	3

Discussion

FLCN is the causative protein in BHD syndrome. Its true function in both physiology and disease is yet to be confirmed. With this project we aimed to establish a method to identify novel binding partners of FLCN, since the function of its binding partners can give more insight in the function of FLCN itself. We generated TAPtagged FLCN and show that we can tandem affinity purify the FLCN complex and identify potential novel binding partners by mass spectrometry. Validation of the binding between FLCN and the identified proteins will confirm if their binding is specific.

Further investigation of putative binding partners

The FLCN protein complex was purified and from this sample two protein bands were analysed by mass spectrometry. Next, we selected the proteins with the highest probability of being specifically co-purified with FLCN (Table 3). All of these proteins are potential binding partners of FLCN. Validating this binding will consume time and resources. Therefore, they should be carefully considered before validation experiments are initiated.

Nucleoprotein TPR

Mass spectrometry, of the >191 kDa protein band co-purified with FLCN (band 1 in Figure 12), suggests nucleoprotein TPR (translocated promotor region) as a putative binding partner of FLCN. TPR is part of the nucleopore complex and plays a role in the transport of proteins and RNA across the nuclear membrane.^{44,45} However, only one peptide unique to nucleoprotein TPR was identified and the protein score of 33 is low, indicating a considerable chance of the detection being a random event. Investing in the validation of the binding between FLCN and TPR would therefore not be first choice.

Elongation factor 1-alpha 1 and 2

From the proteins that were identified in the band of 39 to 51 kDa (band 2 in Figure 12), the two isoforms of eukaryotic elongation factor 1-alpha (eEF1A), eEF1A1 and eEF1A2 have the highest protein scores (Table 3). Both are involved in protein synthesis. eEF1A delivers aminoacylated-tRNA to the ribosome, where it is translated into mRNA. For this, eEF1A has to be GTP bound. When the GTP is hydrolysed to GDP, eEF1A is released from the ribosome (as reviewed by Browne and Proud⁴⁶). In general, a guanine nucleotide exchange factor (GEF) is able to replace GDP by GTP on a GTPase. Conversely, a GTPase-activating protein (GAP) hydrolyses GTP into GDP. It is likely that eEF1a is activated and in-activated by GEFs and GAPs to control its role in protein translation. Recent studies show that FLCN may have both GEF and GAP functions towards Rab proteins.^{47,48} These functions may be a possible link between eEF1A and FLCN. eEF1A1 also plays a role in the nuclear export of proteins, such as the Von Hippel Lindau tumour suppressor (pVHL).⁴⁹ pVHL is the causative protein in Von Hippel Lindau syndrome. Like BHD syndrome, this disorder predisposes to cyst formation and renal cell carcinomas, amongst other symptoms.⁵⁰ eEF1A2 in turn, is a putative oncogene that is expressed in only a few tissues, but is found in multiple types of cancer.⁵¹⁻⁵⁴ There, it promotes invasion and cell migration, mainly through the PI3K-Akt pathway.⁵⁵⁻⁵⁷ eEF1A2 is also involved in phosphatidylinositol signalling.⁵⁸⁻⁶⁰ This is a membrane bound phospholipid that is involved in oncogenesis, cell survival, proliferation, vesicular trafficking and cytoskeleton organisation (as reviewed^{61,62}). The tumour susceptibility of patients with BHD syndrome and the recent suggestion that FLCN may be involved in vesicular trafficking⁶³ also make eEF1A2 an interesting potential binding partner to study further. However, eEF1A is a protein with high abundance in the cell. It composes 1-3% of the protein content, which makes it the second most profuse protein after actin.⁶⁴ This abundance increases the change of eEF1A1 and eEF1A2 being non-specifically co-purified with FLCN. Validation of their binding is therefore essential before drawing any further conclusions. For validation, immunoprecipitation of endogenous eEF1A1 and eEF1A2 from cells and subsequent Western blotting to check if endogenous FLCN is co-purified, could be performed.

Immunoglobulin heavy chain variants

After purifying the FLCN complex, mass spectrometry detected peptides that are present in immunoglobulin heavy chain variable region (IGH@) (Table 3). Immunoglobulins are assembled from multiple genetic loci to generate the extensive variety of antibodies that is required in our immune system. Thus, many variants exist and two are identified in the sample of purified FLCN. Immunoglobulins are produced and subsequently excreted by B lymphocytes. Immunoglobulins are not expected inside other cell types, such as the HK-2 cells, nor on their exterior, when cultured in the absence of B cells and their excreted. Therefore, chances are small that IGH@ is a true binding partner of FLCN. The identified

protein most likely is a contamination of the mouse anti-HA antibody that was used in the HA-IP. The peptides that were detected by mass spectrometry, were compared with a database of human proteins only. Murine antibodies would therefore not be recognised, although they could have been present and have high amino acid sequence homology with human proteins. Another possibility is that the IGH@ originates from the bovine serum that was used to culture the HK-2 cells. The bovine immunoglobulins could bind the cells and be present in the cell lysates. Hence they could be (non-)specifically purified with FLCN. Since the bovine immunoglobulins are only used at the start of the experiments after which many wash steps and purification steps were performed, it is less likely to be the origin of IGH@ contamination. To conclude, IGH@ is an improbable binding partner and does therefore not give rise to further validation experiments.

Alpha tubulin

Based on 3 detected peptides, alpha tubulin was identified in the purified FLCN sample (Table 3). Alpha tubulin has multiple isotypes and this set of peptides matches with isotype 1A, 1B and 1C. Alpha tubulin, in a heterodimer with beta tubulin, is the main constituent of microtubules. Microtubules function as tracks for transportation within the cell. Furthermore, they give shape to the cell, play a role in the positioning of organelles and are involved in cell polarity. One of the microtubule-based organelles, is the cilium (as reviewed⁶⁵). We found that FLCN has a role in ciliogenesis and cell polarity.¹⁹ FLCN is also suggested to play a role in vesicular trafficking⁶³, in which microtubules play an important role.⁶⁶ These overlapping functions make the putative interaction of FLCN and alpha tubulin highly interesting. As the identification of alpha tubulin is based on only 3 peptides with a protein score of 104 (Table 3), this interaction requires validation before further conclusions are drawn.

Alpha-tubulin is known to undergo post translational modification, such as acetylation.⁶⁷ When the PPIs of alpha tubulin are studied, it is therefore important to use a system that allows such modifications. The interaction with FLCN could be validated by immunoprecipitation of endogenous alpha tubulin from human cells and subsequent Western blotting. When the endogenous levels of bound FLCN and alpha tubulin are too low to be detected by the antibodies, (over)expression of tagged forms of one or both proteins can be used to examine their binding.

Further exploration of binding partners when validated

To gain insight into the role of FLCN towards its binding partner, as well as insight into the role of the protein complex in the cell, the characteristics of the complex could be further explored. The subcellular localisation can be studied using immunofluorescence of FLCN and its binding partner. Also, it would also be of interest to identify the other components

of that specific protein complex. For this, a tandem approach can be used in which the first affinity purification or immunoprecipitation is directed towards (tagged) FLCN and the second to its newly identified binding partner. Subsequent mass spectrometry can identify the other proteins in the complex.

Factors that influence the PPI formation can give more insight in the function of FLCN and its binding partner. Binding can, for example, depend on phosphorylation, acetylation, the presence of other molecules such as proteins, nutrients or ATP. As it is impossible to investigate them all, the known function of the binding partner could give rise to prioritising factors to study. When studying alpha-tubulin for example, the difference in binding to the tyrosinated versus the detyrosinated form and the polyglutamylated versus the non-glutamylated form is worth studying. These different post translational modifications influence the recruitment of different motor- and transport molecules to the microtubule network (as reviewed⁶⁸) and could for instance indicate if FLCN is associated with such a transport system.

As the binding of FLCN to other proteins often depends on the presence of FNIP1 and/or FNIP2^{9, 10, 47, 48, 69}, it is worth to evaluate the binding of FLCN and its binding partner in the absence of FNIP1 and FNIP2. This can be achieved in an *in vitro* binding assay or by immunoprecipitation or affinity purification in FNIP1 and/or FNIP2 depleted cell lines. Furthermore, it would be of great interest to compare the characteristics of the binding between mutated FLCN and the binding partner with those of wild type FLCN. This indicates if the binding may be affected in patients with BHD syndrome and may hence play a role in the pathophysiology of the disorder. To study this, an *in vitro* binding assay with truncated forms of FLCN can be performed. However, this method lacks the opportunities for post translational modifications of the proteins. Therefore, a method using cells expressing mutant FLCN is preferred.

Limitations

The method we established has limitations that should be considered when planning future experiments. We used cells that exogenously expressed FLCN, with the advantage of having enough FLCN present for all binding partners to interact. On the other hand, the non-physiological levels of FLCN that result from exogenous expression can lead to spurious complex formation.

False positive results can also occur due to contaminating proteins. The evident contaminants, such as skin and saliva proteins, can easily be eliminated from the list of identified proteins. However, the contaminating proteins have binding partners as well. These can be mistaken for relevant binding partners when identified by mass spectrometry. External keratin contamination can be reduced by wearing proper protective measures and

by diminishing laboratory steps that need to be taken in the experiment. For example, by performing mass spectrometry of the entire purified sample, instead of a single band, one saves performing SDS-PAGE, silver staining and releasing the protein band. However, analysing the entire sample by mass spectrometry would eliminate the possibility to correlate the size of the identified protein with the size of the analysed band. Also, the opportunity to compare silver stained bands with the control sample is lost. Nevertheless, when the control sample is subjected to mass spectrometry too, it creates the opportunity to compare all proteins that are identified in both samples. This way, all proteins present in the control sample can be discarded as possible binding partners. Moreover, proteins with low abundance may not be visible by silver staining, but can be identified by mass spectrometry. Hence, whole sample analysis would be a more sensitive method. In our study, it would have been a better control to compare the bands from purified FLCN with corresponding samples from the EGFP control. Unfortunately, resources did not permit this. The risk of purifying proteins non-specifically underscores the importance of validating binding partners that are identified with our TAPtag system.

In addition, false negative results may occur. The detergent in the cell lysis buffer disrupts the cell membranes by interrupting the phospholipid bilayer and solubilising the lipids and the membrane bound proteins. Non-denaturing detergents, such as the Triton X-100 and octyl glucoside that we used, do not typically interrupt PPIs. However, disruption of the lipid bilayer may disrupt membrane-bound protein complexes. Hence, membrane-bound binding partners may not be found. Furthermore, weak PPIs can be disrupted during the purification process. To avoid these unintentional interruptions, PPIs can be stabilised by treating the cells with a cross-linking agent before harvesting. However, cross-linking will increase the amount of non-specific background. Elution of the protein complex should therefore be done under denaturing conditions, such as the urea elution or low pH elution that we used for the HA-IP in our experiment. As we did not intend to identify all PPIs of FLCN, there was no need for cross-linking in this study.

In our experiments, the cell lines transfected with mutant *FLCN* did not survive (UOK 257), did grow very slowly (HK-2) or did not express the mutated FLCN (RPE). This could be due to the *in vitro* system we used. It is also possible that FLCN1285dupC is toxic to the cells when over-expressed. Nevertheless, other researchers were able to express truncated FLCN in cells by transfecting an expression vector containing FLCN1285dupC.⁷⁰ However, they did use other cell lines (FTC-133 and DLD-1) that are derived from advanced stages of carcinomas. Carcinomas generally are adapted to survive in strenuous conditions, possibly explaining their resistance to the likely toxicity of mutated FLCN. To possibly prevent toxicity, mutant FLCN could be expressed at endogenous levels. For this, bacterial artificial chromosomes, which can hold large inserts and restrict their plasmid copy number, could be used to

harbour mutant TAPtagged *FLCN* as well as the endogenous regulatory sequence for *FLCN* expression. Another possibility is using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9) to first add TAPtag DNA to the endogenous *FLCN* gene in a human cell line and subsequently generate a truncating mutation in the *FLCN* gene. For both methods new cell lines need to be generated, which is labour intensive. However, being able to compare PPIs between wild type and mutant *FLCN* will be a valuable asset in elucidating the pathophysiology of BHD syndrome.

Finally, our experiments were performed in HK-2 cells only. Research by others shows that the effect of *FLCN* on signalling transduction pathways differs greatly between cell lines.⁷¹ As signal transduction depends on the binding of proteins, PPIs may also differ between cell lines. Hence, it is desirable to repeat our experiments in another cell line, such as the UOK 257 cells expressing TAPtagged *FLCN*. Future experiments with TAPtagged *FLCN* as well as validation experiments for putative binding partners, should preferably be done in duplicate, using both HK-2 and UOK257s.

Other research into binding partners and functions of *FLCN*

Studies by other research groups confirm that protein complex purification followed by mass spectrometry is a successful method to identify binding partners of *FLCN*. Tsun *et al.* immunoprecipitated FLAG tagged RagA, -B, -C and D and identified *FLCN* as putative binding partner by mass spectrometry. They confirmed binding of *FLCN* to RagA and RagC by immunoprecipitation of FLAG-*FLCN* from transfected cells. The binding only occurred when FNIP2 was co-transfected.⁴⁸ Dunlop *et al.* identified *FLCN* as putative binding partner of GABARAP after HA-IP of HA-GABARAP and subsequent mass spectrometry. The binding was confirmed in an *in vitro* binding assay and *in vivo* by HA-IP in cells transfected with HA tagged GABARAP and V5 tagged *FLCN*. This binding also only occurred when FNIP1 or FNIP2 was present.⁶⁹ To our knowledge, we are the first to identify binding partners by purifying the *FLCN* complex itself.

Even more studies show that the purification of (tagged forms of) *FLCN*, followed by Western blotting, can confirm its binding to other proteins. As was done for the binding to TDP-43⁷², PKP4^{41, 73} and KIF3a⁷⁴ and again for the Rag proteins by a second research group.⁴⁷ PKP4 and KIF3a were first picked up as possible binding partners by Y2H and subsequently validated by purification of *FLCN* and Western blotting. TDP-43 was shown to co-localise with *FLCN* by immunofluorescence before their interaction was confirmed by immunoprecipitation and Western blotting of tagged *FLCN* and TDP-43.

Reiman *et al.* used another technique to detect pathways in which *FLCN* is involved. They combined the results of a gene expression array and a protein array in cells and tissues

with aberrant *FLCN* expression. This revealed one apoptosis related protein to be differentially expressed. An apoptotic protein assay in cells expressing mutant *FLCN* showed upregulation of two other apoptosis related proteins. This led them to conclude that *FLCN* may have a role in apoptosis and tumour suppression.⁷⁵ These results give rise to further validation experiments. Altogether, multiple techniques can be used to study *FLCN*'s relation to other proteins. We showed our TAPtag system to be one of them. Conveniently, the TAPtag system can be used for further experiments as well.

Future experiments using TAPtagged *FLCN*

The experiments that we performed thus far are just the start of what can be achieved with the TAPtagged *FLCN* system. With currently advancing mass spectrometry techniques, it is possible to subject the complete tandem purified sample to mass spectrometry, instead of just subjecting a single protein band. In this manner, all purified binding partners can be identified and not only those of a certain size. This does not only enable the identification of a wide range of putative binding partners, it also gives insight in the pattern of PPIs. A pattern of multiple proteins from one specific cellular structure, pathway or function strongly suggests that *FLCN* has a role in these as well. Since *FLCN* is known to localise throughout the entire cell⁹, it may be involved in multiple different structures and/or functions. Pattern analysis of all PPIs is a great opportunity to get an overview of all these possible functions. However, the data obtained by whole sample analysis may be complex. To reduce complexity, the tandem purified sample can be enriched first, for example by performing gel-filtration chromatography or density gradient centrifugation. This eliminates all fractions that do not contain *FLCN* and will therefore reduce the complexity of the mass spectrometry results. To make matters even more complex, *FLCN* may form various complexes with different binding partners, so the binding partners that are identified are not necessarily in the same protein complex. This should be taken into account when analysing the data.

To further elucidate *FLCN*'s function in a specific organelle or a certain subcellular localisation, cells can be fractionated prior to tandem affinity purification and mass spectrometry. This allows the identification of binding partners from one specific part of the cell. Next to newly identified cellular structures, it is also worth to explore binding partners in structures that *FLCN* is shown to localise to, such as cilia^{19, 74} and lysosomes.^{47, 76} When the putative binding partners alpha tubulin and eEF1A1 and eEF1A2 can be confirmed, also the microtubules, ribosome and nuclear membrane are of particular interest to study. Furthermore, *FLCN* localises to the nucleus and cytoplasm whereas FNIP1 and FNIP2 localise to the cytoplasm only.⁹⁻¹¹ This may indicate that *FLCN* has a FNIP dependent function in the cytoplasm and a FNIP independent role in the nucleus. Therefore, it is of interest to compare the binding pattern in the cytoplasm with that of the nucleus can be studied by isolating these fractions separately.

Not only the absence or presence of PPIs, but also their quantity is of interest. For relative quantification of proteins in mass spectrometry SILAC (stable isotope labelling by amino acids in cell culture) can be used. Mutant and wild type FLCN could be compared and the influence of factors such as the exposure to drugs or changes in nutrient status of the cell, could be studied. Together, whole sample analysis, comparison of subcellular fractions and PPI quantity comparison give lots of opportunities for further research with TAPtagged FLCN.

Conclusion

We established a TAP method for identifying PPIs for FLCN and used it to identify multiple possible binding partners by mass spectrometry. Both eEF1A and alpha tubulin are potential binding partners worth validating further. Additionally, the TAPtagged FLCN gives many opportunities for future research to further elucidate the function of FLCN and its role in the pathogenesis of BHD syndrome.

Appendix 1: Troubleshooting tandem affinity purification

The method for TAP of FLCN presented in this chapter, was established after optimising the purification steps. The most essential optimisations will be described in this appendix.

Conventional nickel affinity purification

In conventional Ni-AP of TAPtag-FLAG-FLCNwt from HK-2 cells, TAPtag-FLAG-FLCNwt was captured on the nickel beads without being eluted further, whereas TAPtag-EGFP was present in all elutions (Figure A1-A). We hypothesised that not only the TAPtag, but also FLCN itself binds nickel and showed endogenous FLCN from HK-2 cells to non-specifically bind the nickel beads (Figure A1-B). Non-specific binding of proteins to nickel can be prevented by using imidazole in the binding buffer and wash buffer. Therefore, we tested binding- and wash buffers with different concentrations of imidazole (5 mM, 40 mM and 80 mM). The use of 80mM imidazole in the buffers, results in the least endogenous FLCN being captured on the beads (Figure A2-A). Further non-specific binding was fully prevented by incubating the nickel beads with imidazole prior to loading the input onto the beads (Figure A2-B).

Figure A1. FLCN non-specifically binds nickel beads. (A) Western blot of a conventional Ni-AP in HK-2 cells expressing TAPtag-FLAG-FLCNwt or TAPtag-EGFP, probed with anti-HA. TAPtag-FLAG-FLCNwt (left panel) is present in the first elution, but none is released from the beads in the second elution, whereas it is still present on the beads after these elutions. TAPtag-EGFP (right panel) is present in the first elution, in the second elution and to a lesser extent it is left on the beads after the elutions. (B) Western blot of a conventional Ni-AP in HK-2 cells expressing TAPtag-FLAG-FLCNwt or TAPtag-EGFP and in un-transfected HK-2 cells, probed with anti-FLCN (rabbit polyclonal, 1:2000, a kindly provided by prof. A. Pause, McGill University, Montreal, Canada). Endogenous FLCN from the HK-2 cells expressing TAPtag-EGFP is present on the beads after elution. Using a higher film exposure time for the Western blot of un-transfected HK-2 cells shows endogenous FLCN in the elution, indicating that it bound the beads. FLCN is also captured on the beads after elution. Probing only with anti-rabbit (HRP-conjugated goat anti-rabbit polyclonal 111-035-144 Jackson ImmunoResearch 1:30,000) was done as a negative control (lower panel).

Pre-incubating the nickel beads with imidazole, and using higher concentrations of imidazole in the buffers, may not only prevent non-specific binding to the beads, but may also prevent specific binding of the 10His tag to the beads. If this happens, the TAPtag can no longer be pulled down. However, Figure 7 has shown that TAPtag-EGFP and TAPtag-FLAG-FLCNwt are present in the respective Ni-APs, confirming that these 10His tagged proteins were sufficiently purified. Pre-incubating the nickel beads with imidazole and using 80 mM imidazole in the binding- and wash buffer was used for all further experiments.

Figure A2. 80mM imidazole binding buffer and wash buffer combined with pre-incubation of nickel beads with imidazole prevents non-specific binding of FLCN. (A) Western blot of a conventional Ni-AP in HK-2 cells expressing TAPtag-FLAG-FLCNwt or TAPtag-EGFP, using binding buffer and wash buffer with 5mM, 40mM or 80mM imidazole, probed with anti-FLCN. With 80mM imidazole, the least endogenous FLCN binds to the nickel beads (arrows), however, a small amount was bound (dark arrow). The samples from the input, the wash steps and the unbound flow through were used as an internal control for the experiment. (B) Western blot of a conventional Ni-AP in HK2-cells expressing TAPtag-EGFP or TAPtag-FLAG-FLCNwt, probed with anti-FLCN. No endogenous FLCN from the TAPtag-EGFP expressing cells was present in the elution or captured on the beads after elution. The amount of input used for Western blotting was too small to visualize the endogenous FLCN. HK-2 cells expressing TAPtag-FLAG-FLCNwt were used as a control, showing FLCN is purified. Even though, four times less sample of the control was used, because of the over expression of FLCN compared to the cells expressing TAPtag-EGFP, it causes intense bands.

Nickel affinity purification using fast protein liquid chromatography

When preparing samples for FPLC Ni-AP we used our standard lysis buffer containing Triton-X100. This buffer turned out to absorb UV at 280 nm, which interfered with the

wavelength used by the FPLC machine to measure protein concentration. An alternative lysis buffer based on octyl glucoside did not absorb UV at 280 nm, making it compatible with further use in FPLC Ni-AP.

In FPLC Ni-AP of HK-2 cells expressing TAPtag-EGFP we used imidazole concentration gradient of 5 - 300 mM for elution, with collection of 30 fractions of 1 ml. Under these conditions, TAPtag-EGFP was only present in the final two elutions, when visualised by SDS-PAGE and subsequent Coomassie Brilliant Blue staining (data not shown). Since there were no subsequent elution fractions, we could not be certain that all TAPtag-EGFP was eluted. When we changed the imidazole gradient to 5 - 600 mM, TAPtag-EGFP was present in the middle six elution fractions. Later fractions did not contain TAPtag-EGFP, indicating that all was eluted (Figure A3). In further experiments a 5 - 600 mM imidazole gradient was used for elution.

Figure A3. TAPtag-EGFP is present in central elution fractions of FPLC Ni-AP with 5 - 600 mM imidazole gradient for elution. Coomassie Briljant Blue staining after SDS-PAGE of samples from a FPLC Ni-AP of HK-2 cells expressing TAPtag-EGFP. In elution fractions 11 to 16 a band with the size of TAPtag-EGFP is present. This band is not seen in further elution fractions (elution fraction 19 to 30 not shown). As expected, many protein bands are seen in the input, unbound and washes. Elution fractions 5 to 9 show a strong band for a protein heavier than TAPtag-EGFP, which most likely is a non-specifically purified protein.

HA immunoprecipitation

HA-IP of the TAPtag-EGFP containing elutions of a FPLC Ni-AP in HK-2 cells expressing TAPtag-EGFP showed a purified protein band for TAPtag-EGFP, but no other protein bands were seen upon SDS-PAGE and Coomassie Brilliant Blue staining (data not shown). Even though only few proteins are expected to be co-purified with TAPtag-EGFP, this suggests that the EGFP protein complex could have been disrupted during the purification process. To overcome this issue, we used milder conditions, with a lower salt concentration in all buffers and continuously handling the samples at 4°C. Additionally, we replaced the Coomassie Brilliant Blue staining by silver staining, which is a more sensitive method to detect proteins in a SDS-PAGE gel. After applying these changes, we could detect multiple protein bands in the elution of the tandem purification, confirming successful co-purification of proteins along with TAPtag-EGFP (data not shown).

Next, we tandem purified TAPtag-FLAG-FLCNwt from HK-2 cells by FPLC Ni-AP and subsequent HA-IP. Unexpectedly, no TAPtag-FLAG-FLCNwt was detected in the elution and a lot was captured on the anti-HA agarose, indicating that TAPtag-FLAG-FLCNwt was not properly eluted (data not shown). The standard elution buffer we used contained 8 M urea for disrupting PPIs. To optimise the elution, four alternative elution methods were investigated. First, 9 M urea elution was used, because a higher concentration of urea can be more effective in disrupting protein binding. Second, an elution buffer with pH 3 was used, since a low pH can disrupt protein binding too. Third, a buffer containing a high concentration of 3HA peptides (AS-63764 Eurogentec 2.4 mg/ml) was used, for peptide competition. This is based on the principle that an overload of 3HA peptides in the buffer will compete with the binding of the single HA tag in TAPtag-FLAG-FLCNwt and hence releases this tag from the HA-agarose. Fourth, TEV protease was used to cleave the TEV cleavage site that is present in the TAPtag. This would release FLAG-FLCNwt from the TAPtag that is captured on the agarose. Western blots of the first three elution methods, showed that the 9 M urea adequately eluted TAPtag-FLAG-FLCNwt. The low pH elution buffer completely eluted TAPtag-FLAG-FLCNwt, but also showed lower bands, suggesting that TAPtag-FLAG-FLCNwt - and possibly its entire protein complex - was degraded by this buffer. Peptide competition showed hardly any elution of TAPtag-FLAG-FLCNwt (Figure A4-A). The TEV cleaved sample was subjected to SDS-PAGE and silver staining, but no protein band representing released FLAG-FLCNwt was seen (Figure A4-B). Hence, 9 M urea buffer was the only suitable elution buffer to be used in further experiments.

Figure A4. TAPtag-Flag-FLCNwt is effectively released from anti-HA agarose by 9 M urea. SDS-PAGE and (A) Western blots, probed with anti-6His, or (B) silver staining of samples from elutions and from agarose after elution, from HA-IPs of TAPtag-FLAG-FLCNwt containing fractions of FPLC Ni-PD in HK-2 cells expressing TAPtag-FLAG-FLCNwt, using 9 M urea, low pH, 3HA or cleavage by TEV protease to release FLCN from the anti-HA agarose. TAPtag-FLAG-FLCNwt is eluted with 9 M urea and low pH buffer, none is left on the agarose. Three lower bands are present after low pH elution, suggesting degradation of FLCN. FLCN is not released and left on the agarose after elution with 3HA or cleavage from the TAPtag by TEV protease.

Appendix 2: Primer sequences

Table A1. Overview of primers used for Sanger sequencing of created plasmids.

Plasmid	Primer name	Primer sequence
pGEM-Teasy-TAPtag	SP6-20	CTATTTAGGTGACACTATAG
	SP6rev	TATTTAGGTGACACTATA
pGEM-Teasy-FLAG-FLCN	T7fw	TAATACGACTCACTATAGG
	FLCN159fw	GATGAACAGTCGGATGCGTGCG
	FLCN389rev	CTCAGGCTCCGGACACAGG
	FLCN314fw	AATACGTCAGCCACCAGCACC
	FLCN714fw	CGCTGACATCGCTGACAAGTG
	BHD9cF	CCCCGGAAGCTGCCAGTC
	FLCN1435fw	GGCCCCACCATCCTGAATAAG
pTAPtag-FLAG-FLCNwt and pTAPtag-FLAG-FLCN1285dupC	SP6rev	TATTTAGGTGACACTATA
	EGFP-N1-F	GAGGTCTATATAAGCAGAGC
	EGFP-C1-R	AACCATTATAAGCTGCAATAAAC
pTAPtag-EGFP	Flcn314fw	AATACGTCAGCCACCAGCACC
	EGFP-N1-F	GAGGTCTATATAAGCAGAGC
pQCXIP-TAPtag-FLAG-FLCNwt and pQCXIP-TAPtag-FLAG-FLCN1285dupC	FLCN159fw	GATGAACAGTCGGATGCGTGCG
	FLCN389rev	CTCAGGCTCCGGACACAGG
	FLCN314fw	AATACGTCAGCCACCAGCACC
	FLCN714fw	CGCTGACATCGCTGACAAGTG
	BHD9cF	CCCCGGAAGCTGCCAGTC
	FLCN1435fw	GGCCCCACCATCCTGAATAAG
pQCXIP-TAPtag-EGFP	pcDNA5-906fw	CATCCACGCTGTTTGGACCTCC

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Discussion & valorisation

Partly based on:

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Discussion & valorisation

Birt-Hogg-Dubé (BHD) syndrome (MIM 135150) predisposes to fibrofolliculomas, lung cysts, pneumothorax and renal tumours and is caused by mutations in *FLCN* encoding the folliculin protein (FLCN).¹⁻³ Though the disorder is rare, it can cause medical and psychological burden to the patients and hence pressures the health care system. Since BHD syndrome is a genetic disorder, patients may not only be concerned about their own health, but also worry about affected relatives and passing on the disorder to their children. This stresses why developing preventive measures for these patients is important. To date, prevention is not possible, as the pathogenic mechanism of BHD syndrome has not been elucidated yet. Hence, the current clinical focus is on timely detection and treatment of renal tumours. Eventually, we aspire to treat or even prevent the symptoms by aiming at the pathogenic mechanism. This underscores the importance of performing fundamental research into the disorder. Not only BHD syndrome patients may benefit from this research. As all types of renal cell carcinoma can develop in BHD syndrome, it is a good model to study renal cancer in general.

BHD syndrome may be underreported, because it is a rare disorder and not all clinicians are aware of the syndrome. Even when they are, they may not recognise it due to the heterogeneity of clinical manifestations between families, between patients within one family and between patients of different ethnicity (as explained in **chapter 1 and 2**). Moreover, the clinical manifestations (specifically the fibrofolliculomas) may not cause acute morbidity and patients will not always seek medical attention, particularly when health care is expensive for them. Therefore, the incidence of BHD syndrome may be underestimated. With increasing accessibility of advancing medical technologies, more patients are likely look for medical attention in the future. Hence, research into BHD syndrome would benefit more patients than presently thought. Raising clinicians' awareness of the syndrome by publishing this research is important for all patients, as it will increase their chance of having the disorder discovered in a timely fashion.

This thesis addresses the timely detection of renal tumours, research into a therapeutic intervention, as well as the elucidation of the pathogenic mechanism in BHD syndrome. In this chapter, the results of the previous chapters will be considered together and put in perspective of the published literature. This gives rise to new ideas for future studies.

Renal surveillance

In **chapter 2** we evaluate the compliance to surveillance of our cohort of BHD syndrome patients in a retrospective study of 32 cases. Compliance is low. Of 19 patients who commenced surveillance, only 42% underwent renal imaging with recommended frequency.

Most reported reasons for non-compliance to MRI scanning are claustrophobia and not having been reminded. Hence, we recommend educating patients about the possibility of open MRI scanning and explicitly communicating who is responsible for initiating surveillance, as well as sending reminder letters. Furthermore, we noticed that appointing a case manager as point of contact for patients, prevents difficulties they experienced in arranging imaging. These factors influencing compliance remain to be verified. Moreover, we need to verify our experience that BHD syndrome patients do not seem to value the importance of renal surveillance and neither see the significance of the increased risk of renal cancer. Adding a psychological survey to a future prospective study into surveillance might help to further explore these possibilities and potential ways to anticipate them. Such a prospective study could also be used to collect more data on the moment renal tumours are diagnosed (i.e. with baseline imaging, surveillance or interim imaging because of complaints) and treated. A future study will enable further optimisation of surveillance frequency for BHD syndrome patients. For this, a larger cohort of patients and a long follow up period is required. However, assembling a large cohort is difficult, as BHD syndrome is rare. Multi-centre collaboration on national (or even international) level is desirable.

It is important to optimise renal surveillance for timely detection of renal tumours, because this may enable nephron sparing surgery. Preserving healthy renal tissue is essential, as patients stay at risk for developing a second tumour in the contralateral kidney, which may require surgery too.

FLCN and mTOR

In **chapter 3**, we explore rapamycin for treatment of fibrofolliculomas in BHD syndrome, based on the clinical similarities between BHD syndrome and tuberous sclerosis complex (TSC). Both disorders combine renal cysts and tumours with cystic pulmonary lesions and hair follicle tumours.⁴ This clinical resemblance might be reflected in a similar underlying biology. In TSC the signalling of mTOR (mammalian target of rapamycin) is deregulated⁴ and mTOR signalling been reported to be affect in BHD syndrome as well.⁵⁻¹² Hence, we hypothesised that topical rapamycin, which effectively treats skin lesions in TSC, is a potential treatment for the fibrofolliculomas in BHD syndrome. Here, we discuss our results, as well as the data from other groups studying mTOR in BHD syndrome.

mTOR involvement in the maintenance of fibrofolliculomas

In **chapter 3**, we report that topical application of 0.1% rapamycin solution during 6 months is not an effective treatment for fibrofolliculomas in BHD syndrome patients. Rapamycin is an inhibitor of the mTOR cellular signal transduction cascade. Many studies report deregulation of mTOR signalling in mouse models and cell lines that harbour aberrant FLCN expression.⁵⁻¹² This led us to hypothesise that inhibiting mTOR with rapamycin could be an

effective treatment for fibrofolliculomas in BHD syndrome. Our finding that topical rapamycin treatment was not effective could imply mTOR signalling is not upregulated in the fibrofolliculomas. Alternatively, our treatment was not potent enough, due to insufficient penetration in the skin or because the cutaneous concentration was too low to be effective.

Arguing against the latter explanations, the potency of the 0.1% rapamycin solution we used has been confirmed in other studies. It was found to be effective for the treatment of skin papules in familial discoid fibroma and in TSC.¹³⁻¹⁵ This indicates the rapamycin level reached in the skin lesions sufficiently inhibits mTOR signalling underlying these disorders. A recent publication suggests that 0.2% rapamycin gel may be more effective than a 0.1% gel when treating angiofibromas in TSC.¹⁶ However, due to anomalous results and the small sample size of the TSC group that was treated with 0.1% rapamycin gel, conclusions cannot be drawn and it cannot be excluded that the 0.1% gel is equally effective. Nevertheless, it might be worth investigating the efficacy of 0.2% rapamycin gel in treating fibrofolliculomas in BHD syndrome. However, before undertaking a clinical trial, it should be established beyond doubt that upregulation of mTOR signalling causes these fibrofolliculomas.

Our findings suggest that mTOR may not play a role in the maintenance of the fibrofolliculomas; giving clues to researchers to consider shifting directions when studying the pathogenesis of BHD syndrome. Furthermore, it prevents doctors from trying topical rapamycin 0.1% solution as a possible, but ineffective, treatment for their BHD syndrome patients suffering from skin lesions.

Indeed, whilst our trial was being conducted, conflicting data were reported on the dysregulation of mTOR signalling in BHD syndrome.⁹ Since then, more contradictory results have been published. Both up-¹⁷⁻²³ and downregulation²⁴⁻²⁷ of mTOR signalling have been seen upon aberrant FLCN expression. Khabibulin *et al.* show that the mTOR readout phospho-S6 is upregulated upon FLCN knockdown in SAEC cells (human small airway epithelial cells), but is unaffected in HBE cells (human bronchial epithelial cells).¹⁹ These observations suggest that the effect of the used systems can greatly influence the outcome of the study. Moreover, mTOR signalling is affected by many different signals (such as ATP levels, amino acids, growth factors, flow stress) and has various effectors (as reviewed by Laplante and Sabatine²⁸). Due to the diverse functions of mTor it is difficult to study the effect of one single player in its complex signalling cascade.

Additional mTOR studies in BHD syndrome

More recent data have started to provide an explanation for the confusing results on mTOR involvement. FLCN itself has multiple functions that target mTOR in different man-

ners (Figure 1). First, Petit *et al.* found FLCN to bind RagA/B through the GTPase domain of RagA, suggesting FLCN may be a guanine exchange factor (GEF), for loading GTP on RabA/B.²⁴ RabA/B is a small GTPase that forms a heterodimer with RabC/D. When activated, the heterodimer binds mTOR and recruits it to the lysosome where mTOR can be activated (Figure 1A). This study reports downregulation of mTOR upon FLCN knockdown.²⁴ Second, FLCN was identified as GTPase activating protein (GAP), hydrolysing GTP to GDP on RabC/D in order to recruit mTOR to the lysosome²⁵ (Figure 1B). This study by Tsun *et al.* also show that FLCN knockdown results in downregulation of mTOR.²⁵ It may sound contradictory that FLCN enriches one Rab with GTP, whereas it charges the other Rab with GDP. However, it is known that mTOR binding is strongest by GTP loaded RagB in heterodimer with GDP loaded RagC.²⁹ Thus, by its GEF and GAP function, FLCN stimulates mTOR recruitment to the lysosome and hence facilitates its activation. Third, FLCN can stimulate mTOR by increasing the leucine levels in the lysosome.²⁶ FLCN facilitates this by inhibiting the expression of PAT1 (proton-assisted amino acid transporter 1) on the lysosome surface. This decreases the export of leucine from the lysosome and the resulting higher intra-lysosomal leucine levels activate mTOR³⁰ (Figure 1C). In accordance with this function, knockdown of FLCN causes downregulation of mTOR in this report.²⁶ Fourth, FLCN inhibits mTOR by recruiting LKB1 to the cilium. LKB1 phosphorylates AMPK at the basal body which leads to mTOR inhibition²³ (Figure 1D). Accordingly, mTOR activity is reported upon FLCN knockdown in the study describing this function of FLCN. Fifth, FLCN could prevent mTOR activation at the lysosome, by facilitating perinuclear localisation and immobilisation of the lysosome. FLCN can do so by binding RILP at the perinuclear membrane and loading Rab34 onto it.³¹ The presence of Rab34 on the perinuclear membrane causes lysosomal recruitment.³² When lysosomes cluster at the perinuclear region mTOR activation cannot take place³³ (Figure 1E). In summary, FLCN has multiple functions and can modulate mTOR signalling in several ways. Targeting mTOR in BHD syndrome is not an option until we know what part, if any, of the mTOR signalling cascade is involved in the pathogenesis of the syndrome and if up or downregulation causes clinical symptoms.

Interpreting mTOR studies in BHD syndrome

All aforementioned studies measured mTOR signalling by looking at its readouts (phosphorylated) S6 or (phosphorylated) S6-kinase 1 (S6K). Conversely, Wada *et al.* report that FLCN is required for proper mTOR signalling towards the transcription factor TFE3, which regulates mitochondrial biogenesis.²⁷ This is a mTOR mediated signalling cascade that is also influenced by amino acids levels, but has no effect on S6 and S6K. This underlines the complexity of the role of FLCN in mTOR signalling and illustrates that measuring FLCN's influence on mTOR is challenging.

Furthermore, the effects of FLCN on mTOR are triggered by different stimuli. The upregulation of mTOR through cilia happens upon flow stress.²³ The GEF and GAP function of FLCN towards the Rab heterodimer is activated by the presence of amino acids. On the other hand, mTOR inhibition by stimulating the perinuclear localisation of lysosomes occurs under starvation. So, mTOR is upregulated by FLCN in a nutrient-rich state and downregulated upon starvation. Of note, the signalling of amino acid levels to mTOR is independent of hamartin and tuberlin, which are the affected proteins in TSC, a disorder that resembles BHD syndrome. Both disorders combine renal cysts and tumours with cystic pulmonary lesions and hair follicle tumours.⁴ The hamartin-tuberlin complex inhibits canonical mTOR signalling³⁴⁻³⁶ and is involved in signalling low ATP levels to mTOR.³⁷ FLCN, on the other hand, is required for inhibiting mTOR upon high ATP levels.²³ Nevertheless, both FLCN and the TSC complex are required for proper mTOR signalling. This may explain why TSC and BHD syndrome have similar but not identical phenotypes.

As the various mTOR effectors and its multiple affecting stimuli are largely interconnected, it is difficult (if not impossible) to study one single aspect of FLCN's function in this signalling route. For example, to study FLCN's role in mTOR stimulation through the cilia, one could drive cells to ciliate by starving them. However, starvation itself also affects mTOR signalling. Hence, when one aims to study FLCN's role in mTOR signalling, all experimental conditions should be carefully considered and multiple mTOR readouts should be measured.

In order to find out if rapamycin is a potential therapy for BHD syndrome patients, it may be necessary to first elucidate the key function of FLCN that is deregulated in BHD syndrome, before further studying FLCN's general role in mTOR signalling.

Mutant FLCN

To better understand the pathogenesis of BHD syndrome, we investigated the molecular mechanisms underlying key aspects of the disease phenotype, as presented in **chapter 4 and 5**. For this, we used different cell lines having FLCN knockdown or FLCN overexpression and cells transfected with mutant variants of FLCN. We noted both overexpression and knockdown of FLCN disrupt spheroid formation and delay ciliation of cells (**chapter 4**). Hence, the correct level of wild type FLCN seems important for ciliogenesis. Of the mutant variants we used, c.1523A>G (encoding p.Lys508Arg) was the only one for which we could generate a usable cell line, in contrast to c.1285dupC (encoding p.His429ProfsX27), which did not result in a usable cell line, because of low expression of the encoded protein, cell death and/or low growth rate. Here, we explore possible explanations for these intriguing results.

Figure 1. FLCN can stimulate (A-C) and inhibit (D,E) mTOR signalling in different ways. A detailed description can be found in the text.

c.1285dupC

Transduction of human kidney-2 (HK-2) cells with EGFP tagged FLCN1285dupC resulted in cells with a low FLCN1285dupC expression level (**chapter 4**). HK-2 cells transduced with His-HA-FLAG tagged FLCN1285dupC did grow slow and did not show expression at all, nor did retinal pigment epithelium (RPE) cells transfected with this construct. UOK 257 cells³⁸ transfected with His-HA-FLAG tagged FLCN1285dupC did not survive (**chapter 5**). In all, we were not able to generate a cell line stably expressing FLCN1285dupC. A dominant negative or toxic effect of mutant FLCN could explain this observation. However, Nahorski and colleagues did manage to overexpress FLCN1285dupC in the DLD-1 colorectal cancer cell line. It shows normal growth compared to DLD-1 transfected with an empty vector. From this observation, Nahorski et al conclude there is no evidence FLCN1285dupC has a dominant negative effect, but suggest this may be due to the cell line used.³⁹ They also expressed FLCN1285dupC in FTC-133 cells, which are derived from a lymph node metastasis of a thyroid carcinoma.³⁹ The FTC-133 cells do not express endogenous FLCN, whereas DLD-1 cells do. Hence, the presence of endogenous FLCN does not seem to influence the survival of cell lines with FLCN1285dupC. Cell lines derived from advanced

stages of carcinomas generally are adapted to survive in strenuous conditions, which may explain resistance of DLD-1 and FTC-133 cells to a dominant negative effect, as well as to any toxicity of mutant FLCN. However, these potential effects of mutant FLCN may prevent survival of other cell lines. Our HK-2 and RPE cell lines having no or low expression of FLCN1285dupC when surviving, could be due to improper incorporation of the plasmid, resulting in cells containing only the resistance cassette and not the FLCN1285dupC DNA. Since functionally tested mutations in FLCN have been shown to disrupt the stability of the protein³⁹, this may further impair expression of FLCN1285dupC if transfection was successful.

c.1523A>G

Noticeably, UOK257 cells transduced with FLCN mutant variant *c.1523A>G* (encoding p.Lys508Arg) survive, whereas cells with *c.1285dupC*, *c.1844C>G* (encoding p.Tyr463Stop) and *c.764A>G* (encoding p.His255Arg) do not (**chapter 4**). This *c.1523A>G* mutation might not be pathogenic and could be a polymorphism. The mutation was reported in three patients with renal tumours who reportedly had no other symptoms of BHD syndrome.^{40,41} No loss of heterozygosity was detected in two of them; in one it was not tested. To explain this seemingly contradictory observation, the renal tumours in these patient might have been caused by mutations in other renal cancer susceptibility genes. Moreover, another 24 people, not diagnosed with BHD syndrome, were reported to carry *c.1523A>G* as well, according to the ExAC database of protein-coding genetic variations.⁴² Either BHD syndrome was not recognised in these 24 controls, or *c.1523A>G* could be a rare polymorphism. One could speculate that *c.1523A>G* is a (partially) functional polymorphism that does not cause the BHD syndrome phenotype, but can cause renal tumours in combination with mutations in other renal susceptibility genes.

Alternatively, *c.1523A>G* could be a splice site variant, as is predicted by Alamut Visual (*Interactive Biosoftware*, version 2.11), a program that incorporates numerous software prediction tools to predict the pathogenic status of genetic mutations (Figure 2). To experimentally confirm this, a functional assay by reverse-transcription PCR on the patient's RNA could be performed. As no genotype-phenotype correlation has been claimed in BHD syndrome^{40, 43}, one would not expect cells expressing *c.1523A>G* cDNA to behave differently from cells transfected with other mutants. However, *c.1523A>G* being a splice site variant could explain the lack of any pathogenic effect of FLCN1523A>G cDNA. Since splicing is determined by the genomic context, the *c.1523A>G* mutation inserted in the cDNA will not affect splicing. In this case, FLCN1523A>G cDNA in *in vitro* experiments may not have a pathogenic effect whereas other mutants do.

A Original splice site

B Alternative splice site

Figure 2. Mutation c.1523A>G in *FLCN* enhances an alternative 3' acceptor site between nucleotides 1523 and 1524. This acceptor site would delete 91 bases from the mRNA (r.(1433_1523del)). Splicing probability was predicted by SpliceSiteFinder-like, MaxEntScan, NNSPLICE and GeneSplicer and combined by Alamut Visual (*Interactive BioSoftware*, version 2.11) for (A) the original splice site in wild type *FLCN* and (B) the alternative splice site between nucleotide 1523 and 1524 in wild type *FLCN* (upper panel) and in *FLCN* c.1523A>G (lower panel).

In **chapter 4** we also show that overexpression of FLCN1523A>G cDNA does not delay ciliogenesis, whereas overexpression of wild type FLCN does. Furthermore, FLCN1523A>G overexpression only slightly, but significantly, changes the lumen size in spheroid formation, whereas wild type FLCN overexpression as well as knock down severely impaired spheroid formation. This suggests the FLCN1523A>G cDNA may lack physiological functionality. Studies by Hasumi *et al.* suggest at least partial functionality of the murine equivalent of spliced c.1523A>G. They used bacterial artificial chromosome (BAC) recombination technology to express the mutated genomic *Flcn* region in mice with kidney specific *Flcn* knock out. These mice have a partially rescued renal phenotype compared to mice with kidney specific *Flcn* knock out.⁴¹ Additionally, they show polycystic kidneys and/or tumours occur in all heterozygous *Flcn* knock out mice and in only 26% of mice expressing both wild type *Flcn* and p.Lys508Arg, again suggesting a partial functionality of p.Lys508Arg.^{41, 44}

All in all, c.1523A>G could be an innocuous polymorphism. It might also affect splicing, although formal proof for this supposition is lacking. Hence, one should be cautious when interpreting results of experiments using FLCN1523A>G genomic DNA as well as cDNA. Results should not be interpreted as representative for the situation in BHD syndrome patients.

Not only for research purposes, but also for patients and society, it may be worth to further clarify if this variant of *FLCN* is pathogenic. It could prevent patients from being wrongfully diagnosed with BHD syndrome when this variant of *FLCN* is found in a renal tumour again. Consequently, it could prevent psychological burden and medical costs involved with the diagnosis.

FLCN and cilia

As lung, kidney and liver cysts have been reported in BHD syndrome patients^{45, 46}, we hypothesized that BHD syndrome might be a ciliopathy. Ciliopathies are a group of disorders caused by dysfunction of primary cilia, conserved cellular structures that are best understood as antennae. Cyst formation is a hallmark of these ciliopathies.

To test our hypothesis, we investigated the relationship between FLCN and the formation and function of cilia in **chapter 4**. There is strong evidence that cilia are involved in controlling planar cell polarity (PCP).⁴⁷ In polarised epithelia, PCP regulates cell division in plane of the tissue. It ensures that spatial organisation of tissue remains intact. In **chapter 4** we show that knockdown of FLCN results in abnormal ciliation and disrupts PCP in a spheroid model. Our results have been confirmed by other groups that also report aberrant PCP¹⁷ and defects in ciliation²³ in the context of abnormal FLCN levels. We show that knockdown

of FLCN delays ciliogenesis. Once ciliogenesis has commenced, FLCN may no longer have a role, as mature cilia do not have a defective shape or size in cells with less FLCN.

However, recent research suggests that FLCN may also have a role in the functioning of the cilia. FLCN is found to interact with KIF3A²³, which is part of the motor for intraflagellar transport (IFT) that is required for the assembly and maintenance of cilia.⁴⁸⁻⁵⁰ As FLCN binds to the cargo-binding region of KIF3A, it might be a cargo protein for the IFT motor.²³ Possibly consistent with a role in transport, FLCN was found to recruit LKB1 to the cilium.²³ LKB1 is subsequently transported to the basal body, where it phosphorylates AMPK and thereby inhibits mTOR signalling (Figure 1C).²³ If FLCN is indeed an IFT cargo protein, it might regulate the trafficking of signalling molecules, such as LKB1, through the cilium or regulates the level of these molecules in the cilium.

In our study, we noticed β -catenin localises to the cilium and is absent from the cilia upon FLCN knockdown. Ciliary sequestration of β -catenin limits its nuclear translocation and thereby inhibits Wnt signalling.⁵¹ Accordingly, we found β -catenin activation and increased canonical Wnt signalling upon FLCN knockdown. Hence, FLCN may play a role in localising and/or transporting β -catenin and thereby influence Wnt signalling. Intriguingly, Wnt signalling is also known to be activated by LKB1 (as reviewed by Martin-Belmonte⁵²) and is potentially oncogenic (as reviewed by Zhan⁵³). Moreover, LKB1 influences PCP by non-canonical Wnt signalling (as reviewed by Zhan⁵³). Hence, our work together with that of Zhong *et al.*, connects FLCN's role in cilia with mTOR and Wnt signalling through LKB1. Disturbance of ciliary function, mTOR signalling and Wnt signalling have been linked to cyst formation and oncogenesis⁵³⁻⁵⁸ and therefore might contribute to the BHD syndrome phenotype. Interestingly, LKB1, cilia and Wnt have been linked to tumour formation in Peutz-Jeghers syndrome (PJS). PJS is most often caused by mutations in LKB1, which cause ciliary defects⁵⁹ and Wnt was shown to be activated in this syndrome⁶⁰⁻⁶² that is characterised by mucocutaneous pigmentation, gastrointestinal polyps and predisposition to multiple cancers.⁶³

By classifying BHD syndrome as a ciliopathy, the BHD field can now benefit from all studies being done on ciliopathies, to better understand the pathophysiology of BHD syndrome. If a clear-cut role of Wnt signalling in the pathophysiology can be confirmed, new treatment possibilities are raised, as multiple Wnt inhibitors are in clinical development for the treatment of cancer (as reviewed⁶⁴).

Involvement of FLCN in intracellular transport

With regards to the functions of FLCN described above, it is striking that multiple functions of FLCN towards mTOR and the cilia seem to involve transport and localisation of other molecules. FLCN localises mTOR to the lysosome^{24, 25}, it localises the lysosome itself to the

perinuclear region³¹, binds to a main component of the intraflagellar transport motor²³, localises LKB1 to the cilium²³ and regulates the transport of leucine from the lysosome.²⁶ In addition, FLCN was found to be involved in the nuclear export of TDP-43.⁶⁵ Thus far, TDP-43 has not been linked to the pathophysiology of BHD syndrome. Instead, it is involved in regulating autophagy and its cytoplasmatic accumulation and mislocalisation is a hallmark of the neurodegenerative disorders amyotrophic lateral sclerosis and frontotemporal lobar dementia.⁶⁶⁻⁶⁸ However, FLCN's involvement in nucleocytoplasmatic shuttling of TDP-43 again points to a possible role for FLCN in the transport and/or localisation of a broader group of other molecules. In particular, FLCN seems to be involved in energy related transport and localisation, as many effects depend on the presence or absence of amino acids (Figure 1A,B,E) or involves amino acid transport (Figure 1C). Concurrently, FLCN was found to be involved in autophagy⁶⁹, a process that uses vesicular transport and lysosomal degradation to regulate cellular energy maintenance upon nutrient starvation (as reviewed⁷⁰). Furthermore, FLCN having a role in energy related transport is in line with its function in ciliogenesis (**chapter 4**), as the cilium functions as energy sensor and has a role in autophagy.⁷¹ The putative binding partners of FLCN that we identified (**chapter 5**) could further support FLCN's role in transport and/or localisation. Alpha tubulin and both isoforms of eukaryotic elongation factor 1-alpha (eEF1A), eEF1A1 and eEF1A2, were found to be possible binding partners of FLCN. eEF1A is involved in the transport of RNA to the ribosome.⁷² Furthermore, eEF1A1 plays a critical role in the nuclear export of proteins⁷³ and eEF1A2 plays a role in vesicular trafficking through phosphatidylinositol signalling.^{74, 75} In addition, alpha tubulin, together with beta tubulin, is the main constituent of microtubules, which can be regarded as tracks for transportation within the cell (as reviewed⁷⁶). Thus, an increasing body of evidence suggests a role for FLCN in (energy related) transport and localisation of other molecules. This is in line with the function of FLCN's yeast ortholog LST7 (lethal with sec-thirteen protein 7), which has a role in amino acid transport by shuttling general amino-acid permease GAP1 from the Golgi apparatus to the plasma membrane in response to nitrogen, which is an essential component of amino acids and thereby functions in energy related transport.^{77, 78}

FLCN's role in vesicular transport by being a GAP/GEF, or facilitating GAP/GEF interaction with key target proteins

From recent findings, FLCN may orchestrate transport by affecting GTPases as a GEF and/or GAP, or facilitating GAP/GEF reactions. Not only was FLCN suggested to be GAP for the small GTPase RabA/B²⁴ and a GEF for RabC/D²⁵ (as already described in this chapter and shown in Figure 1A and B), FLCN's C-terminal structure is also similar to that of DENN (differentially expressed in normal cells and neoplasia) domain proteins.⁷⁷ DENN domain proteins are known to function as GEFs for Rab GTPases.⁷⁹ Accordingly, FLCN was suggested to be a GEF for Rab35 *in vitro*.⁷⁷ Rab GTPases are essential in vesicle membrane

transport, where they are activated by GEFs.^{80,81} The Rabs on a vesicle coats and organelle membranes determine where the vesicle is transported to and which effector proteins are recruited (as reviewed by Stenmark⁸²). In line with FLCN's DENN domain having an essential function, three pathogenic *FLCN* mutations that were *in silico* analysed, are predicted to cause structural changes in its DENN domain.⁸³

Furthermore, two independent studies show that FLCN interacts with PKP4^{17, 84}, which binds Rab11 and is involved in its subcellular localisation.⁸⁵ Rab11 is known to be hydrolysed by a GAP.⁸⁶ It is not yet known if FLCN functions as this GAP. PKP4 also regulates activation of the small GTPase RhoA, by interacting with its GEF Ect2.⁸⁷ FLCN affects Rho activity too, however both inhibition and activation were observed in FLCN deficient cells.^{17, 84} FLCN could function as Rho GAP or facilitate either the Rho GAP or the GEF Ect2. Finally, eEF1A, the putative binding partners of FLCN that we identified (**chapter 5**), needs to be GTP loaded in order to deliver RNA to the ribosome (as reviewed⁷²).

Alternatively, FLCN may not be a GEF or GAP itself, but rather provide a platform for GDP/GTP loading of Rab GTPases and associated GEFs. FLCN is already known to bind RILP and load it with Rab34, to promote lysosomal clustering at the perinuclear region (as already described in this chapter and shown in Figure 1E). However, a GEF function of FLCN towards Rab34 could not be demonstrated.³¹ One could propose that FLCN (together with RILP) acts as a scaffold for another GEF toward Rab34. Putatively it is a scaffold for GEFs and GAPs towards other Rabs as well, instead of being a GEF/GAP itself. This hypothesis is supported by *in vitro* experiments in which a GEF or GAP function of FLCN could not be confirmed (unpublished data; personal communications with dr. A. Tee, Cardiff University, Cardiff, United Kingdom).

Recently, FLCN's role as a (scaffold for) GAP towards Rab GTPases was further established by Laviolette and colleagues, who showed that FLCN binds Rab7A and the GTPase activity of Rab7A increases when overexpressed together with FLCN. Moreover, FLCN was shown to bind Rab7B, Rab9A and Rab35.⁸⁸ Laviolette *et al.* relate the increased GTPase activity of Rab7a in the presence of FLCN to tumour growth in BHD syndrome, by showing that it affects EGFR signalling. EGFR is mutated or overexpressed in many types of cancer, where it increases proliferation and migration of cells (as reviewed⁸⁹). EGFR signalling is largely regulated by endocytic recycling⁸⁹, in which Rab7A plays a role.⁸² Consistently, FLCN knockout in FTC-133 cells was shown to increase EGFR signalling and EGFR overexpression was shown in BHD syndrome related kidney tumours versus healthy kidney tissue.⁸⁸ Another study showed EGF induced cell growth increased upon FLCN knock down.⁹⁰ From these observations, it is tempting to hypothesise that tumours in BHD syndrome might be caused by increased EGFR signalling and that, therefore, treatment with EGFR inhibitors

might be effective. However, many of the experiments by Laviolette *et al.* were performed in FTC-133 cells, a thyroid cancer cell line that expresses no FLCN and in addition harbours many other carcinogenic mutations.^{91, 92} Hence, the results will have to be confirmed in other cell lines and *in vivo* models. Xenograft FTC-133 tumours in nude mice grow more slowly when treated with an EGFR inhibitor, but do not regress. Therefore, increased EGFR signalling may be a maintaining factor for tumour growth, but not a driver mechanism in BHD syndrome associated renal tumours. Moreover, EGFR upregulation is associated with many cancer types, such as non-small-cell lung cancer, metastatic colorectal cancer and glioblastoma, but not typically with renal cell carcinoma.⁹³ Further studies are required to elucidate the exact influence of FLCN mutations on EGFR signalling and its role in tumour formation in BHD syndrome patients.

Summarising, the key function of FLCN may be localising and/or transporting molecules to the right places in the cell in response to energy levels. FLCN putatively acts as (a scaffold for) GAP and/or GEF for small Rab GTPases. As transport of molecules is important for many functions in the cell, it is challenging– but essential– to explain which functions are disrupted by mutated FLCN in BHD syndrome and how the consequences lead to the patient's phenotype. Ultimately, mutant versus wild type FLCN should be studied in a representative model for BHD syndrome to better understand the underlying molecular pathophysiology.

Future prospects

Currently, treatment of BHD syndrome by targeting its causal pathophysiological mechanism is out of reach. Care for patients focusses on the early detection of renal tumours. In the absence of targeted treatment, a prospective multi-centre study into the moment of diagnosis of renal tumours and pinpointing the reasons for suboptimal compliance could improve patient care.

To be able to develop (preventive) treatment for BHD syndrome, it is crucial to understand how aberrant FLCN eventually leads to symptoms in patients. This exact knowledge is currently lacking. To elucidate the pathogenic mechanism in BHD syndrome, one could compare cells expressing wild type FLCN with cells expressing mutant FLCN. From the research in this thesis we now know that overexpressing FLCN is not a proper model.

Creating a cell line with normal levels of mutant FLCN by using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9) could be a more representative model for studying BHD syndrome. Tumour cells are not an ideal system for this, as they harbour many other (carcinogenic) mutations that may have effects that are not primary related to BHD syndrome. Tumour cells also have an altered

metabolism⁹⁴, which is undesirable when studying FLCN, as we hypothesise that FLCN may act in response to energy levels. On the other hand, primary kidney cells from BHD syndrome patients are an interesting model to create a second mutation (homozygous or compound heterozygous) in, using CRISPR/Cas9, to mimic the initiation of tumorigenesis in BHD syndrome; as loss of heterozygosity was shown to be a frequent event in BHD syndrome related renal tumours.⁹⁵ However, these cells have limited proliferative capacity and require immortalisation for continuous usage. In turn, this will cause additional mutations that may alter the behaviour of the cell line. To overcome this, patient derived induced pluripotent stem cells (iPSCs) are of interest, as they have unlimited proliferative capacity and can differentiate into any cell type.⁹⁶ Kidney differentiated iPSCs could be kept growing to study their natural course and CRISPR/Cas9 could be used to study the effect of a second hit mutation. Patient derived iPSCs with the FLCN mutation corrected could serve as a control with identical genetic background. All cell lines could be compared for readouts such as cilia formation, Wnt, EGFR and mTOR signalling. Any differences could then be confirmed by immunofluorescence on kidney and tumour tissue of patients, for involvement of the identified pathway in the pathogenesis of BHD syndrome. In addition, our TAPtag (see **chapter 5**) can be used in CRISPR/Cas9 manipulated cells to enable an unbiased search for new interactors of FLCN. CRISPR-Cas9 was previously used before to successfully add three hemagglutinin tags to the N-terminus of FLCN.²⁴ Analysing the pattern of protein-protein interactions (PPIs) of FLCN may give insight in the (many) cellular structures, functions and signalling routes that FLCN might be part of. Comparing the (quantity of) PPIs between wild type and mutant FLCN could further indicate which functions of FLCN are the most affected in BHD syndrome.

If the effect of aberrant FLCN on tumour formation is elucidated and key pathways are identified, further studies can focus on targets to stop, reverse or even prevent malignancies in BHD syndrome. Furthermore, BHD syndrome may be a good model for renal tumours in general, as the disorder can cause all types of renal cell carcinoma (RCC). Uncovering the function of FLCN may also give better understanding of the pathobiology of other RCCs. As an example, such discoveries have facilitated treatment in von Hippel Lindau (VHL) disease, a disorder that strongly predisposes to clear cell RCC. The *VHL* gene was found to be mutated or lost in most sporadic clear-cell RCCs as well. Further research showed *VHL* to be involved in hypoxic inducible factor mediated RCC growth, which can be antagonised by multi-targeted tyrosine kinase inhibitors. These drugs proved effective and are now used to treat metastatic renal cancer (as reviewed⁹⁷). Similarly, the research presented in this thesis and the method established in **chapter 5** could contribute to a cascade of discoveries, eventually leading to treatment options for BHD syndrome and possibly for a broader range of renal tumours. Therefore, not only BHD syndrome patients, but also patients suffering from other types of kidney tumours may benefit from research into BHD syndrome.

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7

Summary
Samenvatting

Summary

Birt-Hogg-Dubé (BHD) syndrome is a rare autosomal dominant disorder predisposing to fibrofolliculomas, lung cysts, pneumothorax and renal cancer. The disorder is caused by heterozygous mutations affecting folliculin (FLCN), an evolutionarily conserved protein that is considered a tumour suppressor. Previous research has uncovered multiple roles for FLCN in cellular physiology, yet it remains unclear how these translate to the lesions occurring in BHD syndrome patients. Hence, targeted and preventive treatment is currently out of reach and the clinical focus is on timely detection of renal tumours to enable nephron-sparing surgery if needed. Therefore, surveillance by renal imaging is recommended to be performed every 1-2 years.

In **chapter 2** we retrospectively describe our cohort of BHD syndrome patients, evaluate their compliance to surveillance and identify possibilities to optimise compliance. In our cohort of 32 BHD syndrome patients, 3 previously unpublished mutations in *FLCN* were detected. Fibrofolliculomas are present in 100% of patients, renal cysts in 44% and renal tumours in 9%. Pneumothorax is reported in 13% of patients. Lung cysts are seen in 70% of the 23 patients for whom lung imaging was available. Compliance of BHD syndrome patients to recommended renal surveillance in this cohort is low. At least 61% of patients complied with surveillance and 42% had renal imaging with recommended follow-up frequency. Not being prompted for surveillance and claustrophobia are reported reasons for non-compliance to MRI scanning. We recommend to create a protocol for follow up incorporating explicit communication on responsibility for initiating follow up, sending reminder letters to patients, educating them about imaging methods and limiting obstacles for attending surveillance.

The fibrofolliculomas that occur in BHD syndrome, can be a psychological and social burden to patients and they may therefore seek treatment for them. Current treatments for fibrofolliculomas have high rates of recurrence and carry a risk of complications. A preventive treatment for fibrofolliculomas, aiming at the underlying pathogenic mechanism, would be desirable. Animal models of BHD syndrome have previously shown deregulation of mammalian target of rapamycin (mTOR). Topical use of the mTOR inhibitor rapamycin is an effective treatment for the angiofibroma skin tumours in tuberous sclerosis complex, which is also characterised by mTOR deregulation. In **chapter 3** we aimed to determine if topical rapamycin is an effective treatment for fibrofolliculomas in BHD syndrome. We performed a double blinded, randomised, facial left-right controlled trial of topical rapamycin 0.1% versus placebo in 19 BHD patients, during 6 months. No change in cosmetic status of fibrofolliculomas was reported in the majority of cases for the rapamycin treated (79% by doctors, 53% by patients) as well as the placebo treated facial sides (both 74%). No

significant difference in fibrofolliculoma number or change in size of the fibrofolliculomas was seen. This study provides no evidence that treatment of fibrofolliculomas with topical rapamycin in BHD syndrome results in cosmetic improvement.

To further explore the pathogenic mechanism of BHD syndrome, we took a closer look at its similarities with other disorders. Since BHD syndrome manifests hallmark characteristics of ciliopathies, we speculated that FLCN might also have a ciliary role. In **chapter 4** we show our data that indicate that FLCN localizes to motile and non-motile cilia, centrosomes and the mitotic spindle. Alteration of FLCN levels can cause changes to the onset of ciliogenesis, without abrogating it. In three dimensional culture, abnormal expression of FLCN disrupts polarized growth of kidney cells and deregulates canonical Wnt signalling, which may, at least partly, explain cyst formation and tumour growth in BHD syndrome.

Furthermore, we approached the search for FLCN's functions from a different angle, by establishing an unbiased method to detect protein-protein interactions for FLCN, since the function of its binding partners can give more insight in the function of FLCN itself. In **chapter 5** we show addition of two affinity purification tags to FLCN, followed by chimeric expression in cells. The double tagged FLCN and its interacting proteins were subsequently tandem purified from the cells. The protein complex was analysed by mass spectrometry and several potential binding partners for FLCN were identified. Validation studies will confirm if their binding is specific and may lead to future studies to characterise FLCN's role towards the binding partners, as well as to the role of the protein complex in the cell.

FLCN's function and its role in the pathogenesis of BHD syndrome has not been completely elucidated yet. When we take our data together with the results of other studies regarding FLCN's function, as done in **chapter 6**, it is striking that many functions relate to molecular transport in the cell, in response to energy levels. FLCN putatively has a role in here, acting as a (scaffold for) GAP and/or GEF for small Rab GTPases. Further research is required to evaluate this hypothesis.

Uncovering FLCN's function may give rise to new therapeutic targets in BHD syndrome. Since all types of renal cell carcinoma occur in BHD syndrome, it also provides a unique model to study the pathophysiology of renal cell carcinoma in general.

Samenvatting

Birt-Hogg-Dubé (BHD) syndroom is een zeldzame autosomaal dominante aandoening die risico geeft op fibrofolliculomen, longcysten, pneumothorax en nierkanker. De aandoening wordt veroorzaakt door heterozygote mutaties waardoor het eiwit folliculine (FLCN) wordt aangedaan. FLCN is in de evolutie goed bewaard gebleven en wordt beschouwd als een tumor suppressor. Eerder onderzoek heeft verschillende mogelijke rollen laten zien voor FLCN in de cel. Het is echter nog onduidelijk hoe deze de symptomen van BHD syndroom veroorzaken. Er is daarom op dit moment nog geen (preventieve) behandeling beschikbaar die zich richt op het pathofysiologisch mechanisme. Klinisch richt men zich zodoende op het tijdig opsporen van niertumoren, om niersparend te kunnen opereren als dit nodig is. Er wordt daarom aangeraden om iedere 1-2 jaar nierscreening middels beeldvorming te doen.

In **hoofdstuk 2** beschrijven we ons cohort van BHD syndroom patiënten, beoordelen of ze het nierscreeningsadvies opvolgen en geven mogelijkheden aan om die opvolging te verbeteren. In ons cohort van 32 BHD syndroom patiënten zijn 3 niet eerder gepubliceerde mutaties in *FLCN* gevonden. Fibrofolliculomen kwamen voor bij 100% van de patiënten, niercysten bij 44% en niertumoren bij 9%. Pneumothorax werd door 13% van de patiënten gemeld. De opvolging van het nierscreeningsadvies van BHD syndroom patiënten in ons cohort is laag. Ten minste 61% van de patiënten onderging nierscreening en 42% deed dit met de geadviseerde frequentie. Niet opgeroepen worden voor screening en claustrofobie werden genoemd als redenen voor het niet opvolgen van het advies een MRI scan te ondergaan. We bevelen aan om een protocol voor de follow-up te maken waarin wordt vastgelegd dat duidelijke gecommuniceerd moet worden wiens verantwoordelijkheid het is om de follow-up te initiëren, dat er een herinneringsbrief gestuurd wordt naar patiënten, dat er uitleg moet zijn omtrent de beeldvormingsmethode en dat belemmeringen om screening te ondergaan beperkt moeten worden.

De fibrofolliculomen die voorkomen bij BHD syndromen kunnen psychologisch en sociaal belastend zijn voor de patiënten, waardoor ze behandeling wensen. Met de huidige behandelmethodes kunnen fibrofolliculomen weer terugkomen en is er een risico op complicaties. Een preventieve therapie, gericht op het onderliggende pathogene mechanisme, is wenselijk. Diermodellen van BHD syndroom laten zien dat *mammalian target of rapamycin* (mTOR) ontregeld is. Lokale behandeling met rapamycine, een mTOR-remmer, is effectief tegen de huidtumoren die voorkomen bij tubereuze sclerose, een aandoening die ook gekenmerkt wordt door ontregeling van mTOR. In **hoofdstuk 3** onderzoeken we of lokale behandeling met rapamycine ook effectief is tegen fibrofolliculomen bij BHD syndroom. We hebben een dubbelblinde, gerandomiseerde, links-recht gecontroleerde studie gedaan naar lokale rapamycine 0,1% in vergelijking met placebo, in het gelaat van

19 BHD syndroom patiënten, gedurende 6 maanden. Zowel artsen als patiënten zagen in de meeste gevallen geen cosmetisch resultaat van de behandeling met rapamycine (artsen 79%, patiënten 53%) of met placebo (beiden 74%). Er werd ook geen significant verschil gezien in het aantal of de grootte van de fibrofolliculomen. Deze studie levert daarom geen bewijs voor cosmetische verbetering van de fibrofolliculomen bij BHD syndroom door behandeling met lokale rapamycine.

Om het pathogene mechanisme van BHD syndroom verder te ontrafelen hebben we gekeken naar de overeenkomsten met andere aandoeningen. BHD syndroom heeft symptomen die kenmerkend zijn voor ciliopathiën, waardoor we denken dat FLCN ook een rol heeft in het cilium. In **hoofdstuk 4** laten we zien dat FLCN zich in de bewegende en niet-bewegende cilia, het centrosoom en de kernspoel bevindt. Verandering van het FLCN-niveau vertraagt de start van de ciliogenese, zonder dat deze afgebroken wordt. In drie dimensionale kweken zorgt abnormale FLCN-expressie voor ontregeling van de ruimtelijke oriëntatie van niercellen en van de Wnt signaaltransductieroute, waarmee de vorming van cystes en tumoren in BHD syndroom verklaard zou kunnen worden.

Verder hebben we de zoektocht naar de functie van FLCN benaderd vanuit een ander gezichtspunt, door een methode te ontwikkelen waarmee op onbevooroordeelde wijze eiwitbindingen voor FLCN kunnen worden opgespoord. De functie van haar bindingspartners kan namelijk inzicht geven in de functie van FLCN zelf. In **hoofdstuk 5** laten we zien hoe we twee opzuiveringslabels aan FLCN hebben bevestigd, dit tot expressie brachten in cellen en vervolgens een dubbele opzuivering deden om FLCN en de daaraan gebonden eiwitten te isoleren uit de cel. Dit eiwitcomplex werd vervolgens geanalyseerd met massaspectrometrie, waarmee verschillende potentiële bindingspartners van FLCN werden geïdentificeerd. Validatieonderzoeken zullen uitwijzen of de binding met FLCN specifiek is en kunnen leiden tot vervolgonderzoek naar de rol van het eiwitcomplex in de cel.

De functie van FLCN en haar rol in de pathogenese van BHD syndroom is nog niet volledig opgehelderd. In onze resultaten, samen met de resultaten van onderzoek door anderen, valt op dat veel functies van FLCN gerelateerd zijn aan het transport van moleculen in de cel, als reactie op het energieniveau (**hoofdstuk 6**). FLCN zou hier een rol in kunnen spelen als (platform voor) een GAP en/of een GEF voor kleine Rab GTPases. Vervolgonderzoek is nodig om dit te kunnen bevestigen.

Het ophelderen van de functie van FLCN kan leiden naar nieuwe aangrijpingspunten voor behandeling van BHD syndroom. Doordat alle soorten van niercelcarcinoom voorkomen bij BHD syndroom, is dit ook een bijzonder model om de pathofysiologie van het niercelcarcinoom in het algemeen mee te bestuderen.

A

Addendum

Curriculum vitae

Lists of publications and presentations

Dankwoord

Curriculum vitae

Momenteel ben ik – Marigje Vernooij, geboren 20 november 1983 in Utrecht – in opleiding tot dermatoloog in het Maastricht Universitair Medisch centrum (MUMC+). Hiermee startte ik in december 2013. Na twee perifere stages van elk een half jaar in het Elkerliek ziekenhuis in Helmond (2014-2015) en het Zuyderland Medisch Centrum in Heerlen (2017), ben ik weer terug in Maastricht om de opleiding af te ronden.

Ik heb oog voor nieuwe ontwikkelingen en een voorliefde voor organiseren. In het MUMC+ ben ik actief betrokken bij de ontwikkeling van de Polikliniek van de Toekomst.

De basis voor mijn specialisatie tot dermatoloog én de basis voor dit proefschrift werden gelegd tijdens het VWO aan het Mondriaan College in Oss (1996-2002) en de geneeskunde opleiding die ik volgde aan de Universiteit Maastricht (2002-2009). Mijn coschappen deed ik in Nederland en Thailand. Ik werd geprikkeld door de bijzondere en leerzame buitenlandervaring en ben na mijn afstuderen drie maanden vrijwilligerswerk gaan doen in het Kumasi South Hospital in Ghana. In het laatste half jaar van mijn geneeskunde opleiding deed ik mijn wetenschappelijke stage in het laboratorium dermatologie (2009, MUMC+). Hier ontdekte ik dat fundamenteel en translationeel onderzoek fascinerend is. Met plezier begon ik daarom eind 2009 in hetzelfde laboratorium als promovenda aan het onderzoek dat geresulteerd heeft in dit proefschrift (2009-2013). Het was enorm inspirerend om als promovenda ook twee maanden onderzoek te mogen doen bij de afdeling biochemie aan de Cambridge University (2011). Deze stage werd door zowel de René Vogels stichting als de KWF kankerbestrijding gewaardeerd met een reisbeurs.

Ook buiten het werk daag ik mezelf graag uit. In mijn vrije tijd ben ik een fanatieke sporter en doe ik aan team-triatlon. Dit jaar liep ik de marathon van Rotterdam. Net als het schrijven van dit proefschrift was dat een prachtige uitdaging en ervaring.

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Grants and awards

Best poster award, Science day of GROW research school of Maastricht University, Maastricht, 2012. Poster: *Truncating mutations in folliculin-interacting protein 1 cause familial multiple disoid fibroma*. €500.

Academic scholarship, Dutch Cancer Society (KWF), 2011. Project: the identification of potential interacting partners for folliculin at the department of Biochemistry, University of Cambridge, Cambridge, United Kingdom. €1500.

Travel grant, René Vogels stichting, 2011. Destination: scientific internship Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom. €1300.

Oral and poster presentations

Nierscreening bij patiënten met Birt-Hogg-Dubé syndroom. Oral presentation. 344th Scientific meeting of the Dutch Association for Dermatology and Venereology, Maastricht, 2017.

Een schaap in wolfskleren? Epidermodysplasia verruciformis. Oral presentation. Regional dermatology seminar of the Maastricht University Medical Centre+, Maastricht, 2017.

Weet u het zeker? Subcorneale pustuleuze dermatose. Oral presentation. Regional dermatology seminar of the Maastricht University Medical Centre+, Maastricht, 2016.

Jonge patiënt met bruine maculae, amyloidosis cutis. Oral presentation. Regional dermatology seminar of the Maastricht University Medical Centre+, Maastricht, 2014.

Truncating mutations in folliculin-interacting protein 1 cause familial multiple discoid fibromas. Oral presentation. 14th Scientific meeting of the Dutch Association for Experimental Dermatology, Lunteren, 2013.

Birt-Hogg-Dubé syndroom een ciliopathie. Oral presentation. 326th Scientific meeting of the Dutch Association for Dermatology and Venereology, Maastricht, 2012.

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Tap tagging for folliculin-protein interactions. Poster presentation. 3rd International Birt-Hogg-Dubé syndrome symposium, Maastricht, 2011.

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