What is BHD?

1.1 Birt–Hogg–Dubé syndrome

Birt–Hogg–Dubé (BHD) syndrome (OMIM 0135150) is an autosomal, dominantly inherited, monogenic condition, characterised by the development of benign skin tumours (fibrofolliculomas) on the face and upper torso, pulmonary cysts and pneumothorax (collapsed lung), and predisposition to kidney cancers with clear cell, chromophobic or oncocytic features (Birt et al, 1977).

In 2001, a BHD-associated gene locus was localised to chromosome 17p11.2 (Khoo et al, 2001; Schmidt et al, 2001) and a novel gene, Folliculin (FLCN), was subsequently identified as the inactivated gene in individuals with BHD Syndrome. The FLCN gene codes for a protein of unknown function called folliculin (FLCN).

BHD syndrome was first described in 1977 by three Canadian doctors – Birt, Hogg and Dubé (Birt et al, 1977). To date, approximately 200 families have been reported with pathogenic FLCN mutations (Schmidt et al, 2005; Graham et al, 2005; Toro et al, 2008; Misago et al, 2008; Frohlich et al, 2008; Leter et al, 2008; Woodward et al, 2008).

BHD syndrome may be considered one of the hamartoma syndromes. Hamartoma syndromes are dominantly inherited, predispose to cancer that affects multiple organs, and result in the development of benign tumours, made up from the cell type from which they arise. This association has arisen since BHD syndrome shares many clinical features (i.e. hamartomas, mucosal fibromas, and epithelial tumourigenesis) with other hamartoma syndromes, like Cowden syndrome, Peutz-Jeghers syndrome, and tuberous sclerosis complex (TSC), caused by inactivation of the tumour suppressor genes PTEN, LKB1 and TSC1/TSC2 respectively (Liaw et al, 1997; Marsh et al, 1999; Toro et al, 2002). PTEN, LKB1, and TSC1/2 are members of the mammalian target of rapamycin (mTOR) signalling pathway. The mTOR pathway is a key regulator of cell growth and proliferation (Hay and Sonenberg, 2004) and an increasing amount of evidence suggests that its deregulation is
associated with human diseases, including cancer and diabetes (Landau et al, 2009). The mTOR signalling pathway integrates signals from nutrients and growth factors to regulate many processes, including autophagy, ribosome biogenesis and metabolism (Laplante and Sabatini, 2009).

Loss of gene function is known to be involved in the deregulation of the mTOR signalling pathway and is implicated as a contributing factor to various human diseases, especially various types of cancer (Harris and Lawrence JC, 2003; Fingar and Blenis, 2004; Sarbassov et al, 2005). Current research suggests that the FLCN protein also has a tumour suppressor function and is implicated in the mTOR signalling pathway (Baba et al, 2006; Wang et al, 2009; Baba et al, 2009).

1.2 Clinical manifestations of BHD syndrome:

1.2.1 Benign skin tumours
Benign skin tumours usually develop in patients with BHD after the age of 20 years, as multiple whitish papules. These develop primarily on the face, but can also appear on the neck, ears and the upper torso (Menko et al, 2009). They are benign hair follicle tumours known clinically as fibrofolliculomas. Previously, fibrofolliculomas and trichodiscomas were considered hallmarks of BHD syndrome but recent studies suggest that they may not be distinct histological entities, and that a morphological spectrum of these benign skin tumours may exist, upon which both these two lie (Misago et al, 2009). Birt et al. (1977) originally described the skin manifestations of BHD syndrome to be a triad of fibrofolliculomas, trichodiscomas, and acrochordons. Acrochordons, more commonly referred to as “skin tags”, are common in the general population and may represent a phenotypic variant of fibrofolliculoma. (Fujita et al, 1981; Schulz and Hartschuh, 1999; Vincent et al, 2003).

1.2.2 Pulmonary cysts and spontaneous pneumothorax
The presence of pulmonary cysts in BHD syndrome was first described by Toro et al. (1999) in a study of 152 individuals from 49 families with familial renal neoplasms
syndromes. Three of the thirteen patients who had BHD syndrome exhibited pulmonary cysts, and one of these three patients developed pneumothorax (Toro et al, 1999). Additional cases of pulmonary cysts and spontaneous pneumothorax have since been reported in the literature (Zbar et al, 2002; Toro et al, 2007; Johannesma et al, 2009; Koga et al, 2009; Kluger et al, 2009, Ishii et al, 2009; So et al, 2009; Sundaram et al, 2009; Diamond and Kotloff, 2009) along with two descriptions of bullous emphysema (Schmidt et al, 2001).

A genome wide scan in a large Finnish family with a dominantly inherited predisposition to primary spontaneous pneumothorax (PSP) discovered that the PSP locus mapped to chromosome 17p11, where FLCN maps to (Painter et al, 2005). Screening of FLCN revealed a 4-bp deletion in the first coding exon, resulting in a frameshift of the reading frame and subsequent truncation of the protein. All carriers of the deletion presented with bullous lung lesions. Unlike previously identified mutations in FLCN, the exon 4 deletion seemed to be associated only with PSP, which showed 100% penetrance, suggesting a genotype-phenotype correlation. These results suggest that FLCN may have a significant role in normal pulmonary physiology since its inactivation in this study results in an exclusive PSP phenotype. Because of the strong association between primary spontaneous pneumothorax and BHD syndrome, Painter et al, (2005) suggested that patients with familial PSP should be investigated for increased risk of renal cancer.

Gunji et al, (2007) screened for mutations in FLCN in BHD syndrome patients with multiple pulmonary cysts and recurrent pneumothorax (mean age of first pneumothorax was 30.4 years), but without skin or renal lesions, and identified mutations in five of eight unrelated Japanese patients. All five patients had a family history of the disorder. The authors suggested that isolated pulmonary cysts and pneumothorax may be a milder form of the BHD syndrome and that patients should be monitored for renal or skin lesions.

In ten of 102 Chinese probands with spontaneous pneumothorax, four different mutations in FLCN were identified (Ren et al. 2008). Although only five of the probands reported a
family history of the disorder, CT imaging showed that eight of the probands had family members with either pneumothorax or pulmonary cysts. Two mutation carriers from two different families did not have pulmonary cysts.

Lung anatomy and histology generally appears normal in patients with BHD, and despite the presence of multiple pulmonary cysts, lung function is usually unaffected (Toro et al, 2007). Zbar et al, (2002) identified a 50-fold increase in the risk of pneumothorax for BHD-affected individuals, which was hypothesised to be related to the presence of pulmonary cysts (Toro et al, 2007). Pneumothorax has been reported in BHD syndrome patients as young as seven and sixteen years (Bessis et al, 2006; Ayo et al, 2007). Following a single episode of spontaneous pneumothorax, recurrent events are more common (Toro et al, 2007).

Smoking is an important risk factor for spontaneous primary pneumothorax; however, the role of smoking as a risk factor in BHD has been not been fully clarified (Toro et al, 2007; Ayo et al, 2007).

1. 2. 3. Renal Cell Carcinoma (Kidney Cancer)

Individuals with BHD syndrome are predisposed to develop bilateral multifocal tumours of the kidneys which has implications for clinical management, since regular surveillance of the kidneys is required to monitor tumour growth and size, and surgery is inevitably required to excise these tumours. (Birt et al, 1977; Toro et al, 1999; Zbar et al, 2002). Chromophobe renal cancer and a mixed pattern of chromophobe and oncocytic renal tumours are typical for patients with BHD. However, other histological subtypes can occur, including clear-cell and papillary carcinoma, and several mixed patterns (Pavlovich et al, 2002; Pavlovich et al, 2005; Fahmy et al, 2007; Janitzky et al, 2008; Kluijt et al, 2009). Relatively few patients with BHD and metastatic renal cancer have been described in literature (Kluijt et al, 2009; Souza et al, 2005). Prospective studies using cohorts are required to clarify the exact mechanisms of renal carcinogenesis in BHD syndrome (Toro et al, 2008).
Loss of FLCN expression in sporadic renal RCC suggests that FLCN is involved in the normal cellular functions that regulate growth and proliferation in the kidney and that the aberrant functioning of FLCN is mechanism of pathogenesis in both sporadic and BHD related renal carcinogenesis (Gad et al, 2007; Woodward et al, 2008).

Renal cancer is the most life-threatening complication associated with BHD Syndrome and various studies have reported the incidence of renal cancer in individuals with BHD syndrome, with the mean age of RCC incidence described as 50.4 years (Pavlovich et al, 2002) and the earliest reported age at diagnosis of renal cancer in a patient with BHD syndrome being twenty years old (Khoo et al, 2002). Further difficulties in establishing the risk of renal cancer in BHD patients include the possible under diagnosis of BHD Syndrome, variations (interfamilial or otherwise) in cancer risk due to different types of germline FLCN mutations and differences in genetic background.

1.2.4 Other clinical manifestations

The coincidence of BHD syndrome and a range of non-renal tumours has been documented. Early studies of BHD syndrome suggested an association between BHD and colorectal neoplasia (Hornstein, 1976; Birt et al, 1977; Schachtschabel et al, 1996; Schulz and Hartschuh, 1999) but a subsequent study has not confirmed this: Zbar et al, (2002) found no association between BHD and colonic polyps or CRC in a study involving a large cohort of 111 BHD syndrome patients.

Nevertheless, subsequent observations suggesting that some BHD families are at increased risk of colorectal neoplasia, and that interfamilial differences might be related to allelic heterogeneity or modifier effects, have been made, most notably by Khoo et al, (2002) who reported a high incidence of colorectal polyps and carcinomas in patients with confirmed FLCN germline mutations, indicating that FLCN may be involved in colorectal tumourigenesis. The role of FLCN in 47 unselected colorectal tumours (ten polyps and 37 carcinomas) was evaluated by screening all coding exons of FLCN for mutations and
analyzing 46 of the tumours for loss of heterozygosity (LOH) in the chromosome region surrounding \textit{FLCN} (Kahnoski \textit{et al}, 2003). Alterations in \textit{FLCN} promoter methylation profiles were determined in 23 cases of matched normal/carcinoma tissues. They reported the detection of two novel somatic missense mutations of \textit{FLCN} and LOH in 81\% of primary sporadic colorectal tumours with no change in promoter methylation profile. All mutations were detected in microsatellite stable (MSS) tumours.

In a clinical study of 22 patients from ten unrelated French families with BHD syndrome over five years, Kluger \textit{et al}, (2009) 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 thirteen of twenty cases (65\%). No medullary carcinoma or other thyroid carcinomas were detected. Additionally, colonoscopy procedures failed to detect colorectal carcinoma 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.

Other reported benign and malignant tumours are listed in Menko \textit{et al}, (2009), but, so far, a causal relationship between BHD syndrome and these tumours has not been shown.

\textbf{1.3 \textit{Folliculin} gene}

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

Schmidt \textit{et al}, (2001) performed a genome wide scan in a large BHD kindred (172 members) and also localised the gene to the pericentromeric region of 17p using linkage analysis. Two-point linkage analysis of eight additional families with BHD 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. One additional family, which had histologically confirmed fibrofolliculomas, did not show evidence of linkage to 17p, suggesting genetic heterogeneity.

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. Significantly, this genomic region is associated with a number of diseases (including bladder and breast cancer, Charcot-Marie-Tooth disease and Neurofibromatosis) because of the presence of unstable low-copy number repeat elements. FLCN consists of 14 coding exons (Nickerson et al, 2002) spanning approximately 20 kb of genomic DNA.

1.4 FLCN Mutations

1.4.1 Mutations in FLCN: Knudson’s two-hit hypothesis

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 tumourigenesis results from a germline mutation in a tumour suppressor gene, e.g. FLCN, (the ‘first hit’), but that this alone is not sufficient for tumour development; inactivation of the wildtype allele by a somatic mutation (second hit) is required.

Somatic mutations in the remaining wild-type copy of FLCN and 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 (Vocke et al, 2005).

1.4.1 FLCN mutations

Schmidt et al, (2005) combined mutation analysis from 30 BHD families with previous data obtained from screening FLCN in 30 families with BHD syndrome (Nickerson et al, 2002) and together, these studies have identified germline BHD mutations in 51 (84%) of 61 families with BHD; 27 (53%) of the mutations involved either a cytosine insertion or
deletion in the mononucleotide tract of eight cytosines (C8) in exon 11, which appears to represent a mutation hotspot. A slippage-mediated mechanism during DNA replication was postulated to be the mechanism responsible for these frameshift mutations leading to protein truncation. Notably, most reported mutations were predicted to terminate FLCN prematurely: 22 unique mutations predicted to truncate the protein, including a “hot spot” insertion deletion in a C8 tract, were identified in 84% of BHD kindreds. Further evidence supporting the theory that the C8 tract is a mutation hotspot was provided by Khoo et al, (2002) when haplotype analysis of FLCN identified two germline mutations in exon 11 (c.1733insC and c.1733delC) in three of four BHD families as well as two of four sporadic cases of BHD syndrome.

Two sequence variation databases for FLCN currently exist. Both are hosted online by the LOVD (an open source DNA variation database), where researchers can submit published or unpublished mutations. One Folliculin database (www.flcn.nl/lovd) is curated by Dr Derek Lim (University of Birmingham, UK, European BHD Consortium; Lim et al, 2009) whilst the other is called www.skingenedatabase.com (Wei et al, 2009). There are a total 115 novel variants (including 37 SNPs) including twelve novel unpublished variants/mutations. Seventy are classified as being pathogenic and seven are ‘probably’ pathogenic (i.e. pathogenicity yet to be confirmed).
1.4.1.1 Substitution Mutations

Amino acid substitutions account for approximately 42% of reported FLCN mutations and occur more frequently than other types of mutations throughout exons 11 to 14. Sixteen have been reported as pathogenic (shown in bold), whilst the pathogenicity of the remaining mutations is yet to be determined.

<table>
<thead>
<tr>
<th>Exon</th>
<th>DNA Change</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>c.-487G&gt;C</td>
<td>5'UTR</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
</tr>
<tr>
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<td>c.-302C&gt;T</td>
<td>5'UTR</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
</tr>
<tr>
<td>1</td>
<td>c.-299A&gt;G</td>
<td>5'UTR</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
</tr>
<tr>
<td>1</td>
<td>c.-228+994T&gt;C</td>
<td>Intron</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
</tr>
<tr>
<td>1</td>
<td>c.-228+1368C&gt;A</td>
<td>Intron</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
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<tr>
<td>1</td>
<td>c.-90T&gt;C</td>
<td>5'UTR</td>
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<tr>
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<td>5</td>
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<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
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<td>c.397-14A&gt;G</td>
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<td>7</td>
<td>c.779+113G&gt;A</td>
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<tr>
<td>8</td>
<td>c.871+31C&gt;T</td>
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<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
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<tr>
<td>8</td>
<td>c.871+204T&gt;C</td>
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<td>8</td>
<td>c.871+226C&gt;T</td>
<td>Intron</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
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<tr>
<td>8</td>
<td>c.871+684C&gt;T</td>
<td>Intron</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
</tr>
<tr>
<td>9</td>
<td>c.943G&gt;T</td>
<td>Nonsense</td>
<td>Graham et al., 2005. Am J Respir Crit Care Med 172:39-44</td>
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<tr>
<td>9</td>
<td>c.1062+6A&gt;G</td>
<td>Intron</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
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<td>9</td>
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<td>Palmirotta et al., 2008 Eur J Dermatol 18:382-6</td>
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<td>10</td>
<td>c.1176+31C&gt;T</td>
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<tr>
<td>Exon</td>
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<td>Remarks</td>
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<td>10</td>
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<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
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<tr>
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<td>11</td>
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<td>11</td>
<td>c.1278G&gt;A</td>
<td>Coding SNP</td>
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<td>11</td>
<td>c.1301-59G&gt;A</td>
<td>Intron</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
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<td>12</td>
<td>c.1333C&gt;T</td>
<td>Coding SNP</td>
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<tr>
<td>13</td>
<td>c.1538+121G&gt;A</td>
<td>Intron</td>
<td>Cho et al., 2008. BMC Medical Genetics 9:120-130</td>
</tr>
</tbody>
</table>

Legend: Sequence variations nomenclature is as recommended by the Ad-Hoc Committee for Mutation Nomenclature (AHCMN) (den Dunnen JT and Antonarakis SE [2000], Hum.Mut. 15:7-12).

### 1.4.1.2 Deletion Mutations

Nucleotide deletions are the second most frequently reported type of FLCN mutation, causing a frameshift. No deletion mutants have been reported in exons 1 to 3.

<table>
<thead>
<tr>
<th>Exon</th>
<th>DNA Change</th>
<th>Remarks</th>
<th>Reference</th>
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</table>
1.4.1.3 Duplication Mutations

All eight duplication mutations are pathogenic and result in a frameshift.

<table>
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<tr>
<th>Exon</th>
<th>DNA Change</th>
<th>Remarks</th>
<th>Reference</th>
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1.4.1.4 Indel Mutations

Three indel (insertion/deletion) mutations have been reported and all have been described as being pathogenic.

<table>
<thead>
<tr>
<th>Exon</th>
<th>DNA Change</th>
<th>Remarks</th>
<th>Reference</th>
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1.4.1.5 Insertion Mutations
One insertion mutation of unknown pathogenicity has been reported in exon 12 and results in a frameshift.

<table>
<thead>
<tr>
<th>Exon</th>
<th>DNA Change</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
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</table>

1.5 Folliculin expression

*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. FLCN protein sequence is as follows:

MNAIVALCHFCELHGPRTLFCTEVLHAPLPQDGNEDESPGQGEGQAEEEGGIQMNRSRMRAHSPA EGADEVSSPGPKSDMCRCRSLGYPHYISHDKETSIKYVSHQHPHQPQFVIFVRQACVRSLSCE EVCPREGPIFFGDEQHGFVSHHTFFIKSALRFRQRWYSIITIMMDRIYLINSWPFLLGKVRGIIDE LGKAKLVFEAEQFGCPQRAQRMNTAFTPHLQHRGNAARSLTSLLLWACLHHTSFAWLL KACGSRLTEKLEGAPTEDTLVQMEKLDIEESTWDEAEEEKAPVLPESTEGRELTPGAPA ESSENSGCSWQPRKLPSRKRLHRQVLGAPSFRMLAWHLMGNQWIKSRTDVLVQASFEVLR RTMLPVGCVRIIPYSSQEEAYRCNFLGSLPHVQIPPHVLSSEFAVIVEVHAARSLTPHSVGEEDQ SLSKYEFVVTSGPSVAADRVPILNKIEAALTNQNLSDVVDQCLVCLKEEWMNKVKLVFKFTK VDSRKPEDTQKLGSILGASEEDNVKLLKFWMGLSKTYKSHLMSTVRSPTSES

**Protein Key:**

- **G** Glycine (Gly)
- **A** Alanine (Ala)
- **L** Leucine (Leu)
- **M** Methionine (Met)
- **F** Phenylalanine (Phe)
- **W** Tryptophan (Trp)
- **K** Lysine (Lys)
- **Q** Glutamine (Gln)
- **E** Glutamic Acid (Glu)
- **P** Proline (Pro)
- **V** Valine (Val)
- **I** Isoleucine (Ile)
- **C** Cysteine (Cys)
- **Y** Tyrosine (Tyr)
- **H** Histidine (His)
- **R** Arginine (Arg)
- **N** Asparagine (Asn)
- **D** Aspartic Acid (Asp)
S Serine Ser T Threonine Thr

No transmembrane domains or organelle localization signals have been determined as yet (Warren et al, 2004). FLCN has no significant homology to any known protein, but is highly conserved across species including *Drosophila melanogaster*, *Xenopus tropicalis*, *Mus musculus*, *Rattus norvegicus*, *Caenorhabditis elegans*, *Canis lupus familiaris*, *Bos Taurus*, *Gallus gallus*, *Macaca mulatta* and *Pan troglodytes*, suggesting an important biological role (Nickerson et al, 2002).

*FLCN* mRNA is expressed in: the skin; the distal nephron of the kidney; stromal cells and type 1 pneumocytes of the lung; acinar cells of the pancreas and parotid gland; and epithelial ducts of the breast and prostate. In the brain, *FLCN* mRNA is expressed in neurons of the cerebrum, and Purkinje cells in the cerebellum. *FLCN* mRNA is also detectable in macrophage and lymphocytes in the tonsils and spleen. Tissues with lower levels of *FLCN* mRNA included heart, muscle and liver (Warren et al, 2004). Further evidence supporting the tumour suppressor function of *FLCN* in renal cancer is that *FLCN* mRNA is not detected in renal tumours from BHD patients. Localisation of FLCN to both the nucleus and cytoplasm was determined by fluorescence *in situ* hybridization using epitope-tagged FLCN expressed in HEK293 cells (Baba et al, 2006).
1.6 Folliculin protein interactions

Folliculin-binding protein 1 (FNIP1), was identified in 2006 (Baba et al) as a protein able to interact with and phosphorylate FLCN. FNIP1 was also determined to be able to bind 5′-AMP-activated protein kinase (AMPK), a negative regulator of mTOR and a key protein for energy sensing in cells (Inoki K et al, 2003; Gwinn DM et al, 2008). Baba et al, (2006) demonstrated that both FLCN and FNIP1 are phosphorylated by AMPK. This interaction was also shown to be modified by external influences since treatment with an AMPK inhibitor (compound C), rapamycin or amino acid starvation, affected the phosphorylation status of FLCN, further indicating a role in the mTOR pathway.

FNIP2, a second FLCN-binding protein, was first identified by Hasumi et al, (2008). Takagi et al, (2008) subsequently identified FNIP2 as FNIPL. The accepted nomenclature for this protein is FNIP2, it is homologous to FNIP1 (49% identity, 74% similarity), is conserved
across species, and also binds to AMPK. Interestingly, FNIP1 and FNIP2 are able to form homo- and heterodimers, as well as multimers (Takagi et al, 2008), suggesting a coordinated functional relationship between these proteins.

Significantly, approximately 84% of reported BHD kindreds have $FLCN$ mutations that are predicted to prematurely truncate FLCN (Nickerson et al, 2002; Khoo et al, 2002; Schmidt et al, 2005; Leter et al, 2007). This removes FLCNs ability to interact with FNIP-1 and -2, which suggests that the interaction between FLCN and the FNIPs is functionally important. Recent research (Wang et al, 2009) has shown that serine 62 (Ser62) is the major phosphorylation site in FLCN. Their analysis suggests that Ser62 phosphorylation is indirectly up-regulated by AMPK.

The expression patterns of FLCN, FNIP-1 and -2 in human tissues were determined by Hasumi et al, (2008) using real-time PCR. Expression patterns of FLCN, FNIP1 and FNIP2 were generally similar, and consistently similar in specific tissues such as muscle, nasal mucosa, salivary gland and uvula, suggesting that FLCN, FNIP1 and FNIP2 may cooperate together in those organs. However, FNIP2 expression is higher relative to FNIP1 in fat, liver and pancreas, which suggests that FNIP2 may have a specific function in those metabolic tissues (Hasumi et al, 2008). FLCN/FNIP1 and FLCN/FNIP2 dimers have been shown to co-localize in the cytoplasm in a reticular pattern and binding of FLCN to FNIP1 and -2 is mediated through the C-terminal region of FLCN (Baba et al, 2006; Hasumi et al, 2008; Takagi et al, 2008). Co-expression studies of FLCN, FNIP1 and FNIP2 indicate that both FNIPs regulate the cytoplasmic distribution of FLCN since expression studies have shown that when expressed alone, FNIP2 constructs are distributed within the cytoplasm with condensed features around the nucleus. When FLCN constructs are expressed alone they are found mainly in the nucleus. 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)

The molecular functions of FLCN are poorly understood, but indirect interactions between
FLCN and AMPK within the mTOR signalling networks mediated by FNIP1 and -2 have been firmly established (Baba et al, 2006; Hasumi et al, 2008). However, the functional role of FLCN in mTOR signalling is undetermined since several recent publications have reported opposite impacts on phosphorylated ribosomal protein S6 (p-S6; an indicator of mTOR activation) signalling as a consequence of FLCN downregulation. Two studies recently reported that transient downregulation of FLCN by siRNA in human cell lines results in reduction of phosphorylation of p-S6 (Takagi et al, 2008; Hartman et al, 2009). Reduction of p-S6 was also observed in renal cysts developing in mice heterozygous for FLCN (Hartman et al, 2009). In contrast, kidney-specific homozygous knockout of FLCN results in an increase in phosphorylated p-S6, which contributed to the development of polycystic kidneys (Baba et al, 2008; Chen et al, 2008). This data suggests a role for FLCN in nutrient/energy-sensing mediated through the mTOR signalling pathway.

Further studies in Schizosaccharomyces pombe (S. Pombe) revealed that yeast FLCN homologue and yeast TSC1/2 regulate common downstream targets but have opposing roles, specifically TSC1/2 inhibit the activation of Tor2 and subsequent downstream elements but S.pombe FLCN upregulates the same elements (van Slegtenhorst, 2007). If this relationship between BHD and TSC1/TSC2 is recapitulated in mammalian cells and mTOR is inhibited in cells lacking BHD, there may be important clinical implications for BHD patients.

Recent work using a kidney-targeted FLCN gene inactivation in a mouse model has indicated that the Raf-MEK-Erk pathway, which is activated in many cancers and regulates cell proliferation (Roberts et al, 2007), was activated in FLCN-knockout kidneys (Baba et al, 2008). This suggests that a common upstream effector of these two pathways may be activated by loss of FLCN tumour suppressor function, resulting in cell growth and proliferation within the FLCN-null kidney cell.

1.7 Animal Models of BHD syndrome
Current models provide an excellent basis for drug development. They are also crucial for
more fundamental studies investigating cell biology and protein interactions, which will provide insight into understanding the mechanisms of pathogenesis underlying BHD syndrome \textit{in vivo}.

\textbf{1.7.1 Mouse BHD models}

Several mouse models have been generated: a $FLCN$ homozygous and heterozygous knockout mouse model has been generated that shows mTOR activation in renam cell carcinomas, and that these tumours are multifocal. (Hasumi \textit{et al}, 2009).

A kidney specific knockout mouse model containing a conditional $FLCN$ allele with cadherin 16 (KSP)-Cre transgene which targets $FLCN$ inactivation to the kidney (Baba \textit{M et al}, 2008).

A second kidney specific $FLCN$ knockout mouse model has been generated that develops polycystic kidneys and renal neoplasia (Chen \textit{et al}, 2008). Conditional homozygous inactivation of $FLCN$ in the mouse kidney triggered the development of highly enlarged polycystic kidneys, which led to renal failure and resulted in early death three weeks after birth (Baba \textit{M et al}, 2008; Chen \textit{J et al}, 2008).

A model with targeted inactivation of $FLCN$ using an embryonic stem (ES) cell line has been successfully generated. This ES cell line was created using a gene trap vector technique and contains a $\beta$-geo ($\beta$-galactosidase/neomycin) cassette integrated between exon 8 and 9 in $FLCN$, resulting in a truncated folliculin protein (Hartman \textit{et al}, 2009). These mice developed cysts and tumours in the kidneys (Hartman \textit{et al}, 2009).

\textbf{1.7.2 Rat BHD model}

The Nihon rat is a model of renal carcinoma found in a colony of Sprague-Dawley rats in Japan (Okimoto \textit{et al}. 2004). It contains a single nucleotide insertion in the region of the rat chromosome 10 (an area homologous with human chromosome 17p11.2) producing a frameshift and premature stop codon. In heterozygotes, renal carcinomas develop from early pre-neoplastic lesions, seen as early as three weeks of age, into adenomas by eight
weeks of age, with complete penetrance of this renal carcinoma gene by the age of six months. The renal carcinomas that develop in heterozygotes are predominantly clear cell, as is the case in the Ecker rat, a TSC animal model which carries a single gene mutation in the TSC2 gene as the cause of renal carcinoma. The homozygous mutant condition is lethal at an early stage of fetal life in the Nihon rat. LOH at the FLCN locus in ten of eleven primary renal carcinomas is detectable, fitting the Knudson 2-hit model. Okimoto et al, (2004) concluded that the Nihon rat provides insights into a tumour suppressor gene that is related to renal carcinogenesis and an animal model of human BHD syndrome.

1.7.3 Canine BHD model
Hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis (RCND) is a naturally occurring canine kidney cancer syndrome, originally described in German shepherd dogs. The RCND locus was narrowed to a small region on canine chromosome 5 that overlapped FLCN (Lingaas et al, 2003). The authors described a histidine to arginine mutation in exon 7 of canine FLCN that segregated with the disease phenotype.

1.7.3 Yeast BHD model
van Slegtenhorst et al, (2007) generated a S. pombe BHD model in which the yeast FLCN homologue was reported to activate the mTOR homologue Tor2 (see Section 1.5).

1.7.4 Drosophila BHD Model
Singh et al, 2006 used RNA interference to decrease the expression of Drosophila BHD homologue (DBHDH) and showed that DBHDH was required for male germline stem cells (GSC) maintenance in the fly testis. Subsequent investigation suggested that DBHDH regulates GSC maintenance downstream of, or in parallel to the Janus kinase-signal transducers and activators of transcription (JAK/STAT) and Decapentaplegic (Dpp) signal-transduction pathways. JAK-STAT signalling cascade regulates stem cell renewal or differentiation of renal stem cells whilst the Dpp signal-transduction pathway is crucial for polarisation of cell differentiation in wing development.
Further studies into the *Drosophila* model by Singh and Hou (2009) have shown that over-expression of JAK-STAT signalling results in enlargement of Malphigian Tubules i.e. the *Drosophila* equivalent of kidneys, coupled with an increase in proliferating cells, mitotically active cells and renal stem cells. Aberrant signalling of JAK-STAT signalling could contribute to renal cystic or carcinoma phenotype.

### 1.8 Future Work

Further research is required to characterise the multistep process that leads to the epidermal, pulmonary and renal phenotypes associated with BHD syndrome.

It remains essential to fully determine the role of FLCN in all cellular pathways. Whilst current publications emphasise FLCN's role in the mTOR pathway (Baba *et al*, 2006; Wang *et al*, 2009; Baba *et al*, 2009) recent research has indicated the activation of the Raf (Erk1/2) and JAK-STAT signalling pathways in FLCN null animal models (Baba *et al*, 2008). What role(s) does FLCN fulfil in these pathways? Elucidating the biochemical networks and interactions that FLCN participates in would prove crucial to understanding the mechanism of pathogenesis underlying BHD syndrome.

Characterising the changes that occur in *FLCN* null and *FLCN* heterozygous cells (e.g. gene transcription, protein expression) may provide insight into the causes of phenotypic variation in BHD syndrome, e.g. why only some skin cells generate fibrofolliculomas; whether haploinsufficiency is sufficient for the skin and lung phenotype but loss of heterozygosity is required for renal cell carcinoma.

The exact frequency of renal cysts in comparison with the prevalence in the general population is currently unknown and merits further investigation since benign renal cysts have been documented in patients with BHD syndrome (Toro *et al*, 1999; Kluijt *et al*, 2009). Classically, BHD syndrome has not been associated with the development of cystic kidneys in contrast to TSC and polycystic kidney disease.
Ultimately, understanding these mechanisms will enable the development of drugs and other therapies for individuals with BHD syndrome.

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