What is BHD?

Contents
1. Introduction – Birt–Hogg–Dubé syndrome.................................................................4
  1.1 BHD Families ........................................................................................................5
2. Clinical manifestations of BHD syndrome .................................................................6
  2.1 Fibrofolliculomas ...............................................................................................6
  2.2 Pulmonary cysts and pneumothorax .................................................................6
    2.2.1 Histology ...............................................................................................................6
    2.2.2 Prevalence ...........................................................................................................7
  2.3 Renal cell carcinoma ..........................................................................................8
    2.3.1 Histology ...............................................................................................................8
    2.3.2 Prevalence ...........................................................................................................10
  2.4 Other clinical manifestations ............................................................................10
    2.4.1 Colorectal polyps and colorectal cancer ......................................................10
    2.4.2 Thyroid nodules and cancer ...........................................................................11
    2.4.3 Parotid tumours ...............................................................................................11
    2.4.4 Other manifestations .........................................................................................11
3. Folliculin gene .........................................................................................................13
  3.1 Identification of the FLCN gene .........................................................................13
  3.2 FLCN: mechanism of pathogenesis ...................................................................13
    3.2.1 Tumour suppression .........................................................................................13
    3.2.2 Happloinsufficiency .........................................................................................13
    3.2.3 Dominant negative .........................................................................................14
    3.2.4 Compound heterozygosity ..............................................................................14
  3.3 FLCN mutations ..................................................................................................14
    3.3.1 Base substitution mutations ...........................................................................15
    3.3.2 Deletion mutations .........................................................................................16
    3.3.3 Duplication mutations .....................................................................................18
    3.3.4 Indel mutations ...............................................................................................19
    3.3.5 Insertion mutations .........................................................................................19
    3.3.6 Base substitutions unlikely to be pathogenic .................................................19
    3.3.7 FLCN variants not associated with BHD .......................................................19
4. Folliculin and its interacting partners .....................................................................21
  4.1 Folliculin protein ................................................................................................21
    4.1.1 FLCN structure ...............................................................................................23
    4.1.2 Post-translational modifications of FLCN ...................................................23
4.2. Folliculin-binding proteins .............................................................................................................. 24
  4.2.1 FNIP1............................................................................................................................................. 24
  4.2.2 FNIP2............................................................................................................................................. 25
  4.2.3 Plakophilin-4 .............................................................................................................................. 26
  4.2.4 Rpt4.............................................................................................................................................. 26
  4.2.5 Rag proteins ................................................................................................................................. 26
  4.2.6 ULK1 and GABARAP ................................................... 26
  4.2.7 TDP-43........................................................................................................................................ 26
4.3 Expression pattern and cellular localisation of FLCN................................................................. 27
  4.3.1 mRNA ....................................................................................................................................... 27
  4.3.2 Protein ....................................................................................................................................... 27
5. Folliculin-associated signalling pathways ...................................................................................... 28
  5.1 mTOR signalling ............................................................................................................................ 28
  5.2 AMPK signalling ............................................................................................................................. 29
  5.3 HIF signalling and mitochondrial biogenesis .............................................................................. 30
  5.4 Stress resistance and autophagy .................................................................................................... 31
  5.5 Ras-Raf-MEK-Erk signalling and rRNA synthesis ........................................................................ 31
  5.6 JAK/STAT and TGF-β signalling ................................................................................................. 32
  5.7 RhoA signalling ............................................................................................................................. 32
  5.8 Wnt and Cadherin signalling ......................................................................................................... 32
  5.9 Cell Cycle ..................................................................................................................................... 33
  5.10 Apoptosis ..................................................................................................................................... 33
  5.11 Membrane trafficking .................................................................................................................. 34
  5.12 Stem cell maintenance and pluripotency .................................................................................... 34
  5.13 Ciliogenesis ................................................................................................................................ 34
  5.14 Matrix Metalloproteinase signalling ............................................................................................ 35
6. Cell lines and model organisms for studying BHD syndrome .......................................................... 36
  6.1 Cell lines ..................................................................................................................................... 36
    6.1.1 UOK-257 cell line (mouse)...................................................................................................... 36
    6.1.2 ES cell lines (mouse).............................................................................................................. 36
    6.1.3 Flcn-null rat lines .................................................................................................................... 36
  6.2 Yeast models ................................................................................................................................. 36
  6.3 C. elegans model .......................................................................................................................... 37
  6.4 Drosophila model .......................................................................................................................... 37
  6.5 Mouse models ............................................................................................................................... 38
    6.5.1 FLCN mouse models .............................................................................................................. 38
    6.5.2 FNIP1 mouse models ............................................................................................................ 38
6.6 Rat model .......................................................................................................................... 39
6.7 Dog models .......................................................................................................................... 39
  6.7.1 FLCN .............................................................................................................................. 39
  6.7.2 FNIP2 .............................................................................................................................. 39
7. Future Work ............................................................................................................................ 40
  7.1 Clinical research ................................................................................................................ 40
  7.2 Basic research ................................................................................................................... 40
  7.3 Drugs and therapies .......................................................................................................... 41
8. References .............................................................................................................................. 43
1. Introduction – Birt–Hogg–Dubé syndrome

Birt–Hogg–Dubé (BHD) syndrome (OMIM 135150) is an autosomal, dominantly inherited, monogenic condition, characterised by the development of fibrofolliculomas (benign skin tumours) on the face, head and upper torso, pulmonary cysts and pneumothorax (collapsed lung), and predisposition to renal cell carcinoma (kidney cancers) with clear cell, chromophobe, papillary or oncocytic features. The clinical manifestations of BHD syndrome are discussed in Section 2.

BHD syndrome was described in 1977 by three Canadian doctors – Birt, Hogg and Dubé. Hornstein and Knickenberg had also identified the syndrome in 1975, and it has been suggested that the syndrome be renamed Hornstein-Birt-Hogg-Dubé. In 2001, a BHD-associated gene locus was localised to chromosome 17p11.2 and a novel gene, Folliculin (FLCN), was subsequently identified as being inactivated in individuals with BHD syndrome. The FLCN gene codes for a protein called Folliculin (FLCN), which has a putative tumour suppressor function. To date, based on literature, approximately 612 families have been diagnosed with BHD (See Section 1.1). As of February 2016, 183 different FLCN mutations have been identified. The Folliculin gene and its mutations are described in Section 3.

BHD syndrome shares many clinical features with hamartoma syndromes. Hamartoma syndromes are dominantly inherited, predispose to cancer that affects multiple organs, and result in the development of benign tumours. Such syndromes include Cowden syndrome, Peutz-Jeghers syndrome, and Tuberous sclerosis complex (TSC), caused by inactivation of the tumour suppressor genes PTEN, LKB1 and TSC1 or TSC2 respectively. The hereditary nature of renal cell carcinoma in BHD is also seen in association with mutations in other genes: VHL in VHL syndrome; MET in hereditary papillary renal carcinoma (HPRC); FH in hereditary leiomyomatosis and renal cell cancer (HLRCC); BAP1 and CDKN2B.

The histological features of renal tumours alongside other phenotypes can help differentiate between the forms of hereditary RCC.

Folliculin and its known interacting proteins - FNIP1, FNIP2, PKP4, RPT4 and Rag proteins - are discussed in Section 4. The structure of the C-terminal domain of FLCN suggests that the protein functions as a Rab guanine nucleotide exchange factor (GEF) but experimental evidence suggests it could also be a GTPase-activating protein (GAP). FLCN is subject to a number of post-transcriptional modifications, including phosphorylation and ubiquitylation. FNIP1, FNIP2 and S’-AMP-activated protein kinase (AMPK), are able to phosphorylate FLCN, and all three proteins have also been found to be subject to post-translational modification themselves.

FLCN has been implicated in numerous signalling pathways and cellular processes: mTOR signalling; AMPK signalling; HIF signalling and mitochondrial biogenesis; stress resistance and autophagy; Ras-Raf-MEK-Erk signalling and rRNA synthesis; JAK-STAT and TGF-β signalling; RhoA signalling; Wnt and cadherin signalling; cell cycle; apoptosis; membrane trafficking; stem cell maintenance and pluripotency; ciliogenesis; and matrix metalloproteinase function.

The function of FLCN and its role in these pathways is discussed in detail in Section 5, whilst cell lines and animal models of BHD are described in Section 6. Future avenues of research are considered in Section 7.

Recent BHD reviews include those from Hasumi et al., 2015 which focuses on clinical features, management and molecular aspects, and Schmidt & Lineham 2015 which covers the clinical features, genetics and potential therapies for BHD.
## 1.1 BHD Families

*Updated February, 2016*

<table>
<thead>
<tr>
<th>Country</th>
<th>Number of families</th>
<th>References</th>
</tr>
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<td>227</td>
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</tr>
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<td>Japan</td>
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<td>France</td>
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<td>Benusiglio et al., 2014; Ardiouze et al., 2014; Zenone, 2013; Spring et al., 2013; van Denhove et al., 2011; Vint et al. 2010; Steff et al., 2010; Kluger et al. 2010; Le Guyadec et al. 1998; Unpublished*</td>
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<td>The Netherlands</td>
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<tr>
<td>UK</td>
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<td>Belgium</td>
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<tr>
<td>Finland</td>
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<td>Painter et al. 2005</td>
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<td>India</td>
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<td>Iran</td>
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<td>Portugal</td>
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<tr>
<td>Russia</td>
<td>1</td>
<td>Unpublished*</td>
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<tr>
<td>Somalia</td>
<td>1</td>
<td>Pritchett et al., 2014</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>616</strong></td>
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**Legend:** This is a conservative estimate of the number of BHD families, arranged by country. References were only included where it could be determined that the patients in the cohort were unique and not reported elsewhere in the literature. Thus, the actual number of BHD families is likely to be higher.

* - data from [BHD Symposia](https://example.com)
2. Clinical manifestations of BHD syndrome

2.1 Fibrofolliculomas

Patients with BHD syndrome usually develop benign hair follicle tumours, clinically known as fibrofolliculomas, which appear as multiple whitish papules after the age of 20 (Menko et al., 2009). Fibrofolliculomas develop primarily on the face, but can also appear on the neck, ears and the upper torso (Menko et al., 2009).

Previously, fibrofolliculomas and trichodiscomas (skin-coloured tumours occurring on the upper body) were considered separate hallmarks of BHD syndrome, but studies suggest that they may not be distinct histological entities, and that a morphological spectrum of these benign skin tumours may exist (Fujita et al., 1981; Misago et al., 2009). Several case studies suggest that this spectrum could also be expanded to include perifollicular fibromas (Kamada et al., 2015). In addition Aivaz et al., (2015) reported BHD patients with comedonal and cystic fibrofolliculomas; which can be associated with a range of other dermatological conditions.

The number of fibrofolliculomas per BHD patient can range from under 10 to over 100 (Toro et al., 1999). It has been postulated that fibrofolliculomas arise from sebaceous glands, rather than hair follicles, because epithelial strands within fibrofolliculomas are continuous with the sebaceous gland, and are usually localised to areas with a high concentration of sebaceous glands, such as the peri-nasal area (Claessens et al., 2012; Vernooij et al., 2013). The morphology and histology of various benign hair tumours is discussed in a review from Tallechea et al., 2015.

Retrospective analysis of 62 Asian cases of BHD reported in the literature showed that Asian patients are less likely to develop skin lesions, and that there are only four reported cases of Asian BHD patients developing all three symptoms of BHD (Murakami et al., 2014).

Gijzen et al., (2014) performed a small clinical trial testing Rapamycin as a treatment for fibrofolliculomas. Nineteen BHD patients completed the 6 month double-blind, randomised, facial left-right controlled trial. The number of patients who reported cosmetic improvement with Rapamycin was not significantly more than those patients who reported no improvement at all or improvement with the placebo drug. Additionally, 13 out of 19 patients reported one or more side effect; burning, redness, dryness and itching. Overall, these results suggest that Rapamycin in this form is not an effective treatment for fibrofolliculomas.

Starink et al. (2011) described the syndrome Familial Multiple Discoid Fibromas (FMDF), characterised by the presence of multiple discoid fibromas which resemble fibrofolliculomas and develop on the face and pinnae during childhood or early adulthood. Although clearly a genetic syndrome, FMDF is not linked to the FLCN locus therefore represents a distinct syndrome. Wee et al. (2013) report that an 8 week course of topical rapamycin reduced the size and redness of skin lesions in two siblings with FMDF, suggesting that dysregulated mTOR signalling may contribute to the development of skin lesions.

2.2 Pulmonary cysts and pneumothorax

2.2.1 Histology

BHD patients often develop pulmonary cysts and have an increased risk for pneumothorax (Predina et al., 2011; Zbar et al. 2002). Lung anatomy and histology generally appears normal in individuals with BHD, and despite the presence of multiple pulmonary cysts, lung function is usually unaffected (Toro et al., 2007; Tobino et al., 2012). BHD lung cysts are distinct from those cysts that develop in LAM. BHD patients generally have fewer lung cysts, which are located in the lower lobes of the lungs and can affect male or female FLCN mutation carriers of any age; high resolution CT scans can be used to differentiate between these two and other cystic lung diseases (Ferreira Francisco et al., 2015, Gupta et al., 2015, Ha et al., 2015).
In 12 BHD patients analysed by Tobino et al. (2011), the number of lung cysts were found to vary from roughly 30-400, and the size varied from a few millimetres to more than 2 cm. The cysts were generally irregularly-shaped and most commonly found in the lower medial zone of the lungs (Tobino et al., 2012). Another study confirmed the presence of multiple lung cysts, mainly in the lower lungs, that varied in size and shape (Agarwal et al., 2011). Koga et al. (2009) have hypothesised that these cysts represent an aberrant cystic alveolar formation. Subsequently, Furuya et al. (2012) used histopathological analysis to show that the BHD-associated lung cysts were lined with differentiated pneumocytes and had alveolus-like structures within them, indicating that their histopathology is distinct from non-specific blebs and bullae. The clinical and pathological features of the lung symptoms seen in BHD syndrome were reviewed by Furuya and Nakatani (2013).

A study comparing the lung pathology seen in BHD patients with that seen in spontaneous pneumothorax cases caused by smoking, found distinct histological and radiological differences between the two groups (Fabre et al., 2013). BHD patients generally had numerous (more than 20) punch-out type cysts lined with CK7 and TTF1 expressing pneumocytes, showing no signs of inflammation and basal predominance while the control cohort had fewer cysts showing apical predominance. The control cohort also had a greater prevalence of smoking-related changes such as fibroelastotic scars, respiratory bronchiolitis and emphysema, which BHD patients generally did not.

However, one BHD patient, who was a current smoker, had pulmonary changes more similar to the smoking cohort. This suggests that although smoking does not cause the pulmonary changes seen in BHD, it is likely to exacerbate lung problems in BHD patients increasing the risk of pulmonary tumours and should be strongly discouraged. However, a diagnosis of BHD wasn’t ruled out and TTF1 expression was not clearly reported in the control cohort used in this study, suggesting that further studies are required before clinicians modify their diagnostic approach in these cases (Johannesma et al., 2014c).

Several cases of pulmonary tumourigenesis in BHD patients have been reported (Gunji et al., 2007, Furuya & Nakatani 2013, Nishida et al., 2015) and appear to correlate with a history of smoking. No FLCN second hits have been reported in these tumours and further research is required to determine if there is a link between smoking and pulmonary tumours in BHD patients.

Kumasaka et al., (2014) analysed 229 lung cysts in resections from 50 BHD patients and compared them with 117 lung cysts from 34 PSP patients, comparing samples for number, size, location and presence of inflammation. They found that BHD lung cysts were found in both subpleural and intrapulmonary areas; often found at interlobular septa and 40% had venules; and only showed signs of inflammation if located in the subpleura. The authors suggest that loss of FLCN causes alveoli walls to become weak and vulnerable to disruption by mechanical stress during breathing, which causes cysts to form. Furthermore, based on the observation that inflamed cysts tended to be larger and located in the sub-pleura, they suggest that pneumothorax inflames existing cysts, causing them to grow and subsume neighbouring cysts.

Several recent reviews discuss the differential diagnosis of BHD, and other cystic lung diseases, based on imaging (Ha et al., 2015, Gupta et al., 2015, Richards et al., 2015).

### 2.2.2 Prevalence

The presence of pulmonary cysts in BHD syndrome was first described by Toro et al. (1999). Three of thirteen BHD patients examined had pulmonary cysts, and one of these three patients also developed pneumothorax (Toro et al., 1999).

Pulmonary cysts are the most common manifestation of BHD, seen in up to 90% of patients (Predina et al., 2011), suggesting that FLCN has a significant role in normal lung physiology. Pneumothorax is strongly correlated with number of lung cysts, indicating that the presence of lung cysts may cause pneumothoraces (Johannesma et al., 2014f). It is thought that pulmonary cysts increase the risk of
pneumothorax by rupturing and releasing air into the chest cavity (Furuya and Nakatani, 2013; Johannesma et al., 2014).

Using a cohort of 111 BHD haplotype carriers and 112 family members without the BHD haplotype as controls, Zbar et al. (2002) identified a 50-fold increase in the risk of pneumothorax for BHD-affected individuals (OR = 50.3, adjusted for age). Toro et al. (2007) found that following a single episode of spontaneous pneumothorax, recurrent events were more common.

A study by Houweling et al. (2011) found that 28 of 115 (24%) FLCN mutation carriers had a medical history of pneumothoraces, and by analysing 21 BHD families estimated the life-time risk of pneumothorax among BHD patients to be 29%, with a mean age for the first event of 36 years. It is believed that lung pathology may be the earliest symptom of BHD syndrome, as pneumothoraces have been reported in BHD patients as young as seven, fourteen and sixteen years of age (Bessis et al., 2006; Gunji et al., 2007; Johannesma et al., 2014). However, there is one case of a BHD patient presenting with her first pneumothorax at the age of 73, suggesting that the age of onset can be quite variable (Kunogi Okura et al., 2013).

Cohort studies of primary spontaneous pneumothorax (PSP) patients have found that up to 10% of these patients carry FLCN mutations, often without any skin manifestations or renal tumours (Ren et al., 2008, Johannesma et al., 2015). Suspicion of BHD should be higher in families with a history of PSP (Painter et al., 2005, Gunji et al., 2007, Hayashi et al. 2010, Ding et al., 2015) especially if there is also a history of renal cancer of dermatological growths. Identification of BHD patients based on a PSP presentation enables the patients and families to access regular monitoring which can result in earlier tumour treatment.

A recent study found that 12 in 190 BHD patients (6.3%) suffered 13 episodes of pneumothorax within one month of taking a commercial flight, suggesting that air travel may cause pneumothorax in a small proportion of patients (Postmus et al., 2014). Patients should be aware of the symptoms of a pneumothorax so they can seek medical assistance if required.

There is currently no evidence of a genotype-phenotype correlation regarding the lung manifestations of BHD (Kunogi et al., 2010).

2.3 Renal cell carcinoma

2.3.1 Histology

Individuals with BHD syndrome are predisposed to develop renal cell carcinoma (RCC) (Toro et al., 1999; Zbar et al., 2002). Unlike other genetic disorders with this predisposition, renal tumours associated with BHD syndrome are histologically diverse. A large study analysing the histology of 130 renal tumours resected from 30 BHD patients found that 50% (65/130) were of hybrid chromophobe/oncocytoma histology; 34% (44/130) were chromophobe; 9% (12/130) were clear cell; 5% (7/130) were oncocytoma; and 2% (2/130) were papillary (Pavlovich et al., 2002). This pattern is strikingly different from the spectrum of sporadic renal cell carcinomas, where 75% are of clear cell histology, 10% are papillary, 5% are chromophobe and 3-5% are oncocytic (Pavlovich et al., 2002).

However, in a cohort of 14 BHD patients with RCC, tumours with chromophobe and clear cell characteristics were found to occur most frequently (Houweling et al., 2011). In a second cohort of 33 BHD patients with kidney cancer, although mixed chromophobe/oncocytoma was the most commonly observed tumour type, present in 23/33 patients, three patients (9%) had clear cell tumours (Benusiglio et al., 2014b). Together, these studies suggest that BHD-associated renal cell carcinomas are histologically diverse.

Several other mixed patterns can also occur (Pavlovich et al., 2005; Fahmy et al., 2007; Janitzky et al., 2008; Kluitj et al., 2009) and 15 deaths have been reported in BHD patients due to metastatic renal cancer, all of which were of the clear-cell, hybrid clear-cell/papillary or hybrid clear-cell/chromophobe.
histology (Pavlovich et al., 2005; Toro et al., 2008; Claessens et al., 2010; Houweling et al., 2011; Nakamura et al., 2013).

The International Society of Urological Pathology Tumour Panel recently recommended that as hybrid oncocytic/chromophobe tumour only occurs in three settings, a diagnosis of BHD should be considered when this tumour is found (Srigley et al., 2013). However, the case of a Japanese patient with multiple bifocal chromophobe renal cell carcinomas and capsular angiomyolipomas, but no FLCN mutation, suggests that there is at least one chromophobe RCC predisposition gene to be discovered (Sugimoto et al., 2013).

Renal angiomyolipomas, benign tumours which are most commonly associated with tuberous sclerosis complex, have also recently been identified in two BHD patients (Tobino and Seyama, 2012; Byrne et al., 2012). Tumour histology is non-concordant within families, indicating that there is unlikely to be a genotype-phenotype correlation with regards to tumour histology (Pavlovich et al., 2005).

A study by Gatalica et al. (2009) analysed three tumours of different histology found in the kidney of a suspected BHD patient with a germline FLCN mutation and identified a genotype-phenotype correlation. An oncocytic tumour was found to have a second FLCN mutation; an oncocytic-papillary tumour showed increased methylation of the FLCN promoter and somatic mutation of the MET gene; and a clear-cell tumour had a somatic mutation in the VHL gene and increased VHL promoter methylation. Inactivating mutations in VHL and activating mutations in MET cause clear cell RCC and hereditary papillary RCC respectively (Latif et al., 1993; Olivero et al., 1999), meaning that these results are perhaps unsurprising. However, the authors do not show whether the germline FLCN mutation observed in this patient (c.1062+6C>T) actually affects splicing. Therefore whilst these results are interesting, their significance is uncertain at present.

A more recent study reports the pathological findings of kidney tumours in a series of six Japanese patients with confirmed BHD (Kuroda et al., 2014). Five patients had multifocal tumours, two had bilateral tumours and one patient had a solitary tumour. The tumour series consisted of one tumour of unclassified histology, but with features resembling hybrid chromophobe/clear cell histology; three hybrid oncocytic/chromophobe tumours; one collision tumour consisting of chromophobe; clear cell and papillary tumours; a chromophobe tumour; and a clear cell tumour. All tumours had intertumoral peripheral small papillary tufts (ITPSTs) at the interface between the tumour and normal kidney tissue. The authors suggest that the presence of either collision tumours or multiple tumours of different histology, and ITPSTs – as they were universal in this cohort of patients – might be diagnostic hallmarks of BHD-associated kidney tumours.

It is unclear from which part of the kidney the tumours arise. Due to the high percentage of chromophobe tumours, Pavlovich et al. (2002) initially believed the tumours to arise from the distal nephron. However, two independent studies observed FLCN expression in the proximal tubules of murine kidneys, suggesting this is the site of origin (Chen et al., 2008; Hudon et al., 2010).

Work on a marker panel to distinguish BHD tumours from sporadic RCC is ongoing. Iribe et al., 2015 reported that FLCN-associated hybrid tumours show decreased expression of CK7 compared to sporadic chromophobe RCCs but increased expression of Ksp-cadherin and CD82 compared to sporadic oncocytomas. In addition Furuya et al., 2015 reported that tumours from BHD patients showed markedly reduced expression of FLCN and increased expression of GPNMB. Kato et al. (2016) then reported that BHD-chRCC and HOCTs retained chromosome 17 disomy unlike sporadic tumours which are typically monosomic. So far these markers are only able to distinguish between some of the sporadic and BHD-associated tumour subtype (summarised in the table). The identification of further markers will allow for greater understanding of the underlying biology but also more accurate RCC subtype diagnoses.
## 2.3.2 Prevalence

Renal cancer is the most life-threatening complication associated with BHD syndrome. Previous estimates of RCC prevalence among BHD patients have varied. Houweling et al. (2011) reported that 14 (12%) individuals in a cohort of 115 Dutch BHD syndrome patients developed renal cancer and further analysis of 22 BHD families of Dutch origin found the lifetime risk for RCC to be 16%. However, Pavlovich et al., (2005) and Toro et al. (2008) studied large cohorts of American BHD patients and found the prevalence of RCC to be 34/124 (27%) and 30/89 (34%) respectively. A more recent French study found the prevalence to be 33/124 (27%) (Benusiglio et al., 2014b).

The difference in these estimates may be due to population differences between cohorts, or ascertainment bias: the patients in the Dutch study were recruited predominantly via dermatology clinics, whereas the cohorts in the American and French studies were recruited via both dermatology and urology clinics. Due to these ascertainment differences, the Houweling et al. (2011) estimation is likely to be low, whilst the Pavlovich et al., (2005), Toro et al. (2008) and Benusiglio et al. (2014b) estimations are likely to be high. Therefore, the risk of developing kidney cancer is likely to be between 12 – 34%.

A study of 130 tumours from 30 patients showed the prevalence of multifocal disease to be 60% (18/30) and that of bilateral disease to be 77% (23/30) (Pavlovich et al., 2002).

Somatic FLCN mutations have been reported in sporadic cases of RCC (Khoo et al., 2003; Gad et al., 2007): FLCN mutations were found in 1/39 (2.6%) (Khoo et al., 2003) and 6/92 (6.5%) (Gad et al., 2007) renal tumour samples (five clear cell RCC, one papillary RCC and one oncocytoma), indicating that FLCN mutations only cause a small percentage of sporadic RCCs.

Heterozygous loss of FLCN was also reported to cause the transformation of an oncocytoma to a high grade oncocytic carcinoma. However, there were additional genomic rearrangements seen in this tumour, suggesting that additional factors also contributed towards disease progression in this patient (Sririntrapan et al., 2014).

### 2.4 Other clinical manifestations

Fibrofolliculomas, pulmonary cysts, pneumothorax and renal cell carcinoma are the only confirmed manifestations associated with BHD syndrome. At present there is no evidence of a genotype-phenotype correlation with respect to the lung, skin, or kidney manifestations of BHD (Schmidt et al., 2005; Toro et al., 2008). Studies have indicated that other manifestations may also be linked to BHD, but these have yet to be confirmed. These manifestations are discussed below.

#### 2.4.1 Colorectal polyps and colorectal cancer

Early studies suggested an association between BHD syndrome and colorectal neoplasia (Hornstein, 1976; Birt et al., 1977; Schachtschabel et al., 1996; Schulz and Hartschuh, 1999). However, this has been subject to some debate, and a subsequent study by Zbar et al. (2002) found no association between BHD and colonic polyps or colorectal cancer (CRC) in a study involving a large cohort of 111 BHD syndrome patients.

Nevertheless, Khoo et al. (2002) reported a high incidence of colorectal polyps and CRC in BHD patients with confirmed FLCN germline mutations, suggesting that some BHD families are at increased risk of...
colorectal neoplasia, and indicating that FLCN may be involved in colorectal tumourigenesis. Another study, by Nahorski et al. (2010), found that 10 BHD patients out of the 149 assessed had CRC or colorectal polyps. This was linked to the c.1285dupC exon 11 mutation, suggesting patients with this particular mutation are more at risk of developing CRC. Interestingly, the BHD patients identified by Khoo et al. (2002) who had colonic polyps also had an exon 11 mutation (c.1285delC), suggesting a possible genotype-phenotype correlation.

Additionally Guda et al., 2015 reported the presence of FLCN mutations in CRC samples from three African American patients. However it was not determined if these mutations were germline or sporadic. As FLCN is thought to be a general tumour suppressor it is possible that sporadic mutations in a range of tissues, in conjunction with other tumourgenic mutations, may result in tumour formation.

2.4.2 Thyroid nodules and cancer

In a five year clinical study of 22 patients from ten unrelated French families with BHD syndrome, Kluger et al. (2010) attempted to define the characteristics of pulmonary, thyroid, renal and colorectal manifestations associated with BHD syndrome more clearly. Notably, thyroid nodules and/or cysts were identified by ultrasound in 13 of 20 cases (65%). No thyroid carcinomas or colorectal carcinomas were detected in any patient. The high prevalence of thyroid nodules in this study is interesting, but crucially the lack of a control group does not enable the authors to assess the significance of these results. No genotype-phenotype correlation was observed in this study. Benhammou et al. (2011) also identified thyroid pathology in 4 out of 11 patients studied, 3 of whom had hypothyroidism and one with a benign thyroid nodule.

One case report describes a BHD patient with thyroid cancer (Benusiglio et al., 2014). Loss of heterozygosity of FLCN was observed in the tumour, but other genetic lesions or causes of thyroid cancer were not investigated, meaning a causal link between this patient’s BHD and their thyroid cancer cannot be conclusively proven. A second case study describes a BHD patient who had thyroid cancer, but as the thyroid tumour predated the patient’s BHD diagnosis, the authors did not attribute this tumour to the patient’s BHD syndrome or investigate a link between the two (Yamada et al., 2014).

Somatic mutation of FLCN, TSC2 and TP53 were found in a sporadic case of anaplastic thyroid cancer that was successfully treated with everolimus for 18 months, at which point the tumour developed a mutation in the mTOR gene and became resistant to treatment (Wagle et al., 2014). This suggests that somatic FLCN mutation – together with other mutations that activate mTOR signalling – can cause additional types of cancer to renal cell carcinoma, and make tumours sensitive to treatment with mTOR inhibitors.

2.4.3 Parotid tumours

At least eight cases of parotid tumours have been reported in patients with a FLCN mutation (Liu et al., 2000; Schmidt et al., 2005; Palmirotta et al., 2008; Maffé et al., 2011; Lindor et al., 2012; Pradella et al., 2013) and it is of note that the parotid tumours analysed by Lindor et al. (2012) and Pradella et al. (2013) were oncocytic, a characteristic commonly seen in BHD kidney tumours. However, there is currently not sufficient statistical evidence to conclusively associate parotid tumours with BHD syndrome.

2.4.4 Other manifestations

BHD syndrome has also been associated with melanoma in several reported cases (Toro et al., 1999; Khoo et al., 2002; Menko et al., 2009; Sempau et al., 2010; Cocciolone et al., 2010; Houweling et al., 2011; Mota-Burgos et al., 2013).
Additionally, adrenal carcinoma may also be a low risk manifestation of BHD, with up to five cases having been reported in the literature (Raymond et al., 2014).

There have been three reported cases of rhabdomyoma formation in patients with BHD; one patient developed the a cardiac rhabdomyomas (Bondavalli et al., 2015), a second reported a previous laryngeal rhabdomyoma (Toro et al., 2008) and the third was found to have a rhabdomyoma in the parathyroid gland (Mikesell et al., 2014).

Other reported benign and malignant tumours are listed by Menko et al. (2009) and Houweling et al. (2011), but, so far, a direct relationship between BHD syndrome and these tumours has not been shown.

A recent report from Kapoor et al., (2015) hypothesises a link between BHD and intracranial vascular pathologies (aneurysms and hematomas). Although there is no firm link the authors suggest an association with BHD based on aberrant HIF-1α and MMP9 activity.
3. Folliculin gene

3.1 Identification of the FLCN gene

Folliculin (GENBANK accession # BC015687) was mapped to the BHD locus by a genome wide linkage analysis using polymorphic microsatellite markers in a large Swedish family with BHD Syndrome. This study found evidence of linkage to 17p12-q11.2 (Khoo et al., 2001). Subsequent haplotype analysis defined a candidate interval between the two flanking markers, D17S1791 and D17S798 (Khoo et al., 2001).

Schmidt et al. (2001) also performed a genome wide scan in a large BHD kindred (172 members) and localised the gene to the pericentromeric region of 17p using linkage analysis. Two-point linkage analysis of eight additional families with BHD syndrome produced a maximum LOD score of 16.06 at D17S2196. Haplotype analysis identified critical recombinants and defined the minimal region of non-recombination as being within an interval of less than four cM between D17S1857 and D17S805 on chromosome 17p11.2.

The FLCN gene was ultimately identified when Nickerson et al. (2002) narrowed the critical region for the BHD locus to a 700-kb segment on 17p11.2. This genomic region contains a number of unstable low-copy number repeat elements which are subject to aberrant recombination events, causing deletions and duplications of the region (Stankiewicz and Lupski, 2002). Deletions within this region cause Smith-Magenis Syndrome (SMS) (Lucas et al., 2002), while duplications cause Charcot-Marie-Tooth Syndrome 1A (Roa et al., 1991). Interestingly, while the FLCN gene is often heterozygously deleted in SMS, patients do not seem to develop any of the symptoms of BHD (Truong et al., 2010). However, there has been one reported case of an SMS patient who suffered three pneumothoraces as a child (Truong et al., 2010).

3.2 FLCN: mechanism of pathogenesis

BHD is inherited in an autosomal dominant manner, but the mechanism through which loss of a single FLCN allele leads to pathogenesis is not fully elucidated.

3.2.1 Tumour suppression

Knudson’s two-hit hypothesis states that tumour formation is initiated by biallelic inactivation of a tumour suppressor gene, and that both inherited and sporadic cancers can arise as a result of mutations in the same gene (Knudson 1971). Inherited predisposition to cancer results from a heterozygous mutation in a tumour suppressor gene, e.g. FLCN, (the ‘first hit’), but alone is not sufficient for tumour development; inactivation of the wild-type allele by a somatic mutation (the ‘second hit’) is required.

Somatic mutations in the remaining wild-type copy of FLCN or loss of heterozygosity at chromosome 17p11.2 have been identified in BHD-associated renal tumours, supporting Knudson’s “two-hit” hypothesis and a tumour suppressor role for FLCN in the kidney (Vocke et al., 2005).

3.2.2 Haploinsufficiency

Haploinsufficiency describes the situation where a single allele of a gene cannot make a sufficient amount of protein to allow normal cell function or growth.

In a study of five BHD patients, van Steensel et al. (2007) found no evidence of somatic mutations or loss of heterozygosity in fibrofolliculomas, suggesting haploinsufficiency is sufficient to cause benign tumour growth in the skin. This was supported by Bønsdorff et al. (2008), who, in a study of the canine equivalent of BHD syndrome, found ‘second hit’ FLCN mutations in kidney tumours, but not in skin nodules.

Nahorski et al. (2011) found that eight out of ten BHD-associated truncated FLCN proteins were unstable, suggesting that the majority of mutant FLCN proteins are degraded. Furthermore,
Benhammou et al. (2011) found 4 families carrying a deletion of FLCN exon 1, which ablated the transcription of this FLCN allele. Both of these studies suggest that FLCN may be haploinsufficient in some contexts.

3.2.3 Dominant negative

Several studies have reported stable expression of truncated FLCN protein (Menko et al., 2013; Laviolette et al., 2013; Luijten et al., 2013). Menko et al. (2013) report the stable expression of FLCN protein in a renal tumour resected from a BHD patient, despite both FLCN alleles carrying a truncating mutation. This suggests that some mutations can produce a truncated FLCN protein, which may have altered or dominant negative function. Furthermore, Luijten et al. (2013) report that ciliogenesis was not rescued in UOK-257-2 cells, which expresses a reconstituted FLCN allele, when compared with the isogenic FLCN-null UOK-257 cell line. This suggests that the mutant form of FLCN present in UOK-257 cells may prevent the reintroduced FLCN protein from functioning in ciliogenesis.

3.2.4 Compound heterozygosity

One study has shown that compound heterozygosity of FLCN and PTEN is the likely cause of oncocytic tumour growth in a Cowden Syndrome patient and a BHD patient (Pradella et al., 2013). The authors found that a BHD-associated parotid tumour carries a somatic PTEN deletion in addition to a germline FLCN mutation, while the Cowden-associated thyroid tumour carries a somatic FLCN deletion in addition to a germline PTEN deletion. The tumours analysed in this study were both oncocytic and displayed characteristic mitochondrial hyperplasia. No mutations in mitochondrial genes, which can cause sporadic oncocytic tumours, were found and array CGH showed that there was no chromosomal instability in the BHD-associated parotid tumour. Although large duplications of chromosomes 5 and 7 were observed in the thyroid tumour, no extensive chromosomal instability was seen in this tumour. Thus, the authors conclude that compound heterozygosity of FLCN and PTEN causes oncocytic tumorigenesis specifically in the context of cancer predisposition syndromes.

3.3 FLCN mutations

FLCN consists of 14 exons (Nickerson et al., 2002) spanning approximately 20 kb of genomic DNA. Nickerson et al. (2002) were the first to identify mutations in the FLCN gene. They screened nine BHD families and found that eight had truncating mutations (seven frameshift, one nonsense) in FLCN, five of which were in exon 11. Screening of an additional 53 BHD families found that 22 had mutations in exon 11, suggesting it is a mutation hotspot (Nickerson et al., 2002).

Schmidt et al. (2005) screened a further 30 families and after combining the mutational data, found that 53% of the FLCN mutations involved either a cytosine insertion or deletion in the mononucleotide tract of eight cytosines (C8) in exon 11. Further evidence supporting the theory of a mutation hotspot was provided by Khoo et al. (2002) when two FLCN germline mutations in exon 11 (c.1733insC and c.1733delC) were identified in three of four BHD families, as well as one of four sporadic cases of BHD syndrome. Nickerson and colleagues suggested that the frameshift mutations might be caused by a slippage-mediated mechanism during DNA replication (Nickerson et al., 2002). The majority of variants were predicted to introduce a premature stop codon into FLCN and therefore to result in protein truncation (Schmidt et al., 2005). It is unclear whether the truncated FLCN is targeted for degradation, or remains in the cell with altered or dominant negative function.

A number of large deletions in folliculin have been identified in families whom had been clinically diagnosed with BHD but had not been shown to have a mutation using DNA sequencing techniques (Benhammou et al., 2011, Ding et al., 2015). Benhammou et al., 2011 proposed a second mutation hotspot in the non-coding exon 1, which was found to contain the putative FLCN promoter, in their cohort but Ding et al., 2015 found a greater number of truncating mutations in their cohort.

There are two publicly available sequence variation databases for FLCN, which consolidate all identified FLCN mutations. Both are hosted online by the Leiden Open (source) Variation Database.
(LOVD), where researchers can submit published or unpublished mutations. The Folliculin Sequence Variation Database is curated by Dr Derek Lim (University of Birmingham, UK; Lim et al., 2010) and currently contains 150 FLCN variants, as of February 2016. The second database is called www.skingenedatabase.com (Wei et al., 2009). Collating FLCN mutational data and combining this with clinical data is important as it allows the spectrum of mutations and genotype-phenotype correlations to be identified. This will help further the understanding of the causes of BHD syndrome and may lead to stratified management of BHD patients, should a genotype-phenotype correlation become evident.

The following tables detail FLCN mutations as described in the Folliculin Sequence Variation Database (up to February 2016). Sequence variation nomenclature in the tables below is as recommended by the Ad-Hoc Committee for Mutation Nomenclature (AHCMN) (den Dunnen JT and Antonarakis SE, 2000).

### 3.3.1 Base substitution mutations

Nucleotide substitutions that are likely to be pathogenic account for approximately 36% of reported FLCN pathogenic mutations.

<table>
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</tr>
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### 3.3.2 Deletion mutations

Deletion mutations account for 41% of reported pathogenic *FLCN* mutations.

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<td>Lim et al., 2010</td>
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<td>Woodward et al., 2008; Palmirotta et al., 2008; Kluger et al., 2010</td>
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</tr>
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<td>Furuya and Nakatani, 2013</td>
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<td>Kumasaka et al., 2014</td>
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</tr>
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<td>Frameshift</td>
<td>van Steensel et al., 2007</td>
<td></td>
</tr>
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<td>c.1408_1418del</td>
<td>Frameshift</td>
<td>Schmidt et al., 2005; Toro et al., 2008</td>
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### 3.3.3 Duplication mutations

Duplication mutations account for 17% of all pathogenic FLCN mutations and most result in a frameshift.

<table>
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<th>Reference</th>
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<td>4</td>
<td>c.199dupG</td>
<td>Frameshift</td>
<td>Iribe et al., 2015</td>
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<td>c.340dupC</td>
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<tr>
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<td>c.347dupA</td>
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<td>Toro et al., 2008; Palmirotta et al., 2008</td>
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<td>Frameshift</td>
<td>Benusiglio et al., 2014</td>
</tr>
<tr>
<td>7</td>
<td>c.655dupG</td>
<td>Frameshift</td>
<td>Letter et al., 2008</td>
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<td>c.689dupT</td>
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<td>Kumagase et al., 2014</td>
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<td>9</td>
<td>c.997_998dup</td>
<td>Frameshift</td>
<td>Kunogi et al., 2010</td>
</tr>
<tr>
<td>10-11</td>
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<td>Frameshift</td>
<td>Benhammo et al., 2011</td>
</tr>
<tr>
<td>11</td>
<td>c.1285dupC</td>
<td>Frameshift</td>
<td>Nickerson et al., 2002; Khoo et al., 2002; Kawasaki et al., 2005; Lamberti et al., 2005; Murakami et al., 2007; van Steensel et al., 2007; Letter et al., 2008; Toro et al., 2008; Ren et al., 2008</td>
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<td>Frameshift</td>
<td>Toro et al., 2008</td>
</tr>
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<td>Woodward et al., 2008</td>
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<td>Gunji et al., 2007; Koga et al., 2009</td>
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<td>c.1347_1353dup</td>
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<td>c.1372dupC</td>
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<td>Kluger et al., 2010</td>
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<td>c.1426dupG</td>
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<td>Schmidt et al., 2005</td>
</tr>
<tr>
<td>13</td>
<td>c.1487_1490dup</td>
<td>Frameshift</td>
<td>Schmidt et al., 2005</td>
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</tbody>
</table>
3.3.4 Indel mutations

Six indel (insertion/deletion) mutations have been reported to be associated with BHD, accounting for 5% of pathogenic FLCN mutations.

<table>
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<th>DNA Change</th>
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<th>Reference</th>
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<td>c.319_320delinsCAC</td>
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<tr>
<td>6</td>
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<td>c.573delGinsT</td>
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<td>c.610_611delinsTA</td>
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<td>c.632_633delinsC</td>
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<td>Nickerson et al., 2002; Schmidt et al., 2005</td>
</tr>
<tr>
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<td>c.871+3_c.871+4delinsTCCAGAT</td>
<td>Splice site</td>
<td>Unpublished</td>
</tr>
<tr>
<td>12</td>
<td>c.1323delinsGA</td>
<td>Frameshift</td>
<td>Leter et al., 2008; Kluijt et al., 2009</td>
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</tbody>
</table>

3.3.5 Insertion mutations

One insertion mutation of unknown pathogenicity has been reported in exon 14 and results in a frameshift.

<table>
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<th>DNA Change</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
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<td>14</td>
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<td>Furuya et al., 2012</td>
</tr>
</tbody>
</table>

3.3.6 Base substitutions unlikely to be pathogenic

Four base substitutions have been reported in individuals with BHD, but are unlikely to be pathogenic.

<table>
<thead>
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<th>DNA Change</th>
<th>Remarks</th>
<th>Reference</th>
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<tr>
<td>3</td>
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<td>Intronic</td>
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<tr>
<td>7</td>
<td>c.726A&gt;T</td>
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<td>9</td>
<td>c.1062+5G&gt;A*</td>
<td>SNP</td>
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<tr>
<td>11</td>
<td>c.1198G&gt;A**</td>
<td>Missense</td>
<td>Lim et al., 2010; Nahorski et al., 2011</td>
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</tbody>
</table>

Legend: * - individuals carry another mutation that is pathogenic, + - variant does not segregate with disease

3.3.7 FLCN variants not associated with BHD

34 variants have been reported in FLCN, but are not associated with BHD so are unlikely to be pathogenic.

<table>
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<th>Reference</th>
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</tr>
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<td>1</td>
<td>c.-299C&gt;T</td>
<td>5'UTR</td>
<td>Cho et al., 2008</td>
</tr>
<tr>
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<td>c.-228+1368G&gt;T</td>
<td>Intron</td>
<td>Cho et al., 2008</td>
</tr>
<tr>
<td>1</td>
<td>c.229+994A&gt;G</td>
<td>Intron</td>
<td>Cho et al., 2008</td>
</tr>
<tr>
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<td>c.-90A&gt;G</td>
<td>5'UTR</td>
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<td>Location</td>
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<td>Intron</td>
<td>Cho et al., 2008</td>
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<td>Cho et al., 2008</td>
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<td>Cho et al., 2008</td>
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<td>Cho et al., 2008</td>
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<td>Cho et al., 2008</td>
</tr>
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<td>c.1176+39G&gt;A</td>
<td>Intron</td>
<td>Cho et al., 2008</td>
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<tr>
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<td>c.1176+68G&gt;C</td>
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<td>Cho et al., 2008</td>
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<tr>
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<td>Cho et al., 2008</td>
</tr>
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<td>c.1176+179A&gt;G</td>
<td>Intron</td>
<td>Cho et al., 2008</td>
</tr>
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<td>c.1177-165C&gt;T</td>
<td>Intron</td>
<td>Cho et al., 2008</td>
</tr>
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<td>c.1269C&gt;T</td>
<td>SNP</td>
<td>Cho et al., 2008</td>
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<td>c.1433-38A&gt;G</td>
<td>Intron</td>
<td>Cho et al., 2008</td>
</tr>
<tr>
<td>13</td>
<td>c.1538+121C&gt;T</td>
<td>Intron</td>
<td>Cho et al., 2008</td>
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4. Folliculin and its interacting partners

4.1 Folliculin protein

*FLCN* is predicted to encode the 579 amino acid protein *FLCN* (64kDa), consisting of a short hydrophobic N-terminal sequence, one N-glycosylation site, three myristoylation sites and a glutamic acid-rich coiled coil domain centrally located in the protein (Nickerson et al., 2002). Moreover, *FLCN* is observed to be phosphorylated (Baba et al., 2006; Dephoure et al., 2008; Gauci et al., 2009; Piao et al., 2009; Wang et al., 2010) and ubiquitinated (Danielsen et al., 2011; Wagner et al., 2011). Further details regarding these modifications can be found in Section 4.1.2.

One isoform, which is 342 amino acids in length (Uniprot ID: Q8NFG-2) and consists of the N-terminal 290 amino acids of *FLCN* plus an additional 52 amino acids not found in the *FLCN* protein, has been observed in four cDNA libraries (Refseq ID: NM_144606.5; Ensembl ID: ENST00000389169). How widely expressed this isoform is, or how it functions, is currently unknown. An additional isoform of 197 amino acids has been predicted (Uniprot ID: Q8NFG4-3) which consists of the N-terminal 133 amino acids of *FLCN* plus an additional 64 amino acids which are not found in the *FLCN* protein. However, there is no experimental evidence to confirm the existence of this isoform in vivo.

The protein sequence of the canonical *FLCN* isoform is as follows:

```
MNAIVALCHFCELHGPRTLFCETVLHAPLPGDGNDSPGQGGEQAEEGGGQMNSRMRAHSFAEGASVESSSP
GPKKSDMCEGCRSLAAGHPGYISHDKETSIKYVSHQHPQFLSIVRQACVRSLSCECVPCRGEPPIFFGDQHEGFV
FSHTFFIKDSLRGQVRWSQITMMDRIYLIINSWPFLGLKVRGIIDELQGKALKVFAEEAQFEGPCQRAQRMHATPF
LHQRNGAARSLTLSDNWLACLHTSFAWLLKACGSLTEKLEAGETDTELQMEKLANDLEEEESWDNSEA
EEEEKAPVLPSETEGLRTGPQAESSGLCSSWQPRKLPVFKSLRHMQRQVGLAPSFRMLAWHVMGONQVIWKS
RDVDLVQSAFEVLRMLPVGCRVIIYSSQYEEAYRCNFLGSLPHVQIPHPHLSEFAVIVEVHAARSLHPVGCED
DQSLSKYEFVVTSGSPVAADRGPTLKNIEALTNQNLSVDVDQCQLCLKEEMNKVVLFKFTKVDSPKEDT
QKLILLSILGASEEDNVKLKFWMTGLSKYKSHLMSTVRSPTASESRN
```

List of amino acids, their abbreviations and details.

Nahorski et al. (2011) performed *in silico* evolutionary analysis on the *FLCN* gene and found that *FLCN* was under strong purifying selection, meaning that the sequence evolved more slowly at the protein level than the average gene, in particular between codons 100-230, suggesting an important function for this N-terminal region.

No transmembrane domains or organelle localisation signals have been determined within the *FLCN* sequence (Warren et al., 2004). The sequence has no significant homology to any known protein, but it is highly conserved across species including *Canis lupus familiaris*, *Bos Taurus*, *Mus musculus*, *Rattus norvegicus*, *Gallus*, *Xenopus tropicalis*, and *Drosophila melanogaster*, suggesting an important biological role (Nickerson et al., 2002). Sequence alignment shows this conservation.
Alignment made using MUSCLE (Edgar, 2004), manually edited using Jalview (Waterhouse et al., 2009) and displayed with Clustal colours. Alignments performed by Angela Pacitto.
4.1.1 FLCN structure

In 2012, Nookala et al. (2012) described the structure of the C-terminal domain of FLCN, determined by X-ray crystallography (PDB ID: 3V42). Residues 341-579 of FLCN were found to form a β-sheet with helices packed on one side, followed by an all helical region. This fold is remarkably similar to that of DENN-domain proteins, which function as Rab guanine nucleotide exchange factors (GEFs). As such, Nookala et al. (2012) proposed that FLCN may function as a Rab GEF, and thus play a role in membrane trafficking.

Residues 104 – 266 of FLCN are predicted to form a domain similar to that in found yeast which is required for Golgi to plasma membrane transport (PFAM ID: PF11704). Interestingly the majority of this region falls within the region that Nahorski et al. (2011) found to be highly evolutionarily conserved and is in a region of homology with the S. pombe homologue of FLCN, BHD1 (see alignment in Section 6.2) (van Sleutenhorst et al., 2007).

Nookala et al. (2012) also analysed the N-terminal domain of FLCN using secondary structure prediction programmes. The domain is predicted to form a longin domain, which is present in other DENN-domain containing proteins and found in proteins involved in membrane trafficking. Work is currently underway to determine the structure of this region of FLCN. The N-terminal 85 amino acids are predicted to form a metal-ion-binding module. The N- and C-terminal domains of FLCN are connected by a 40 amino acid disordered linker region. This region contains a bipartite tryptophan fingerprint (WD-WQ) motif, which has been shown to be a binding motif for kinesin light chain 1, which is an intracellular trafficking protein (Dodding et al., 2011).

4.1.2 Post-translational modifications of FLCN

FLCN phosphorylation was first identified by Baba et al. (2006), when multiple FLCN bands were seen on Western blots. Additionally, FLCN was identified in a screen of phosphorylated peptides (Gauci et al., 2009). Further research has shown that serine 62 (ser62) is a phosphorylation site in FLCN and that it is indirectly up-regulated by AMPK (Wang et al., 2010). FLCN also appears to be phosphorylated at ser302 by unknown kinases downstream of mTORC1 (Piao et al., 2009). Since mTORC1 is known to be indirectly down-regulated by AMPK, this process could be associated with a feedback mechanism that regulates mTOR signalling. Indeed, mTORC1 was subsequently found to phosphorylate ser62 and ser73 of FLCN (Yu et al., 2011).

Phosphorylation of these residues appears to be cell-cycle dependent: ser62 and ser73 become phosphorylated as the cell cycle progresses, with maximum phosphorylation seen during the mitotic phase (Dephoure et al., 2008; Laviolette et al., 2013). FLCN with phosphorylated ser62 and ser73 residues shows reduced stability, suggesting this modification is important for cell cycle regulation (Laviolette et al., 2013). Additionally, S302 is highly phosphorylated during G1 phase (Dephoure et al., 2008).

ULK1 inhibits FLCN’s interaction with GABARAP by phosphorylating three novel phosphorylation sites in the C-terminus of FLCN at S406, S537 and S542 (Dunlop et al., 2014). Mapping these residues onto the crystal structure of FLCN’s C-terminal DENN domain shows that they are all on the solvent-exposed surface of the protein, and are thus accessible for phosphorylation (Dunlop et al., 2014). ULK1 was still able to dissociate the interaction of GABARAP and a triple serine-to-alanine FLCN mutant in vivo, suggesting that other phosphorylation sites in FLCN, GABARAP or the FNIP proteins also control this interaction (Dunlop et al., 2014). Two further ULK1 phosphorylation sites were identified at FLCN S316 and T317, but these are poorly conserved between species (Dunlop et al., 2014).

FLCN has also been found to be ubiquitinated on lysine 206 and 559 (Wagner et al., 2011). Danielsen et al. (2011) also noted FLCN ubiquitinylation, but did not delineate specific residues. The relevance of these post-translational FLCN modifications is yet to be determined, however.
4.2. Follculin-binding proteins

4.2.1 FNIP1

FLCN-interacting protein 1 (FNIP1), was identified in 2006 (Baba et al., 2006) as an evolutionarily conserved protein that interacts with and phosphorylates FLCN. FNIP1 also binds AMPK, which is a negative regulator of mTOR and a key protein for energy sensing in cells (Inoki et al., 2003; Gwinn et al., 2008). Baba et al. (2006) demonstrated that both FLCN and FNIP1 are phosphorylated by AMPK. This interaction between FNIP1 and FLCN was also shown to be modified by additional factors, since treatment with an AMPK inhibitor (compound C), rapamycin or amino acid starvation affected the phosphorylation status of FLCN, further indicating a role for FLCN in energy sensing and the mTOR pathway. FNIP1 has also been shown to be phosphorylated by mTORC1 (Yu et al., 2011) and is ubiquitinatated at lysine 161 (Wagner et al., 2011).

FNIP1 and FNIP2 are required for FLCN’s localisation to lysosomes during amino acid starvation, where FLCN interacts with the Rag proteins in order to activate mTORC1 signalling once amino acid levels are restored (Petit et al., 2013; Tsun et al., 2013). FLCN’s interaction with and activation of the Rag proteins is also facilitated by FNIP1 and FNIP2 (Petit et al., 2013; Tsun et al., 2013).

Zhang et al. (2012) identified a divergent DENN domain in FNIP1 and FNIP2, similar to that of FLCN. Using X-ray crystallography Pacitto et al. (2015) confirmed that the yeast orthologue Lst4 does contain a structural DENN-family protein. Lst4 forms a 1:1 heterodimer with the yeast orthologue of FLCN, Lst7, and like the mammalian proteins this heterodimer localises to the vacuolar membrane to act on TOR signalling (Péli-Gulli et al. 2015).

Behrends et al. (2010) have suggested that FNIP1 is also involved in autophagy, the process by which cellular components are degraded. FNIP1 was found to interact with GABARAP, a member of the ATG8-family of proteins which are required for autophagosomal development. Moreover, knockdown of FNIP1 led to an increase in autophagosome production. However, this perceived increase could reflect an accumulation of autophagosomes due to a block in a later step of the pathway.

Two independent studies published in 2012 suggest that FNIP1 is required for B cell development in mice (Park et al. 2012; Baba et al. 2012). Using flow cytometry, Park et al. (2012) observed an increase in p-S6R in FNIP1-null pre-B cells, suggesting an increase in mTOR-mediated metabolism. In addition, AMPK activation failed to inhibit this phosphorylation in FNIP1-null B cells, which indicates that FNIP1 may be important for AMPK to inhibit mTOR (Park et al. 2012). In contrast Baba et al. (2012) found that their FNIP1-null mice did not show a change in the levels of mTOR and p-S6R in pro-B cells, but did exhibit a marked increase in pre-B cell apoptosis (Baba et al. 2012).

Park et al. (2012) suggest FNIP1 loss leads to energy stress, causing the B cell arrest, whereas Baba et al. (2012) suggest FNIP1 loss leads to increased apoptosis. The differences observed between these studies may be due to the different methods used to generate the FNIP1-null mice (described in 6.3.2). However, Baba et al. (2012) also showed that FLCN knockout mice have the same B cell phenotype as FNIP1 knockout mice, indicating the FLCN gene may also function in B cell development.

Park et al. (2014) show that invariant Natural Killer T (iNKT) cells failed to develop normally in FNIP1-null mice. Although stage 0,1 and 2 cells were found, very few mature stage 3 iNKT cells were found in these mice, suggesting the block happened at some point between stage 2 and stage 3. Mitochondriar mass was reduced, ATP levels were low, cell size was increased and mTOR signalling was dysregulated in FNIP1-null iNKT cells. BrdU pulse experiments showed that FNIP1-null cells over-proliferated in stage 3 of iNKT cell development, making cells vulnerable to apoptosis, as shown by an increase in Caspase 3-positive cells. Together, this suggests that dysregulated mTOR signalling leads to higher energy consumption, meaning that cells do not have the required energy reserves for proliferation and maturation to stage 3, and die somewhere between stage 2 and 3. However, mTOR...
dysregulation is not fully responsible for this phenotype, as *in vivo* treatment of pups, beginning in utero, did not rescue the iNKT cell phenotype.

*FNIP1* has been shown to be alternatively spliced during the later stages of mesenchyme differentiation (Venables et al., 2013), further suggesting that FNIP1 is important for B cell differentiation and development. In primary fibroblasts, a shorter isoform of FNIP1 (Uniprot: Q8TF40) – lacking the final 84 bps of exon 6, corresponding to amino acids 208-235 – predominates. Reprogramming of these fibroblasts to induced pluripotent stem cells (iPSCs) caused the longer, canonical, isoform of FNIP1 to predominate. This splicing pattern was fully reversed upon *in vitro* differentiation of iPSCs into fibroblasts, where the shorter isoform was again observed.

Reyes et al. (2014) reported that Fnip1 also has a role in specification of mammalian skeletal muscle fibres. Loss of Fnip1 resulted in increased numbers of Type 1 muscle fibres, increased AMPK activation and increased expression of the AMPK-target and coactivator PGC1α. Further work is required to fully understand the role of Fnip1 in muscle development and specification.

Sequence analysis across species shows that two thirds of the alternatively spliced exons found in this study (including *FNIP1* exon 6) arose during the emergence of jawed vertebrates, indicating that alternative splicing of these genes may play an important role in the evolution and physiology of jawed vertebrates (Venables et al., 2013).

### 4.2.2 FNIP2

*FNIP2*, a second FLCN-interacting protein, first designated KIAA1450 by Nagase et al. (2000), was found by sequencing clones obtained from a size-fractionated human brain cDNA library. Hasumi et al. (2008) subsequently identified it as FNIP2, with Takagi et al. (2008) identifying it as FNIP1-Like soon after. Additionally, Komori et al. (2009) cloned mouse FNIP2 (which they called MAPO1), and found that it is required for apoptosis triggered by O6-methylguanine mispairing in DNA. This was confirmed by further studies which found that both FNIP2 and *FLCN* were necessary for the induction of apoptosis by N-Nitroso-N-methylurea (Lim et al., 2012; Sano et al., 2013).

*FNIP1* and FNIP2 are required for *FLCN*’s localisation to lysosomes during amino acid starvation, where FLCN interacts with the *Rag proteins* in order to activate *mTORC1* signalling once amino acid levels are restored (Petit et al., 2013; Tsun et al., 2013). FLCN’s interaction with and activation of the *Rag* proteins is also facilitated by FNIP1 and FNIP2 (Petit et al., 2013; Tsun et al., 2013).

FNIP2 may also be a membrane trafficking protein, as it carries a divergent DENN domain similar to that of FLCN (Zhang et al., 2012).

FNIP2 be required for myelination in the central nervous system in mammals, as Weimaraner dogs homozygous for a FNIP2 mutation showed dysmyelination of the spinal cord and developed a tremor phenotype at 12-14 postpartum (Pemberton et al., 2013). FNIP2 expression in oligodendrocytes is controlled by *Sox10*, a known regulator of oligodendrocyte terminal differentiation (Pemberton et al., 2013).

The accepted nomenclature for this protein is FNIP2, and it is homologous to FNIP1 (49% identity, 74% similarity). As with FNIP1, it is conserved across species and binds AMPK (Hasumi et al., 2008; Takagi et al., 2008). *In vitro* kinase assays also suggest that FNIP2 is phosphorylated by AMPK (Takagi et al., 2008). FNIP1 and FNIP2 are able to form homo- and heterodimers, as well as multimers (Takagi et al., 2008), suggesting a functional association between these two proteins.

Binding of FLCN to both FNIP1 and FNIP2 is mediated specifically through the C-terminal region of FLCN (Baba et al., 2006; Hasumi et al., 2008; Takagi et al., 2008). In BHD syndrome, the majority of mutations are predicted to introduce a premature stop codon into *FLCN*, and therefore result in a protein truncation (Schmidt et al., 2005). However, it is unclear whether truncated FLCN is targeted for nonsense-mediated decay, or remains in the cell with altered function. Whatever the outcome,
truncating mutations that result in the loss of the C-terminus of FLCN will abolish its ability to interact with FNIP1 and FNIP2, which suggests that this interaction is functionally important.

An interchangeable role for FNIP1 and FNIP2 alongside FLCN in tumour suppression has been suggested by Hasumi et al., (2015). FNIP1 and FNIP2 were found to be partially functionally redundant with either able to interact with FLCN and inhibit tumourigenic growth in mouse kidneys. A complete loss of both FNIP1 and FNIP2 in the kidneys lead to the development of tumours and aberrant mTOR signalling.

4.2.3 Plakophilin-4

Plakophilin-4 (PKP4, also known as p0071) has been identified as a FLCN-interacting protein by yeast-2-hybrid analysis and co-immunoprecipitation studies (Nahorski et al., 2012; Medvetz et al., 2012). PKP4 has been associated with cytokinesis, intercellular junction formation and RhoA signalling, which is discussed in more detail in Sections 5.7 and 5.8. Through the yeast-2-hybrid screen, it is thought that the head domain of PKP4 binds FLCN. The region of FLCN which is involved in the interaction with PKP4 is currently unknown.

4.2.4 Rpt4

Regulatory particle triple-A ATPase 4 (Rpt4) was found to interact with FLCN in both Drosophila and human cells (Gaur et al., 2013). Rpt4 is a component of the 26S proteasome and is required for rRNA synthesis (Ottosen et al., 2002; Fätyol and Grummt, 2008), which is discussed in more detail in Section 5.5. FLCN interacts with Rpt4, preventing its association with rDNA and thus inhibiting rRNA synthesis (Gaur et al., 2013).

4.2.5 Rag proteins

Folliculin interacts with the Rag proteins at the cytosolic surface of lysosomes, and this interaction is dependent on FNIP1 and FNIP2 (Martina et al., 2014, Petit et al., 2013; Tsun et al., 2013). Petit et al. showed FLCN specifically interacts with the GTPase domain of RagA in HeLa cells, suggesting it may function as a guanine nucleotide exchange factor for this protein. However, Tsun et al. showed that the FLCN-FNIP2 complex acts as a GTPase-activating protein towards RagC and RagD in HEK293T cells, catalysing the hydrolysis of RagC bound GTP to GDP. The interaction between FLCN and the Rag proteins was found to activate mTORC1 signalling in response to amino acid stimulation, as shown by increased S6K1 phosphorylation (Martina et al., 2014, Petit et al., 2013; Tsun et al., 2013). Activation of mTORC1 by FLCN led to the phosphorylation of Ser211 of the transcription factor TFE2 and Ser311 of TFE3, which regulates the expression of lysosomal genes and autophagy genes (Martina et al., 2014, Petit et al., 2013).

4.2.6 ULK1 and GABARAP

FLCN enhances basal autophagic flux through its interactions with ULK1 and GABARAP (Dunlop et al., 2014). This interaction is mediated by FNIP1 and FNIP2 and inhibited by ULK1, which inhibits FLCN’s interaction with GABARAP by phosphorylating three novel phosphorylation sites at S406, S537 and S542. BHD patient FLCN mutations that truncate the C-terminal end of the protein show reduced binding to GABARAP, suggesting that reduced autophagy is likely to contribute to renal tumorigenesis.

4.2.7 TDP-43

Xia et al., (2015) has identified a role for FLCN in the cytosolic translocation and aggregation of the RNA/DNA binding protein TDP-43. Although TDP-43 is usually shuttled between the nucleus and the cytosol, enhanced translocation out of the nucleus and cytosol aggregation is associated with neuronal loss in ALS and FTLD. Xia et al. report that overexpression of FLCN, which directly interacts with TDP-43, results in enhanced TDP-43 translocation into the cytosol and the formation of stress granules. In contrast a depletion of FLCN increased nuclear deposition and dissociates TDP-43 from other stress granule proteins in the cytosol.
TDP-43 has also found to differentially splice FNIP1 (De Conti et al., 2015) with TDP-43 depletion resulting in the exclusion of FNIP1 exon 7. Further research is required to determine the impact of these interactions and any variations in isoform production on either ALS, FTLD or BHD pathology.

4.3 Expression pattern and cellular localisation of FLCN

4.3.1 mRNA

Warren et al. (2004) studied the expression of FLCN mRNA in both normal and neoplastic human tissue and found that in normal cells FLCN was expressed in the skin, the distal nephron of the kidney, stromal cells, type 1 pneumocytes of the lung, acinar cells of the pancreas, parotid gland, epithelial ducts of the breast and prostate, areas of the brain and in macrophages and lymphocytes in the tonsils and spleen. Tissues with no FLCN mRNA expression included the heart, muscle and liver (Warren et al., 2004). The fact that FLCN mRNA is not detected in BHD-associated renal tumours provides further evidence that FLCN has tumour suppressor function in the kidney.

The expression patterns of FLCN, FNIP1 and FNIP2 in human tissues were determined by Hasumi et al. (2008) using real-time PCR. The expression patterns of these proteins were generally similar, and consistently high in specific tissues, such as muscle, nasal mucosa, salivary gland and uvula, suggesting that FLCN, FNIP1 and FNIP2 may work together in these organs. However, FNIP2 expression was higher relative to FNIP1 in fat, liver and pancreas, which suggests that FNIP2 may have a specific function in these metabolic tissues (Hasumi et al., 2008).

Hudon et al. (2010) characterised the tissue distribution of murine FLCN expression. It was found to have a similar expression pattern to human FLCN, with high expression in the kidneys, lungs and spleen. Interestingly, no FLCN expression was seen in the mouse epidermis, correlating with the lack of skin lesions in FLCN-null mice.

4.3.2 Protein

FLCN/FNIP1 and FLCN/FNIP2 dimers have been shown to co-localise in the cytoplasm in a reticular pattern (Baba et al., 2006; Hasumi et al., 2008; Takagi et al., 2008). Co-expression studies of N-terminal tagged FLCN, FNIP1 and FNIP2 indicate that both FNIPs regulate the cytoplasmic distribution of FLCN (Baba et al., 2006; Hasumi et al., 2008; Takagi et al., 2008). When tagged FNIP2 constructs are expressed alone, FNIP2 is seen to be distributed within the cytoplasm of cells, showing more condensed features around the nucleus. Conversely, when tagged FLCN constructs are expressed alone, they appear to be found mainly in the nucleus (Takagi et al., 2008). However, when FNIP2 and FLCN are co-expressed they co-localise together in the cytoplasm in a reticular pattern, which is similar to the co-localisation of FNIP1 and FLCN (Baba et al., 2006; Hasumi et al., 2008; Takagi et al., 2008).

Three studies have shown that although expressed diffusely under basal conditions, FLCN is rapidly recruited to the cytosolic surface of lysosomes during amino acid depletion in a FNIP1- (Petit et al., 2013) or FNIP2- (Tsun et al., 2013) dependent manner. Upon restimulation with amino acids, FLCN rapidly dissociates from the lysosome (Martina et al., 2014, Petit et al., 2013; Tsun et al., 2013).

Nahorski et al. (2012) also studied the co-localisation of FLCN and PKP4 throughout different phases of the cell cycle. During interphase, FLCN and PKP4 were shown to co-localise most strongly at cell junctions, with a more dispersed co-localisation throughout the cytoplasm, whereas during cytokinesis the proteins co-localised at the midbody. Additionally, Gaur et al. (2013) showed that FLCN co-localises with RPT4 in the nucleolus.

Taken together, these studies suggest that FLCN may be widely expressed, and subsequently recruited to sub-cellular locations by its interacting partners.
5. Folliculin-associated signalling pathways

5.1 mTOR signalling

FLCN and AMPK interaction, mediated by FNIP1 and FNIP2, has been shown to regulate mTOR signalling (Baba et al., 2006; Hasumi et al., 2008). The mTOR pathway is a key regulator of cell growth, proliferation and metabolism (Harris and Lawrence, 2003; Hay and Sonenberg, 2004; Wullschleger et al., 2006) and an increasing amount of evidence suggests that its deregulation is associated with human diseases, including cancer (Sarbassov et al., 2005; Landau et al., 2009; Montero et al., 2014).

Early studies in fission yeast, Schizosaccharomyces pombe (S. pombe), show that FLCN homologue, BHD1 (Lst7) activates Tor2 (van Slegtenhorst et al., 2007). However, subsequent studies in mammalian models show that FLCN inhibits mTOR signalling (discussed below). This discrepancy may be explained by the fact that BHD1 only corresponds to the N-terminal region of vertebrate FLCN (see Section 6.2 for alignment).

Furthermore, deletion of the Drosophila homologue of FLCN, DBHD, led to a reduction of Tor signalling in Drosophila larvae, suggesting that DBHD activates Tor signalling, but the resultant starvation phenotype was partially rescued by human FLCN, indicating that there is some functional overlap between the two genes, with respect to mTOR signalling (Liu et al., 2013).

The functional role of FLCN in mTOR signalling in mammals is unclear since several publications have reported contradictory effects of FLCN loss on phosphorylated ribosomal protein S6 (p-S6R), an indicator of mTOR activation. Three studies reported that transient downregulation of FLCN by siRNA in human cell lines results in reduction of phosphorylation of p-S6R (Takagi et al., 2008; Hartman et al., 2009; Bastola et al., 2013). Reduction of p-S6R was also observed in renal cysts developing in mice heterozygous for FLCN (Hartman et al., 2009).

In contrast, kidney-specific homozygous knockout of FLCN resulted in an increase in phosphorylated p-S6R, which contributed to the development of polycystic kidneys (Baba et al., 2008; Chen et al., 2008). Additionally, activation of mTOR signalling in the kidney tumours of mice heterozygous for FLCN was noted by Hasumi et al. (2009).

Furthermore, in 2014 the same group found that biallelic FLCN inactivation in murine heart muscle causes cardiac hypertrophy, cardiac dysfunction and significantly reduces lifespan compared to wild type littermates. FLCN deletion led to overexpression of PGC1A, leading to increased mitochondrial mass and high intracellular ATP levels (Hasumi et al., 2014). This led to a reduction in AMPKα phosphorylation at T172, and subsequent mTORC1 dysregulation. In support of this model, Rapamycin rescued the heart disease phenotype of these mice.

This discrepancy between mouse models could be due to differences in sample preparation and/or the gene targeting strategies used between studies. However, Hudon et al. (2010) noted that a loss of FLCN expression in their heterozygous FLCN knockout mouse resulted in both elevated and reduced levels of p-S6R, depending on the cellular context - which may account for the differing results observed in the earlier mouse models. Furthermore, FLCN loss seems to have highly cell-specific outcomes – for example, knockdown of FLCN increases mTOR signalling in SAEC cells but has no effect on mTOR signalling in HBE cells (Khabibullin et al., 2014).

More recently, two Japanese studies have also reported a strong expression of p-mTOR and p-S6R in cyst-lining epithelial cells from BHD patient lungs, suggesting that heterozygous loss of FLCN leads to a dysregulation of mTOR signalling which, in turn, causes lung cyst formation (Furuya et al., 2012; Nishii et al., 2013). Additionally, there is currently no treatment for BHD syndrome, a Japanese patient with metastatic kidney cancer responded better to the mTOR inhibitor Everolimus, than to Tyrosine Kinase inhibitor treatment, suggesting that mTOR inhibition may prove an effective treatment for BHD-associated kidney tumours (Nakamura et al., 2013).
However, three recent studies have shown that FLCN is recruited to the cytosolic lysosome surface during amino acid depletion, where it interacts with the Rag proteins (Martina et al., 2014; Petit et al., 2013; Tsun et al., 2013). FLCN’s interaction with RagA/B was FNIP1-dependent (Petit et al., 2013), whilst FLCN’s interaction with RagC/D was FNIP2-dependent (Tsun et al., 2013), suggesting that FLCN’s interacting proteins modify its function. FLCN was found to specifically interact with the GTPase domain of RagA, suggesting it may function as a GEF towards RagA/B (Petit et al., 2013), whilst FLCN-FNIP2 complex was found to act as a GAP towards RagC/D (Tsun et al., 2013). In all three studies, FLCN was found to activate mTORC1 signalling in response to amino acid stimulation, as shown by increased S6K1, TFEB and TFE3 phosphorylation (Martina et al., 2014; Petit et al., 2013). In support of a role for FLCN in activating mTOR signalling in some conditions, a clinical trial showed that Rapamycin was not an effective treatment for fibrofolliculomas (Gijezen et al., 2014).

Loss of FNIP1 leads to an increase in mTOR signalling and a block in B cell and iNKT-cell development in FNIP1-null mice, suggesting that FNIP1 can inhibit mTOR signalling (Park et al., 2012).

### 5.2 AMPK signalling

FLCN interacts with AMPK via FNIP1 and/or FNIP2, and regulates mTOR signalling (Baba et al., 2006; Hasumi et al., 2008; Takagi et al., 2008). Phosphorylation at S62 of FLCN increases the FLCN-FNIP complex’s affinity for AMPK, while phosphorylation at S302 decreases FLCN’s affinity for AMPK (Piao et al., 2009; Wang et al., 2010). AMPK is an important energy sensing protein, which inhibits anabolic growth via mTOR signalling and stimulates autophagy to promote cell survival when energy supply is low (Alers et al., 2012).

Loss of FLCN in mouse embryonic fibroblasts (MEFs), leads to the constitutive activation of AMPK is constitutively activated (Yan et al., 2014). This ultimately activates HIF signalling and leads to metabolic changes consistent with the Warburg Effect within FLCN-null cells. A nonphosphorylatable FLCN S62A mutant was unable to bind and inhibit AMPK, meaning that FLCN-FNIP binding to AMPK is required for its inhibition.

Possik et al., (2014) also found that deletion of flcn-1 in nematode worms leads to constitutive activation of AMPK, which causes a subsequent increase in autophagic flux and intracellular ATP levels. This leads to increased stress resistance and protection from apoptosis in C. elegans nematodes, mouse embryonic fibroblasts, and the FLCN-null FTC-133 thyroid carcinoma cell line (Possik et al., 2014). The flcn-1 null nematodes are also more resistant to hyperosmotic stress due to increased glycogen storage, an additional result of the increased AMPK signalling (Possik et al., 2013).

Taken together, these studies suggest that FLCN inhibits AMPK signalling. However, two recent studies have shown that FLCN may also activate AMPK signalling in certain conditions.

Using a mouse model where FLCN had been deleted in alveolar epithelial type II cells (AECs) using a Cre-lox system, Goncharova et al., (2014) found that loss of FLCN in AECs led to increased apoptosis and cell permeability due to loss of E-cadherin expression, subsequent LKB1 dysregulation and consequent AMPK downregulation. The AMPK activator, AICAR, and constitutively active AMPK were able to increase cell survival in FLCN-null cells, and AICAR improved alveolar surface tension in vivo. These mice showed pulmonary developmental defects and impaired lung function consistent with a role for FLCN in branching morphogenesis.

Mice lacking FLCN in their hearts developed cardiac hypertrophy and died at 3 months. AMPKα phosphorylation at T172, was shown to be reduced in the heart cells of these mice. AMPK inactivation was caused by increased ATP levels following PGC1α overexpression and increased mitochondrial biogenesis (Hasumi et al., 2014).

FLCN loss has since been shown to have highly cell-specific outcomes and knockdown of FLCN led to reduced AMPK signalling in HBE cells, but had no effect on in SAEC cells (Khabibullin et al., 2014).
FNIP1 has been found to be required for AMPK to inhibit mTOR signalling in murine B cells by (Park et al., 2012). FNIP1 deletion in iNKT cells lead to increased mTOR signalling despite mitochondrial mass and intracellular ATP levels being reduced, suggesting that AMPK’s ability to inhibit mTOR was faulty in these cells (Park et al., 2014). FNIP1 deletion in muscle tissue also results in increased AMPK and PGC1a activity associated with a shift in muscle fibre specification towards the mitochondria-dense type I fibres (Reyes et al., 2014).

FLCN and FNIP2 activate AMPK to trigger apoptosis in response to N-Nitroso-N-methyleurea treatment and FLCN and AMPK act in opposition to regulate FNIP2 protein stability (Lim et al., 2012, Sano et al., 2013).

In addition to FLCN affecting AMPK signalling, AMPK has also been shown to phosphorylate FLCN, FNIP1 and FNIP2 (Baba et al., 2006; Hasumi et al., 2008, Takagi et al., 2008, Wang et al., 2010), indicating that FLCN and AMPK signalling might form a feedback loop.

### 5.3 HIF signalling and mitochondrial biogenesis

Using the RCC cell line, UOK257, Preston et al. (2011) demonstrated that FLCN inhibits hypoxia-inducible factor (HIF) signalling. Additionally, HIF-1a expression is increased in cystic lung tissue resected from a BHD patient, also suggesting that under normal conditions, FLCN inhibits HIF signalling (Nishii et al., 2013). The HIF signalling pathway regulates a number of different genes that are involved in angiogenesis, erythropoiesis, cell survival and metastasis (Semenza, 2012). Interestingly, increased vascularisation of sub-pleural cysts expressing increased amounts of HIF-1a has been observed (Nishii et al., 2013).

Preston et al. (2011) observed that increased HIF-signalling in FLCN-null cells lead to the increased activity of glycolytic enzymes, thus causing these cells to favour glycolytic rather than lipid metabolism, as seen in the Warburg effect (Warburg, 1956). The resulting increased lactate production could also be activating a second HIF-independent hypoxia pathway mediated by NDRG3 (Lee et al., 2015).

The Warburg effect states that cancerous cells produce energy by glycolysis and lactic fermentation in the cytosol rather than by pyruvate oxidation in mitochondria (Warburg, 1956). Thus, Warburg also postulated that cancer could be interpreted as a mitochondrial disease. Indeed, Klomp et al. (2010) found that the loss of FLCN in BHD-associated renal tumours resulted in mitochondrial dysfunction, as indicated by an increased expression of the mitochondrial genes PGC1a and TFAM in these tumours. Additionally, it has been shown that loss of FLCN Hasumi et al. (2012) or FNIP1 (Reyes et al., 2014) in murine muscle tissues results in an increase in mitochondrial gene expression and a subsequent metabolic shift towards mitochondrial oxidative phosphorylation, and this is regulated by PGC1A.

A study by Yan et al. (2014) drew many of these observations together. They found that AMPK is constitutively activated in FLCN-null mouse embryonic fibroblasts, leading to increased PGC1A activity and a consequent increase in mitochondrial biogenesis and ROS production. This ultimately activates HIF signalling and leads to metabolic changes consistent with the Warburg Effect within FLCN-null cells. Re-addition of FLCN into this cell line reduced the number of colonies formed in a soft agar assay, indicating that the metabolic transformation as a result of FLCN-loss provides these cells with a tumorigenic advantage.

Biallelic FLCN inactivation in murine heart muscle causes cardiac hypertrophy, cardiac dysfunction and significantly reduces lifespan compared to wild type littermates (Hasumi et al., 2014). This effect was mediated by PGC1a, as double FLCN/PGC1a cardiac knock out animals did not show this phenotype (Hasumi et al., 2014). FLCN deletion led to overexpression of PGC1a, leading to increased mitochondrial mass and high intracellular ATP levels, which ultimately led to AMPK inactivation and mTORC1 hyperactivity (Hasumi et al., 2014).
Conversely, biallelic inactivation of FNIP1 leads to reduced mitochondrial mass and lower basal ATP levels in INKT cells (Park et al., 2014).

Interestingly, mitochondrial hyperplasia is a common characteristic of oncocyctic tumours, and is commonly observed in tumours resected from BHD patients (Lindor et al., 2012; Pradella et al., 2013; Raymond et al., 2014).

5.4 Stress resistance and autophagy

Loss of the C.elegans FLCN homologue, flcn-1, led to a 21% increase in lifespan (Gharbi et al., 2013). The increased longevity observed in these FLCN-null animals was due to increased resistance to stress, and was dependent of HIF signalling, but not insulin signalling (Gharbi et al., 2013). Induction of autophagy also increases longevity in C. elegans (Schiavi et al., 2013).

Possik et al. (2014) used the same C. elegans model as Gharbi et al., to investigate the role of flcn-1 in metabolic stress, and also found that loss of flcn-1 led to increased stress resistance, although the authors did not observe increased lifespan. They found that flcn-1 deletion led to constitutive activation of the C. elegans homologue of AMPK, aak2, which led to higher autophagic flux, higher levels of intracellular ATP, thus increasing stress resistance by inhibiting apoptosis (Possik et al., 2014). These findings were replicated in mouse embryonic fibroblasts, suggesting that this pathway is evolutionarily conserved in mammals (Possik et al., 2014).

Increased autophagy was observed in DBHD-null Drosophila (Liu et al., 2013) and FLCN inhibits the activity of TFEB and TFE3 (Martina et al., 2014, Petit et al., 2013), which are transcription factors for autophagy genes, suggesting that FLCN may inhibit autophagy. Additionally, FNIP1 has been found to interact with the autophagy protein GABARAP (Behrends et al., 2010), further suggesting a role for FLCN in this process.

FLCN also inhibits autophagy induction by inhibiting the accumulation of LC3B, and promoting the accumulation of LC3C (Bastola et al., 2013). These experiments were conducted in 786-O cells, which are a model for VHL syndrome, and showed that FLCN is partially responsible for the tumour suppressor action of VHL in ccRCC. Additionally, two studies have found that when treated with the chemotherapy Paclitaxel or radiotherapy, autophagy was induced via increased MEK-ERK signalling in FLCN-null but not FLCN-expressing cells, as shown by increased numbers of autophagosomes, increased levels of LC3B and decreased levels of p62 (Zhang et al., 2013, Zhang et al., 2014).

Taken together, these results suggest that FLCN usually inhibits autophagy. However, autophagic flux was found to be reduced in the heart tissues of mice with FLCN deleted in their cardiac cells, as shown by reduced AMPKa phosphorylation at T172, and reduced ULK1 phosphorylation at S555 (Hasumi et al., 2014). Furthermore, FLCN enhances basal autophagic flux through its interaction with the autophagy proteins GABARAP and ULK1 (Dunlop et al., 2014). Dunlop et al. (2014) also show that reduced autophagy is likely to contribute to renal tumorigenesis in BHD, as autophagy is reduced in patient tumours – as measured by increased SQSTM1 and GABARAP expression – and BHD patient FLCN mutations that truncate the C-terminal end of the protein show reduced binding to GABARAP.

Thus, FLCN’s role in autophagy is not straightforward.

5.5 Ras-Raf-MEK-Erk signalling and rRNA synthesis

Research in mice has shown that the Ras-Raf-MEK-ERK pathway, which regulates cell proliferation and is dysregulated in many cancers (Roberts and Der, 2007), is activated in FLCN-null kidneys (Baba et al., 2008; Hudon et al., 2010). This suggests that an upstream effector of this pathway may be activated by loss of FLCN, resulting in cell growth and proliferation within the FLCN-null kidney cell. Gaur et al. (2013) found that heterozygous loss of Drosophila DBHD enhanced the lethal phenotype of flies carrying a hyper-active allele of Ras1, indicating that FLCN also inhibits the Ras-Raf-MEK-Erk signalling pathway in Drosophila.
Gaur et al. (2013) also found that FLCN interacts with Rpt4 in the nucleolus to inhibit rRNA synthesis in both Drosophila and human cells and that increased rRNA synthesis is required for Ras-Raf-MEK-Erk stimulated oncogenic growth in Drosophila. Therefore, the hyperplastic growth seen in BHD syndrome may be due, in part, to an increase in Ras-Raf-MEK-Erk signalling caused by the overexpression of rRNA.

Zhang et al. (2013) and (2014), found that when treated with the chemotherapy Paclitaxel or radiotherapy, autophagy was induced via increased MEK-ERK signalling in FLCN-null but not FLCN-expressing cells.

Heterozygous loss of FLCN was also reported to lead to dysregulated MEK-ERK signalling in a high grade oncocytic carcinoma. However, there were additional genomic rearrangements seen in this tumour, suggesting that additional factors may have contributed to this signalling dysregulation (Sirintrapun et al., 2014).

5.6 JAK/STAT and TGF-β signalling

Singh et al. (2006) used RNA interference to reduce the expression of the Drosophila FLCN homologue (DBHD), and showed that DBHD was required for male germline stem cell (GSC) maintenance in the fly testis. Subsequent investigation suggested that DBHD regulates GSC maintenance downstream of, or in parallel to, the JAK/STAT and Decapentaplegic (Dpp) signal-transduction pathways.

The JAK-STAT signalling cascade regulates stem cell renewal and differentiation (Harrison, 2012). Singh and Hou (2009) hypothesised that over-expression of JAK-STAT signalling results in the enlargement of the Malphigian tubules, the Drosophila equivalent of the kidneys, coupled with an increased number of proliferating cells, mitotically active cells and putative renal stem cells. Thus, aberrant JAK-STAT signalling could contribute to the renal cystic/carcinoma phenotype observed in BHD syndrome.

Dpp is the Drosophila homologue of the vertebrate bone morphogenetic proteins (BMPs), which are members of the TGF-β superfamily – a signalling system that is crucial for regulating a variety of cellular functions such as stem cell renewal (Ying et al., 2003). Three independent studies have shown that FLCN regulates TGF-β signalling: firstly, Hong et al., (2010a) used microarray analysis to show that FLCN regulates several components of the TGF-β pathway in UOK257 cells that were xenografted into nude mice. Secondly, Cash et al. (2011) observed a general loss of TGF-β mediated transcription (including pro-apoptotic mediator BIM) and chromatin modifications in FLCN-null murine ES cells. FLCN-null ES cells also failed to form normal day 12 embryoid bodies, indicative of a loss of TGF-β signalling. And finally FLCN knockdown has been shown to increase TGF-β expression in HBE cells, and decrease TGF-β signalling in SAEC cells (Khabibullin et al., 2014).

5.7 RhoA signalling

Nahorski et al. (2012) and Medvetz et al. (2012) identified an interaction between FLCN and Plakophilin-4 and reported opposing effects of FLCN loss on RhoA signalling in vitro. Nahorski et al. (2012) observed that cells deficient in FLCN have increased RhoA expression and activity. These FLCN deficient cells also displayed faster cell migration, which was reversed when RhoA signalling was blocked using a ROCK inhibitor. However, Medvetz et al. (2012) report that RhoA signalling is inhibited in FLCN-null cells, and that these cells display a slow cell migration phenotype compared to wild-type cells. Additionally, the authors used a conditional mouse FLCN allele to delete FLCN in the epidermis. These mice showed a hyper-proliferative epidermal phenotype, similar to that of other Rho signalling gene knock out mice.

5.8 Wnt and Cadherin signalling

Reiman et al. (2012) used microarray analysis to screen for targets of FLCN and found a preponderance of differentially expressed Wnt and Cadherin signalling genes in FLCN-null FTC-133 cells. The Cadherin pathway regulates cell-cell junction formation and has been recently linked to BHD as FLCN loss
reduces the amount of E-cadherin at adherens junctions (Nahorski et al., 2012) and causes increased desmosome formation (Medvetz et al., 2012). Furthermore, E-cadherin expression was found to be mis-localised in BHD-associated kidney tumours (Kuroda et al., 2014) and FLCN-null murine alveolar epithelial type II cells (Goncharova et al., 2014) and cell-cell adhesion is increased in HBE cells (Khabibullin et al., 2014) suggesting that E-cadherin dysregulation might contribute to BHD pathogenesis in both kidney and lung tissues.

Loss of FLCN in mouse lung epithelial cells has also been shown to lead to the dysregulation of cell-cell contacts and formation of gaps between cells (Krymskaya et al., 2010). This was attributed to the mis-localisation of β-catenin (Krymskaya et al., 2010), a component of the Wnt signalling pathway, which transduces messages from the cell surface to the nucleus and has roles in embryonic development and tumorigenesis (Klaus and Birchmeier, 2008). More recently, FLCN has been shown to regulate planar cell polarity by sequestering B-catenin in cilia (Luijten et al., 2013).

Both pathways have been previously implicated in cancer, including renal cancer (Jeanes et al., 2008; Hsu et al., 2012) and these results suggest they may play a role in BHD-associated tumorigenesis.

### 5.9 Cell Cycle

Consistent with its putative tumour suppressor role, FLCN has been shown to directly regulate cell cycle progression.

FLCN has been shown to inhibit cyclin D1 expression through an unknown mechanism (Kawai et al., 2013). Cyclin D1 is required to promote G1 to S transition, suggesting that FLCN functions to halt this transition.

FLCN phosphorylation changes throughout the cell cycle, with S302 phosphorylation highest during G1 phase, and ser62 and ser73 phosphorylation highest during mitosis (Dephoure et al., 2008; Laviolette et al., 2013). Laviolette et al. (2013) showed that FLCN acts to slow down cell cycle progression and becomes phosphorylated at ser62 and ser73 as the cell cycle progresses. The authors also show that ser62 and ser73 phosphorylation reduces FLCN’s stability, suggesting that FLCN may be degraded during mitosis in order to allow cells to divide (Laviolette et al., 2013).

### 5.10 Apoptosis

Cash et al. (2011) reported that FLCN-null ES cells were resistant to cell-intrinsic apoptosis due to disruption of TGF-β signalling and consequently a reduction in the expression of Bim, a pro-apoptotic Bcl-2 protein. Microarray analysis has demonstrated that FLCN upregulates the expression of a number of apoptosis genes (Reiman et al., 2012): CASP1, which induces apoptosis; and HtrA2 and SMAC/Diablo which are both part of the mitochondrial apoptotic pathway (Verhagen et al., 2002; Martínez-Ruiz et al., 2008). Interestingly, decreased SMAC/Diablo expression has been linked to poor prognosis in RCC (Mizutani et al., 2005). Furthermore, loss of flcn-1 in C. elegans causes increased stress resistance due to apoptosis being repressed (Possik et al., 2014).

Together, these findings demonstrate a tumour suppressor role for FLCN, whereby it activates apoptosis. Conversely, Baba et al. (2012) used a FNIP1 constitutive mouse knock out and a conditional FLCN mouse knock out to show that both FNIP1 and FLCN interact with the Bcl2 family in order to inhibit apoptosis in B cells. Furthermore, FNIP1 protects iNKT cells from apoptosis during proliferation and maturation (Park et al., 2014).

FNIP2 was identified in a gene trap screen to identify clones that were resistant to MNU-induced apoptosis (Komori et al., 2009). Further studies from the same group showed that AMPK phosphorylation is required for MNU-apoptosis, and occurs in a FLCN and FNIP2 dependent manner (Lim et al., 2012) and that FLCN and AMPK act in opposition to regulate FNIP2 protein stability (Sano et al., 2013). Taken together, it is possible to deduce the following model: under normal conditions, the FNIP2 protein is maintained in equilibrium at a low level by FLCN, which stabilises FNIP2, and AMPK


which phosphorylates FNIP2, causing it to be degraded by the proteasome and thus preventing apoptosis (Sano et al., 2013). Upon treatment with MNU, AMPK is phosphorylated by both FLCN and FNIP2 (Lim et al., 2012), AMPK dissociates from the FLCN-FNIP2-AMPK complex, thus stabilising FNIP2 and allowing apoptosis to proceed (Sano et al., 2013).

5.11 Membrane trafficking

The S. cerevisiae homologue of FLCN, LST7, is part of the cerasiae complex required for Golgi to plasma membrane transport (Roberg et al., 1997), which provided early evidence that FLCN functions in membrane trafficking (Nahorski et al., 2011).

The subsequent discovery that the C-terminal portion of FLCN forms a non-canonical DENN domain suggested that FLCN may be a Rab-GEF and have a role in membrane trafficking (Nookala et al., 2012). Subsequent experiments confirmed that FLCN has GEF activity towards the Rab35 GTPase in vitro. Rab35 is involved in early endocytic trafficking, recycling events and cytokinesis, and these results suggest that FLCN may also function in these pathways. FLCN has also been shown to down-regulate the expression of Rab27b (Hong et al., 2010a; Klomp et al., 2010; Reiman et al., 2012), which is also a membrane trafficking protein (Gomi et al., 2007).

FLCN has been shown to sequester β-catenin in cilia (Luijt et al., 2013) and to preclude TFE3 from the nucleus of stem cells (Betschinger et al., 2013), further hinting that FLCN’s role may be to shuttle other proteins within the cell. FLCN has also been reported to contain a WE/WD binding motif for the Kinesin 1 motor protein (Dodding et al., 2011) and to regulate the accumulation of LC3B and LC3C, which are required for autophagosome double membrane formation and cargo uptake (Bastola et al., 2013).

Of further interest, a study by Zhang et al. (2012) used FLCN’s divergent DENN domain as a template to search for other non-canonical DENN domain proteins. Six such proteins were found – C9orf72, SMCR8, NPR2, NPR3 and FNIP1 and FNIP2, suggesting that FNIP1 and FNIP2 may also be membrane trafficking proteins (Zhang et al., 2012).

5.12 Stem cell maintenance and pluripotency

RNAi knock down of the Drosophila FLCN homologue (DBHD), show that DBHD is required for germline stem cell (GSC) maintenance in the fly testis (Singh et al. 2006).

Hong et al., (2010b) found that FLCN inactivation increases the activity of TFE3 – a transcription factor that has previously been suggested to be an oncogene in renal cell carcinoma (Sidhar et al., 1996) – in BHD renal tumours compared to matched kidney tissue.

TFE3 maintains stem cell pluripotency by activating the transcription of a range of pro-pluripotency genes. Betschinger et al. (2013) showed that FLCN, together with FNIP1 and FNIP2, acts to exclude TFE3 from the nucleus, thus allowing stem cells to exit pluripotency and become poised to differentiate. FLCN’s effect on pluripotency seemed to be either downstream of independent of TSC2 and mTOR signalling (Betschinger et al., 2013).

Venables et al., (2013) showed that the FLCN interacting protein, FNIP1, is alternatively spliced during late mesoderm differentiation of fibroblasts. In primary and derived fibroblast cultures, a shorter isoform of FNIP1 lacking amino acids 208-235 predominates, whilst in iPSCs the longer canonical form predominates. Alternative splicing of FNIP1 is controlled by MBNL1, which is required for muscle and heart development (Kalasota et al., 2008; Lin et al., 2006).

5.13 Ciliogenesis

Luijt et al. (2013) show that FLCN localises to primary cilia in HK2 cells and motile cilia in primary HNE cells. Interestingly, both knock down and overexpression of FLCN in HK-2 cells led to fewer cells with functional cilia at 72 hours. However, after 96 hours, the numbers of cilia tended towards
wildtype in both the FLCN-depleted and overexpressed experiments, suggesting that FLCN may control the onset of ciliogenesis but is less important thereafter.

These data, when considered with the observation that cystic lesions are a hallmark of ciliopathies, suggest that BHD may also be a ciliopathy.

5.14 Matrix Metalloproteinase signalling

Conflicting results have suggested that the metalloproteinases, particularly matrix metalloproteinase 9 (MMP9), which are known to cause lung cyst formation in a range of cystic lung diseases may also promote lung cyst formation in BHD syndrome.

Hayashi et al. (2010) showed immunohistochemical staining of MMP9 in alveolar epithelial cells, macrophages and neutrophils lining cysts in a confirmed case of BHD, but crucially did not perform this experiment in matched healthy lung tissue or in control lung tissue making the significance of this finding unclear. More recently, Pimenta et al. (2012) also found that that macrophages and neutrophils lining cysts walls were stained positively for MMP9 in a suspected case of BHD. Additionally, their patient – who was initially diagnosed with LAM – was treated with the metalloproteinase inhibitor doxycycline with some success, as measured by improved pulmonary function (Pimenta et al., 2012). However, genetic testing was not performed in this patient, meaning that BHD cannot be conclusively diagnosed, so these results must be treated with caution.

Conversely, Niishi et al. (2013) also analysed MMP9 expression in lung cysts of a BHD patient and did not find any increase in expression compared to control lung tissue. Thus the role of MMP9 is BHD lung cyst formation is still uncertain and needs to be investigated in a larger data set of confirmed BHD cases, and including appropriate controls.

Aberrant MMP activity has also been linked to several vascular pathologies (Maradni et al., 2013) and has been suggested to provide a link between BHD and a case series of patients with cranial vascular pathologies (Kapoor et al., 2015).

Mice lacking FLCN in their alveolar epithelial type II cells displayed an increased inflammatory response in vivo, as measured by increased MMP3 and MMP9 expression (Goncharova et al., 2014).

Heterozygous loss of FLCN was also reported to lead to increased MMP9 expression in a high grade oncocytic carcinoma. However, there were additional genomic rearrangements seen in this tumour, suggesting that additional factors may have contributed to this signalling dysregulation (Sirintrapun et al., 2014).
6. Cell lines and model organisms for studying BHD syndrome

Model systems are crucial for fundamental studies, providing insight into the mechanisms of pathogenesis underlying BHD syndrome in vivo. Current model systems can provide an excellent base for drug development.

Contact information regarding these model organisms can be found in Lab Essentials: BHD Animal Models.

The following sections outline the published cell lines and model organisms currently used in BHD syndrome research.

6.1 Cell lines

6.1.1 UOK-257 cell line (mouse)

The FLCN-null UOK257 cell line was isolated from a clear-cell renal tumour of a BHD patient showing somatic loss of 17p (Baba et al., 2006). A FLCN-expressing isogenic cell line, UOK257-2, was established by reintroduction of the FLCN gene using a lentivirus (Baba et al., 2006).

6.1.2 ES cell lines (mouse)

Cash et al., (2011) produced a homozygous Flcn-null mouse embryonic stem (ES) cell line. A variety of other Flcn-specific ES cell lines including gene traps (RRX115, GC0351) and conditional knockouts (IMPC) are available commercially.

6.1.3 Flcn-null rat lines

Seven Flcn-null cells lines were derived from a RCC in a 10-month old male Nihon Rat (see below, Matsumoto et al., 2009).

6.2 Yeast models

van Slegtenhorst et al. (2007) identified the S. pombe FLCN homologue, BHD1, and used homologous recombination to generate a novel deletion strain. BHD1 only has homology with the N-terminal region of the vertebrate protein (see alignment), but these experiments suggest that yeast FLCN is important for amino acid homeostasis, and potentially activates the mTOR homologue Tor2.

The S. cerevisiae orthologues of FLCN and FNIP1/2 are Lst7 and Lst4 respectively. Péli-Gulli et al., (2015) found that Lst7 and Lst4 form a complex that mediates amino acid stimulation of TORC1 signalling.
Legend: Alignment of *S. pombe* and human folliculin. *S. pombe* folliculin only aligns to the N-terminal region of human folliculin.

Alignment made using MUSCLE (Edgar, 2004), manually edited using Jalview (Waterhouse et al., 2009) and displayed with Clustal colours. Alignments performed by Angela Pacitto.

### 6.3 C. elegans model

A strain of *C. elegans* with a deletion in *F22D3.2*, the *C. elegans* homologue of *FLCN*, is available from the *C. elegans* Gene Knockout Consortium. This mutant strain (*flcn-1*) has been characterised by Gharbi et al. (2013), who showed that the deletion leads to a deletion of exon 5 and exon 6 in the mutant cDNA. *Flcn-1* worms show increased longevity compared with wildtype worms, living for 25 days rather than 21, and a mild developmental delay. *Flcn-1* worms also showed increased thermoresistance at 35°C, with 50% of *flcn-1* worms still alive after 10 hours at 35°C, while most wildtype worms had died at this stage.

Other studies with this model found that although *flcn-1* null worms were more resistant to oxidative and temperature stresses (Possik et al., 2014), and hyperosmotic stress (Possik et al., 2015), which seemed to be mediated by the *C. elegans* homologue of AMPK, *aak-2*. However, they did not see increased lifespan in these animals. The reason for this discrepancy is currently unclear.

### 6.4 Drosophila model

Liu et al., (2013) used a homologous recombination strategy to replace exon 1 of the *Drosophila* *FLCN* homologue, *DBHD*, with a white gene marker, resulting in a null DBHD allele. The mutant phenotype was only evident in flies homozygous for the DBHD exon 1 deletion. DBHD-null flies did not develop into adulthood, but remained in a small, early larval form for an extended period of up to three weeks, as opposed to three days in wildtype and heterozygous flies. This starvation phenotype was partially rescued by human FLCN, suggesting that the two proteins have some overlapping function. Dietary leucine also partially rescued the phenotype, allowing flies to reach the larval stage, although they still died during metamorphosis, suggesting that reduced mTOR signalling was partially responsible for this phenotype.
6.5 Mouse models

Mouse models of both FLCN and FNIP1 have been generated and are described below:

6.5.1 FLCN mouse models

Baba et al., (2008) used gene targeting to generate a conditional Flcn, with loxP sites flanking exon 7. Deletion of exon 7 using Cre-recombinase produces a frameshift and generates a premature stop codon in exon 8. Homozygous deletion of Flcn in kidneys using a kidney-specific Cre-driver allele caused these mice to develop polycystic kidneys and to subsequently die of renal failure at 3 weeks post-partum.

Chen et al. (2008) also used gene targeting to generate a conditional Flcn allele, with loxP sites flanking exons 3 – 4 of the gene. This deletion removes the start codon, and is thus predicted to completely prevent the expression of Flcn. The authors demonstrated that constitutive homozygous deletion of Flcn resulted in embryonic lethality at E3.5-E8.5. The authors also found that homozygous deletion of Flcn specifically in kidneys caused mice to develop enlarged, polycystic kidneys and to subsequently die of renal failure at 3 weeks post-partum. Renal cyst cells cultured in vitro were able to form tumours when implanted into nude mice (Wu et al., 2015).

Hartman et al., (2009) generated a Flcn-null allele using gene trap technology: a β-galactosidase/neomycin (β-geo) cassette was integrated between exons 8 and 9 of the FLCN gene, resulting in a truncated form of the Flcn protein. No homozygous Flcn mutant mice were identified and heterozygous Flcn mutant mice developed renal cysts and neoplasia at 3-6 months, similar to those found in BHD patients.

Hasumi et al., (2009) crossed mice carrying their previously developed Flcn<sup>Flox</sup> allele (Baba et al., 2008) with those carrying a β-actin-Cre construct to produce mice with ubiquitous Flcn knockout. Homozygous deletion of Flcn caused embryonic lethality before E9.5. Heterozygous Flcn knockout mice developed renal cysts and tumours of varied histology from around 12 months. The tumours showed vastly reduced Flcn expression, but increased activation of the PI3K-AKT-mTOR pathway.

Hudon et al., (2010) also generated a Flcn-null allele with a β-geo cassette insertion between exons 8 and 9 of Flcn, and showed that homozygous deletion of Flcn caused embryonic lethality before E8.5. Heterozygous Flcn knockout mice developed renal cysts and tumours, which were found to not express Flcn, suggesting that the wild-type allele had been inactivated in these tumours.

Chen et al., (2015) crossed mice carrying their previous developed Flcn<sup>Flox</sup> allele (Chen et al., 2008) with those carrying a Sglt2-Cre construct to produce mice with homozygous knockout of Flcn only in the kidney proximal tubules. These mice develop renal cysts and hyperplasia in the first 6 months, and renal tumours from around 6 months. The authors reported the development of multiple and often mixed histologies. Heterozygous mice rarely develop cysts or tumours indicating a low tumourigenicity.

6.5.2 FNIP1 mouse models

Fnip1-null mice have been generated by N-ethyl-N-nitrosourea (ENU) mutagenesis, which caused a 32bp deletion in exon 9 of Fnip1. These mice are viable and fertile, but display a block in B cell development (Park et al., 2012) and altered muscle fibre subtype specification (Reyes et al., 2014).

Two targeted Fnip1-knockout mouse models were also developed by Baba et al. (2012). One carries loxP-sites flanking exon 6, and the other carries a β-geo cassette insertion in intron 2. Since there were no phenotypic differences between the two models, they were used interchangeably during this study (Baba et al., 2012). These Fnip1 knockouts were both viable and fertile, however, they too displayed a block in B cell development.
6.6 Rat model

The “Nihon” model of renal cell carcinoma (RCC) was found in a Sprague-Dawley strain of rats (Okimoto et al., 2004). These rats contain a single nucleotide insertion within exon 3 of *Flcn* (c.462_463insC), producing a frameshift and premature stop codon within the gene. The homozygous mutant is lethal at an early stage of embryonic development. However, in heterozygotes, renal carcinomas develop from pre-neoplastic lesions as early as three weeks post-partum, then into adenomas by eight weeks post-partum, with all rats presenting with symptoms by six months. The renal carcinomas that develop in heterozygotes are largely composed of clear cells. Loss of heterozygosity at the *Flcn* locus was observed in ten of eleven primary renal carcinomas examined, supporting the Knudson 2-hit hypothesis (Okimoto et al., 2004).

Further characterisation of the Nihon rat was conducted by Kouchi et al. (2006), where they described the extra-renal lesions seen in the endometrium, salivary glands and cardiac tissue of this model. Rescue experiments demonstrated that re-introduction of the *Flcn* gene could rescue the embryonic lethality of the homozygous mutants, as well as suppress the renal carcinogenesis seen in heterozygous rats (Togashi et al., 2006).

6.7 Dog models

6.7.1 FLCN

Originally described in 1985, hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis (RCND) is a naturally occurring canine kidney cancer syndrome in German shepherd dogs (Lium and Moe, 1985). As the name suggests, RCND is characterised by bilateral, multifocal tumours in the kidney and firm nodules within the skin, thus showing similarity to human BHD syndrome.

The RCND locus was located to a small region on canine chromosome 5 that overlapped with *Flcn* (Linggaas et al., 2003). The authors described a histidine to arginine mutation (H255R) in exon 7 of canine *Flcn* that segregated with the disease phenotype and appears to be homozygous lethal (Linggaas et al., 2003).

Researchers also observed a loss of heterozygosity within the renal cysts and tumours of juvenile and adult dogs respectively, indicating a tumour suppressor function for *Flcn* according to the Knudson two-hit hypothesis (Bønsdorff et al., 2008; Bønsdorff et al., 2009).

6.7.2 FNIP2

Pemberton et al. show that the Weimaraner breed of dog carries a *Fnip2* truncating mutation, present at a carrier frequency of 4.285%. Animals carrying biallelic *Fnip2* mutations show defective myelination of the spinal cord, which increased over time, although never reached wild type levels. Clinically, these animals develop a tremor phenotype around 12-14 days postpartum, which abates at 3-4 months of age.

The Chow Chow breed of dog is prone to a similar recessive tremor phenotype caused by hypomyelination of the spinal cord. Although direct sequencing was not performed in this breed, cross breeding experiments show that that Chow mutation is likely to be allelic with the Weimaraner mutation, suggesting that a *Fnip2* mutation also causes this phenotype in Chows (Pemberton et al., 2013).
7. Future Work

7.1 Clinical research

To date, around 600 families have been reported to have BHD (Section 1.1). However, BHD syndrome is believed to be under-diagnosed; partly because it is so rare that doctors are often unfamiliar with it, and partly because phenotypic variation can make it difficult to identify (Menko et al., 2009). For example, Kunogi et al. (2010) sequenced the FLCN gene in 36 patients who presented with multiple lung cysts of unknown cause, and found that 25 carried FLCN mutations. Of these 25 individuals, 21 only displayed pulmonary symptoms at the time of the study (Kunogi et al., 2010). Thus, although these individuals do have BHD, the lack of skin lesions or renal disease makes it difficult to clinically diagnose these patients. Additionally, a recent report described the case of an individual with all three manifestations of BHD, but who had a de novo FLCN mutation, suggesting that a diagnosis of BHD should be considered for patients with one or more of the syndrome’s symptoms, even in the absence of a family history (Menko et al., 2013).

Collating epidemiological data from different studies into a central database would help to ascertain the true prevalence of BHD syndrome. This research would help to identify any genotype-phenotype correlation, population differences, or genetic modifiers that may not be obvious in individual cohorts. More knowledge about the epidemiology of BHD syndrome would help with the genetic counselling and management of patients, and may also lead to the development of stratified treatments for patients with BHD syndrome.

7.2 Basic research

Although recent research, such as the elucidation of the C-terminal structure of FLCN indicating that it is a DENN domain protein with GEF activity, has provided important insights into FLCN’s function, more research is required to comprehensively catalogue the normal cellular functions of FLCN. Understanding its normal function is crucial to understanding how FLCN mutation or loss can lead to the epidermal, pulmonary and renal phenotypes associated with BHD syndrome.

The majority (95%) of FLCN mutations that are associated with BHD truncate the protein by introducing a frameshift (45%), deleting a splice site (19%), introducing a nonsense mutation (18%), deleting or duplicating a large portion of the gene (10%) or by removing the start codon (3%) (Sections 3.3.1-3.3.5). However, it is unknown whether the resultant aberrant protein is degraded, remains in the cell with altered function or exerts a dominant negative effect. Indeed, it has recently been shown that FLCN is expressed in a BHD renal tumour with a different truncating FLCN mutation in each copy of the gene, suggesting that at least one of these mutations does not lead to the complete inactivation of the protein (Menko et al., 2013). The significance of this finding is currently unknown.

There are six missense mutations that are currently reported to be associated with BHD syndrome (Section 3.3.1). Although it is likely that these amino acid substitutions are pathogenic, as they are associated with disease, it is unknown how these mutations affect FLCN function in order to cause the disease. Determining how these missense mutations affect the FLCN protein may shed light on which residues or domains of the protein are particularly important for its correct function and structure.

The identification that the C-terminus binds both FNIP1 and FNIP2 (Baba et al., 2006; Hasumi et al., 2008; Takagi et al., 2008), and has Rab GEF activity (Nookala et al., 2012), demonstrates that this region is important for FLCN function, meaning that truncating mutations that delete the C-terminus are likely to be detrimental. Although initial studies suggest that FLCN acts as a GEF for Rab35 in vitro, it remains to be seen whether FLCN has GEF activity towards other GTPases, although as FLCN does bind the GTPase domain of RagA, making it likely that it acts as a GEF for this and other proteins in vivo (Petit et al., 2013). The first 85 amino acids of the protein contain a metal-ion binding motif (Nookala et al., 2012) although FLCN has thus far not been observed to bind any metals. Thus, the function of the N-terminal region is currently unknown.
Localisation experiments have observed FLCN in both the cell nucleus and the cytoplasm (Takagi et al., 2008). Further experiments by Nahorski et al. (2012) show that FLCN co-localises with binding partner PKP4 at cell junctions during interphase, and at the mid-body during cytokinesis, while Gaur et al. (2013) show that FLCN co-localises in the nucleolus with Rpt4. How FLCN is recruited to the correct location has not yet been elucidated.

Two of the proteins known to bind FLCN - FNIP1 and FNIP2 - are themselves poorly characterised. PKP4, Rpt4 and the Rag proteins have recently been shown to bind FLCN and it is likely that many additional proteins bind to FLCN. The function of the FLCN-FNIP1, -FNIP2, -PKP4 and -Rpt4 complexes are also an area of future work. Characterising FLCN’s interactions with other proteins, and the functions of FLCN-containing protein complexes, will provide insight into how FLCN mutation or loss affects cellular function. These interacting proteins may modulate the phenotype of BHD syndrome and thus affect its variability.

FLCN has been implicated in numerous signalling pathways and cellular processes, including:

- mTOR signalling (Baba et al., 2006; Baba et al., 2008; Hasumi et al., 2008, Petit et al., 2013; Tsun et al., 2013);
- AMPK signalling (Baba et al., 2006; Hasumi et al., 2008; Takagi et al., 2008; Yan et al., 2014);
- HIF signalling and mitochondrial biogenesis (Klopp et al. (2010); Preston et al., 2011; Hasumi et al., 2012; Nishii et al., 2013);
- stress resistance and autophagy (Behrends et al., 2010; Gharbi et al., 2013; Bastola et al., 2013);
- Ras-Raf-MEK-Erk signalling and RNA synthesis (Baba et al., 2008; Hudon et al., 2010; Gaur et al., 2013);
- JAK-STAT and TGF-β signalling (Singh et al., 2006; Hong et al., 2010a; Cash et al., 2011);
- Rhoa signalling (Nahorski et al., 2012; Medvetz et al., 2012);
- Wnt and cadherin signalling (Krymskaya et al., 2010; Nahorski et al., 2012; Medvetz et al., 2012; Reiman et al., 2012);
- cell cycle (Kawai et al., 2013; Laviolette et al., 2013);
- apoptosis (Komori et al., 2009; Cash et al., 2011; Lim et al., 2012; Baba et al., 2012; Reiman et al., 2012; Sano et al., 2013);
- membrane trafficking (Nahorski et al., 2011; Nookala et al., 2012; Zhang et al., 2012);
- stem cell maintenance and pluripotency (Singh et al., 2006; Hong et al., 2010b; Betschinger et al., 2013);
- ciliogenesis (Luijten et al., 2013); and
- matrix metalloproteinase function (Hayashi et al. 2010; Pimenta et al., 2012; Nishii et al. 2013).

However, FLCN’s role in these processes has not been fully elucidated. Thus, fully characterising the role of FLCN in these and additional processes, will provide insight into how FLCN haploinsufficiency or loss causes BHD syndrome.

7.3 Drugs and therapies

There is currently no treatment available for any of the symptoms for BHD syndrome. However, more clinical research and a better understanding of the basic biology will facilitate development of drugs and other therapies to treat, and eventually cure, BHD syndrome.

For example, screening drug libraries could identify novel therapeutic compounds. Additionally, candidate drugs, already known to target a relevant pathway (e.g. mTOR signalling) or a phenotypically similar disorder (e.g. TSC or VHL), could be tested. If FDA approved drugs proved effective in the treatment of BHD they could be rapidly repurposed. Indeed, the antibiotic mithramycin can inhibit the growth of FLCN-null cells and might, therefore, prove to be an effective treatment for BHD-associated RCC (Lu et al., 2011). Additionally, work from the same lab showed that RNAi knock down of Slingshot2 (SSH2) lead to the activation of apoptosis genes Caspase 3 and Caspase 7 and decreased cell viability in FLCN-null cells, but not FLCN-expressing isogenic cells, suggesting that therapies targeting SSH2 activity might also prove to be an effective treatment for these cancers (Lu et al., 2013). Similarly,
combined autophagy inhibition and Paclitaxel treatment promoted apoptosis specifically in FLCN-null cells, but not FLCN-expressing cells (Zhang et al., 2013).

Recently there has been more interest in developing RCC treatments specifically targeted to certain tumour subtypes. Although most of this work has focused on clear cell RCC treatments, increased understanding between aberrant pathways and effective drugs will also impact on treatment in BHD cases.

The NIH recently announced a new clinical trial (NCT02504892) for the mTOR inhibitor everolimus in BHD patients with RCC. Everolimus is an approved treatment for sporadic metastatic RCC but has not been specifically trialled in BHD-associated and sporadic chromophobe RCC.

Gene and stem cell therapy hold much promise in the treatment of a wide variety of disorders. Early experiments show that FLCN function can be successfully reinstated in FLCN-null cells (Wong and Harbottle, 2013), meaning that in the future it may be possible to re-introduce a functional copy of the FLCN gene into FLCN-null or -heterozygous cells, either preventatively or curatively. However, these technologies are still developing, and currently there is only one FDA-approved gene therapy and only one FDA-approved stem cell treatment – Glybera to treat lipoprotein lipase deficiency and cord blood stem cell transplant to treat blood disorders and cancers - available, although a number of both types of treatment are currently undergoing clinical trial.
8. References


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**Further reading:** the majority of the literature relating to this document can be found in the BHD Literature Database.