Birt-Hogg-Dubé syndrome

Discussion & valorisation

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Discussion & valorisation

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

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

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

Renal surveillance

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

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

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

FLCN and mTOR

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

mTOR involvement in the maintenance of fibrofolliculomas

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

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

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

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

Indeed, whilst our trial was being conducted, conflicting data were reported on the dysregulation of mTOR signalling in BHD syndrome.[9] Since then, more contradictory results have been published. Both up-[17-23] and downregulation[24-27] of mTOR signalling have been seen upon aberrant FLCN expression. Khabibulin *et al.* show that the mTOR readout phospho-S6 is upregulated upon FLCN knockdown in SAEC cells (human small airway epithelial cells), but is unaffected in HBE cells (human bronchial epithelial cells).[19] These observations suggest that the effect of the used systems can greatly influence the outcome of the study. Moreover, mTOR signalling is affected by many different signals (such as ATP levels, amino acids, growth factors, flow stress) and has various effectors (as reviewed by Laplante and Sabatine[28]).Due to the diverse functions of mTor it is difficult to study the effect of one single player in its complex signalling cascade.

Additional mTOR studies in BHD syndrome

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

ners (Figure 1). First, Petit *et al.* found FLCN to bind RagA/B through the GTPase domain of RagA, suggesting FLCN may be a guanine exchange factor (GEF), for loading GTP on RabA/B.[24] RabA/B is a small GTPase that forms a heterodimer with RabC/D. When activated, the heterodimer binds mTOR and recruits it to the lysosome where mTOR can be activated (Figure 1A). This study reports downregulation of mTOR upon FLCN knockdown.[24] Second, FLCN was identified as GTPase activating protein (GAP), hydrolysing GTP to GDP on RabC/D in order to recruit mTOR to the lysosome[25] (Figure 1B). This study by Tsun *et al.* also show that FLCN knockdown results in downregulation of mTOR.[25] It may sound contradictory that FLCN enriches one Rab with GTP, whereas it charges the other Rab with GDP. However, it is known that mTOR binding is strongest by GTP loaded RagB in heterodimer with GDP loaded RagC.[29] Thus, by its GEF and GAP function, FLCN stimulates mTOR recruitment to the lysosome and hence facilitates its activation. Third, FLCN can stimulate mTOR by increasing the leucine levels in the lysosome.[26] FLCN facilitates this by inhibiting the expression of PAT1 (proton-assisted amino acid transporter 1) on the lysosome surface. This decreases the export of leucine from the lysosome and the resulting higher intra-lysosomal leucine levels activate mTOR[30] (Figure 1C). In accordance with this function, knockdown of FLCN causes downregulation of mTOR in this report.[26] Fourth, FLCN inhibits mTOR by recruiting LKB1 to the cilium. LKB1 phosphorylates AMPK at the basal body which leads to mTOR inhibition[23] (Figure 1D). Accordingly, mTOR activity is reported upon FLCN knockdown in the study describing this function of FLCN. Fifth, FLCN could prevent mTOR activation at the lysosome, by facilitating perinuclear localisation and immobilisation of the lysosome. FLCN can do so by binding RILP at the perinuclear membrane and loading Rab34 onto it.[31] The presence of Rab34 on the perinuclear membrane causes lysosomal recruitment.[32] When lysosomes cluster at the perinuclear region mTOR activation cannot take place[33] (Figure 1E). In summary, FLCN has multiple functions and can modulate mTOR signalling in several ways. Targeting mTOR in BHD syndrome is not an option until we know what part, if any, of the mTOR signalling cascade is involved in the pathogenesis of the syndrome and if up or downregulation causes clinical symptoms.

Interpreting mTOR studies in BHD syndrome

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

Furthermore, the effects of FLCN on mTOR are triggered by different stimuli. The upregulation of mTOR trough cilia happens upon flow stress.[23] The GEF and GAP function of FLCN towards the Rab heterodimer is activated by the presence of amino acids. On the other hand, mTOR inhibition by stimulating the perinuclear localisation of lysosomes occurs under starvation. So, mTOR is upregulated by FLCN in a nutrient-rich state and downregulated upon starvation. Of note, the signalling of amino acid levels to mTOR is independent of harmartin and tuberin, which are the affected proteins in TSC, a disorder that resembles BHD syndrome. Both disorders combine renal cysts and tumours with cystic pulmonary lesions and hair follicle tumours.[4] The hamartin-tuberin complex inhibits canonical mTOR signalling[34-36] and is involved in signalling low ATP levels to mTOR.[37] FLCN, on the other hand, is required for inhibiting mTOR upon high ATP levels.[23] Nevertheless, both FLCN and the TSC complex are required for proper mTOR signalling. This may explain why TSC and BHD syndrome have similar but not identical phenotypes.

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

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

Mutant FLCN

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

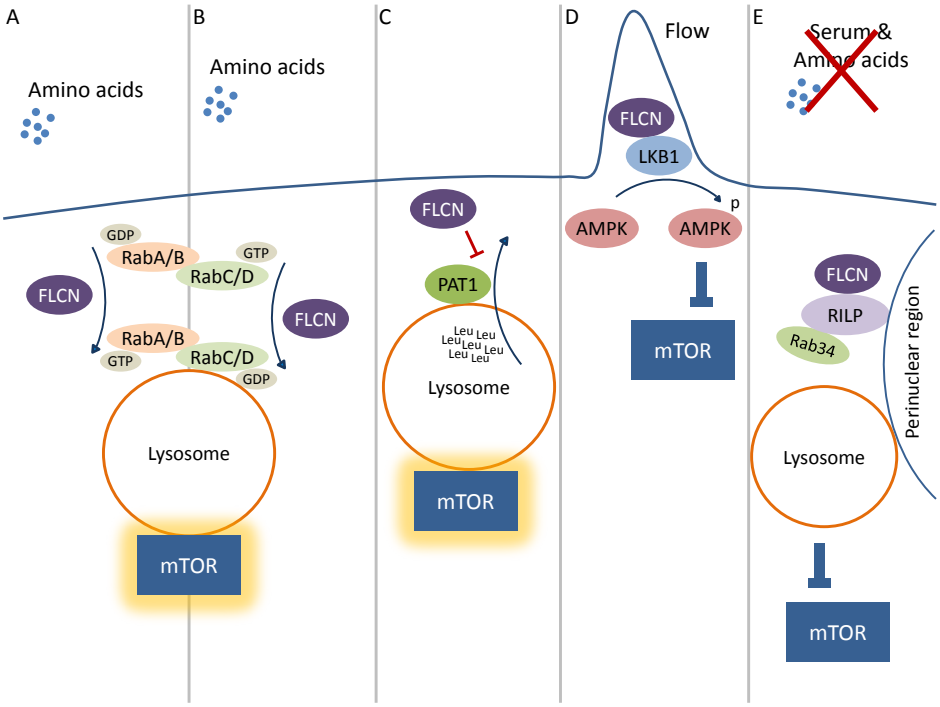

Figure 1. FLCN can stimulate (A-C) and inhibit (D,E) mTOR signalling in different ways. A detailed description can be found in the text.

c.1285dupC

Transduction of human kidney-2 (HK-2) cells with EGFP tagged FLCN1285dupC resulted in cells with a low FLCN1285dupC expression level (**chapter 4**). HK-2 cells transduced with His-HA-FLAG tagged FLCN1285dupC did grow slow and did not show expression at all, nor did retinal pigment epithelium (RPE) cells transfected with this construct. UOK 257 cells[38] transfected with His-HA-FLAG tagged FLCN1285dupC did not survive (**chapter 5**). In all, we were not able to generate a cell line stably expressing FLCN1285dupC. A dominant negative or toxic effect of mutant FLCN could explain this observation. However, Nahorski and colleagues did manage to overexpress FLCN1285dupC in the DLD-1 colorectal cancer cell line. It shows normal growth compared to DLD-1 transfected with an empty vector. From this observation, Nahorski et al conclude there is no evidence FLCN1285dupC has a dominant negative effect, but suggest this may be due to the cell line used.[39] They also expressed FLCN1285dupC in FTC-133 cells, which are derived from a lymph node metastasis of a thyroid carcinoma.[39] The FTC-133 cells do not express endogenous FLCN, whereas DLD-1 cells do. Hence, the presence of endogenous FLCN does not seem to influence the survival of cell lines with FLCN1285dupC. Cell lines derived from advanced

stages of carcinomas generally are adapted to survive in strenuous conditions, which may explain resistance of DLD-1 and FTC-133 cells to a dominant negative effect, as well as to any toxicity of mutant FLCN. However, these potential effects of mutant FLCN may prevent survival of other cell lines. Our HK-2 and RPE cell lines having no or low expression of FLCN1285dupC when surviving, could be due to improper incorporation of the plasmid, resulting in cells containing only the resistance cassette and not the FLCN1285dupC DNA. Since functionally tested mutations in FLCN have been shown to disrupt the stability of the protein[39], this may further impair expression of FLCN1285dupC if transfection was successful.

c.1523A>G

Noticeably, UOK257 cells transduced with FLCN mutant variant c.1523A>G (encoding p.Lys508Arg) survive, whereas cells with c.1285dupC, c.1844C>G (encoding p.Tyr463Stop) and c.764A>G (encoding pHis255Arg) do not (**chapter 4**). This c.1523A>G mutation might not be pathogenic and could be a polymorphism. The mutation was reported in three patients with renal tumours who reportedly had no other symptoms of BHD syndrome.[40, 41] No loss of heterozygosity was detected in two of them; in one it was not tested. To explain this seemingly contradictory observation, the renal tumours in these patient might have been caused by mutations in other renal cancer susceptibility genes. Moreover, another 24 people, not diagnosed with BHD syndrome, were reported to carry c.1523A>G as well, according to the ExAC database of protein-coding genetic variations.[42] Either BHD syndrome was not recognised in these 24 controls, or c.1523A>G could be a rare polymorphism. One could speculate that c.1523A>G is a (partially) functional polymorphism that does not cause the BHD syndrome phenotype, but can cause renal tumours in combination with mutations in other renal susceptibility genes.

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

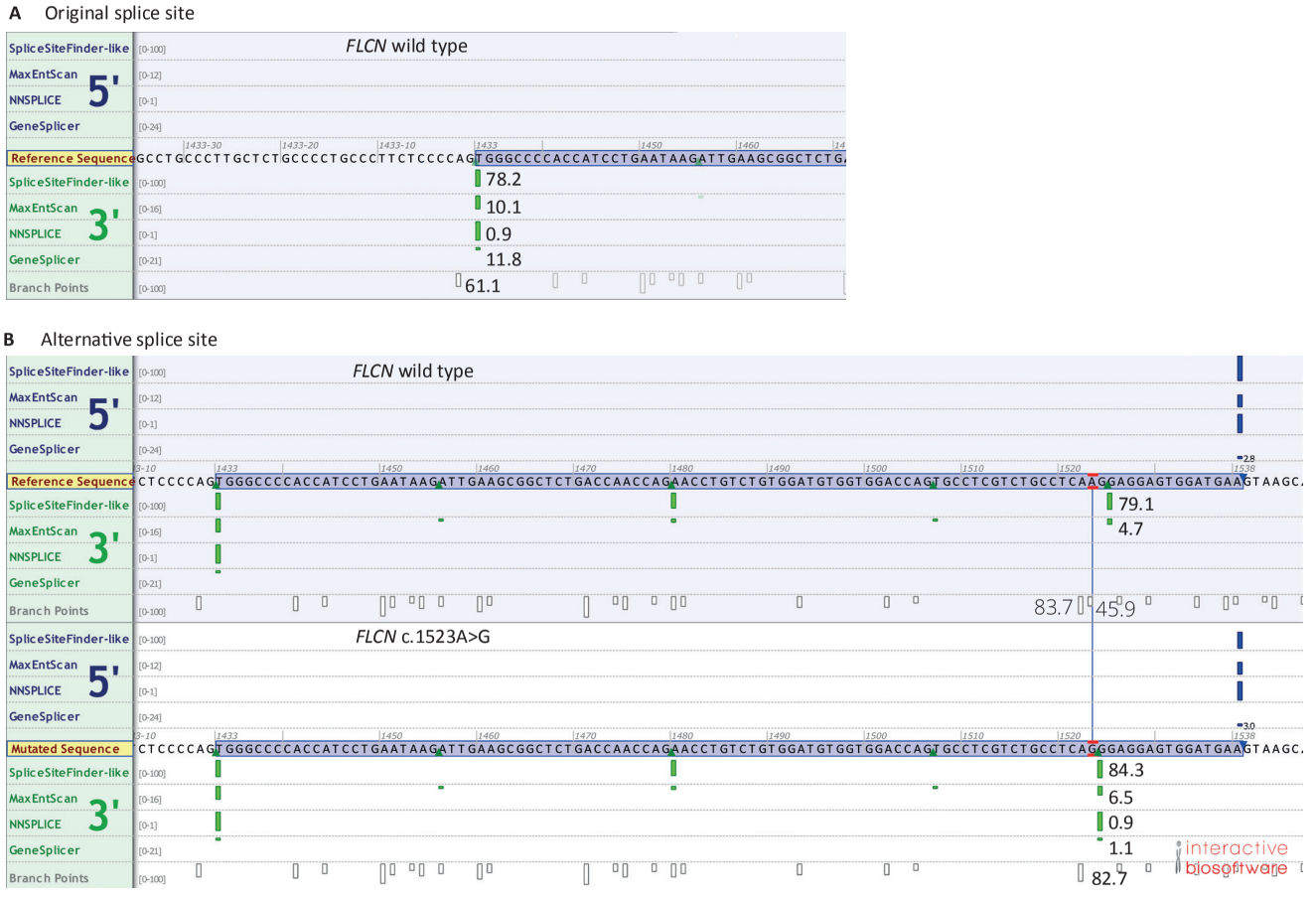

Figure 2. Mutation c.1523A>G in *FLCN* enhances an alternative '3 acceptor site between nucleotides 1523 and 1524. This acceptor site would delete 91 bases from the mRNA (r.(1433_1523del)). Splicing probability was predicted by SpliceSiteFinder-like, MaxEntScan, NNSPLICE and GeneSplicer and combined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

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

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

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

FLCN and cilia

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

To test our hypothesis, we investigated the relationship between FLCN and the formation and function of cilia in **chapter 4**. There is strong evidence that cilia are involved in controlling planar cell polarity (PCP).[47] In polarised epithelia, PCP regulates cell division in plane of the tissue. It ensures that spatial organisation of tissue remains intact. In **chapter 4** we show that knockdown of FLCN results in abnormal ciliation and disrupts PCP in a spheroid model. Our results have been confirmed by other groups that also report aberrant PCP[17] and defects in ciliation[23] in the context of abnormal FLCN levels. We show that knockdown

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

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

In our study, we noticed ß-catenin localises to the cilium and is absent from the cilia upon FLCN knockdown. Ciliary sequestration of ß-catenin limits its nuclear translocation and thereby inhibits Wnt signalling.[51] Accordingly, we found ß-catenin activation and increased canonical Wnt signalling upon FLCN knockdown. Hence, FLCN may play a role in localising and/or transporting ß-catenin and thereby influence Wnt signalling. Intriguingly, Wnt signalling is also known to be activated by LKB1 (as reviewed by Martin-Belmonte[52]) and is potentially oncogenic (as reviewed by Zhan[53]).Moreover, LKB1 influences PCP by non-canonical Wnt signalling (as reviewed by Zhan[53]). Hence, our work together with that of Zhong *et al.*, connects FLCN's role in cilia with mTOR and Wnt signalling through LKB1. Disturbance of ciliary function, mTOR signalling and Wnt signalling have been linked to cyst formation and oncogenesis[53-58] and therefore might contribute to the BHD syndrome phenotype. Interestingly, LKB1, cilia and Wnt have been linked to tumour formation in Peutz-Jeghers syndrome (PJS). PJS is most often caused by mutations in LKB1, which cause ciliary defects[59] and Wnt was shown to be activated in this syndrome[60-62] that is characterised by mucocutaneous pigmentation, gastrointestinal polyps and predisposition to multiple cancers.[63]

By classifying BHD syndrome as a ciliopathy, the BHD field can now benefit from all studies being done on ciliopathies, to better understand the pathophysiology of BHD syndrome. If a clear-cut role of Wnt signalling in the pathophysiology can be confirmed, new treatment possibilities are raised, as multiple Wnt inhibitors are in clinical development for the treatment of cancer (as reviewed[64]).

Involvement of FLCN in intracellular transport

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

perinuclear region[31], binds to a main component of the intraflagellar transport motor[23], localises LKB1 to the cilium[23] and regulates the transport of leucine from the lysosome.[26] In addition, FLCN was found to be involved in the nuclear export of TDP-43.[65] Thus far, TDP-43 has not been linked to the pathophysiology of BHD syndrome. Instead, it is involved in regulating autophagy and its cytoplasmatic accumulation and mislocalisation is a hallmark of the neurodegenerative disorders amyotrophic lateral sclerosis and frontotemporal lobar dementia.[66-68] However, FLCN's involvement in nucleocytoplasmatic shuttling of TDP-43 again points to a possible role for FLCN in the transport and/or localisation of a broader group of other molecules. In particular, FLCN seems to be involved in energy related transport and localisation, as many effects depend on the presence or absence of amino acids (Figure 1A,B,E) or involves amino acid transport (Figure 1C). Concurrently, FLCN was found to be involved in autophagy[69], a process that uses vesicular transport and lysosomal degradation to regulate cellular energy maintenance upon nutrient starvation (as reviewed[70]). Furthermore, FLCN having a role in energy related transport is in line with its function in ciliogenesis (**chapter 4**), as the cilium functions as energy sensor and has a role in autophagy.[71] The putative binding partners of FLCN that we identified (**chapter 5**) could further support FLCNs role in transport and/or localisation. Alpha tubulin and both isotypes of eukaryotic elongation factor 1-alpha (eEF1A), eEF1A1 and eEF1A2, were found to be possible binding partners of FLCN. eEF1A is involved in the transport of RNA to the ribosome.[72] Furthermore, eEF1A1 plays a critical role in the nuclear export of proteins[73] and eEF1A2 plays a role in vesicular trafficking through phosphatidylinositol signalling.[74, 75] In addition, alpha tubulin, together with beta tubulin, is the main constituent of microtubules, which can be regarded as tracks for transportation within the cell (as reviewed[76]). Thus, an increasing body of evidence suggests a role for FLCN in (energy related) transport and localisation of other molecules. This is in line with the function of FLCN's yeast ortholog LST7 (lethal with sec-thirteen protein 7), which has a role in amino acid transport by shuttling general amino-acid permease GAP1 from the Golgi apparatus to the plasma membrane in response to nitrogen, which is an essential component of amino acids and thereby functions in energy related transport.[77, 78]

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

From recent findings, FLCN may orchestrate transport by affecting GTPases as a GEF and/or GAP, or facilitating GAP/GEF reactions. Not only was FLCN suggested to be GAP for the small GTPase RabA/B[24] and a GEF for RabC/D[25] (as already described in this chapter and shown in Figure 1A and B), FLCN's C-terminal structure is also similar to that of DENN (differentially expressed in normal cells and neoplasia) domain proteins.[77] DENN domain proteins are known to function as GEFs for Rab GTPases.[79] Accordingly, FLCN was suggested to be a GEF for Rab35 *in vitro*.[77] Rab GTPases are essential in vesicle membrane

transport, where they are activated by GEFs.[80, 81] The Rabs on a vesicle coats and organelle membranes determine where the vesicle is transported to and which effector proteins are recruited (as reviewed by Stenmark[82]). In line with FLCN's DENN domain having an essential function, three pathogenic *FLCN* mutations that were *in silico* analysed, are predicted to cause structural changes in its DENN domain.[83]

Furthermore, two independent studies show that FLCN interacts with PKP4[17, 84], which binds Rab11 and is involved in its subcellular localisation.[85] Rab11 is known to be hydrolysed by a GAP.[86] It is not yet known if FLCN functions as this GAP. PKP4 also regulates activation of the small GTPase RhoA, by interacting with its GEF Ect2.[87] FLCN affects Rho activity too, however both inhibition and activation were observed in FLCN deficient cells.[17, 84] FLCN could function as Rho GAP or facilitate either the Rho GAP or the GEF Ect2. Finally, eEF1A, the putative binding partners of FLCN that we identified (**chapter 5**), needs to be GTP loaded in order to deliver RNA to the ribosome (as reviewed[72]).

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

Recently, FLCN's role as a (scaffold for) GAP towards Rab GTPases was further established by Laviolette and colleagues, who showed that FLCN binds Rab7A and the GTPase activity of Rab7A increases when overexpressed together with FLCN. Moreover, FLCN was shown to bind Rab7B, Rab9A and Rab35.[88] Laviolette *et al.* relate the increased GTPase activity of Rab7a in the presence of FLCN to tumour growth in BHD syndrome, by showing that it affects EGFR signalling. EGFR is mutated or overexpressed in many types of cancer, where it increases proliferation and migration of cells (as reviewed[89]). EGFR signalling is largely regulated by endocytic recycling[89], in which Rab7A plays a role.[82] Consistently, FLCN knockout in FTC-133 cells was shown to increase EGFR signalling and EGFR overexpression was shown in BHD syndrome related kidney tumours versus healthy kidney tissue.[88] Another study showed EGF induced cell growth increased upon FLCN knock down.[90] From these observations, it is tempting to hypothesise that tumours in BHD syndrome might be caused by increased EGFR signalling and that, therefore, treatment with EGFR inhibitors

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

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

Future prospects

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

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

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

metabolism[94], which is undesirable when studying FLCN, as we hypothesise that FLCN may act in response to energy levels. On the other hand, primary kidney cells from BHD syndrome patients are an interesting model to create a second mutation (homozygous or compound heterozygous) in, using CRISPR/Cas9, to mimic the initiation of tumorigenesis in BHD syndrome; as loss of heterozygosity was shown to be a frequent event in BHD syndrome related renal tumours.[95] However, these cells have limited proliferative capacity and require immortalisation for continuous usage. In turn, this will cause additional mutations that may alter the behaviour of the cell line. To overcome this, patient derived induced pluripotent stem cells (iPSCs) are of interest, as they have unlimited proliferative capacity and can differentiate into any cell type.[96] Kidney differentiated iPSCs could be kept growing to study their natural course and CRISPR/Cas9 could be used to study the effect of a second hit mutation. Patient derived iPSCs with the FLCN mutation corrected could serve as a control with identical genetic background. All cell lines could be compared for readouts such as cilia formation, Wnt, EGFR and mTOR signalling. Any differences could then be confirmed by immunofluorescence on kidney and tumour tissue of patients, for involvement of the identified pathway in the pathogenesis of BHD syndrome. In addition, our TAPtag (see **chapter 5**) can be used in CRISPR/Cas9 manipulated cells to enable an unbiased search for new interactors of FLCN. CRISPR-Cas9 was previously used before to successfully add three hemagglutinin tags to the N-terminus of FLCN.[24] Analysing the pattern of protein-protein interactions (PPIs) of FLCN may give insight in the (many) cellular structures, functions and signalling routes that FLCN might be part of. Comparing the (quantity of) PPIs between wild type and mutant FLCN could further indicate which functions of FLCN are the most affected in BHD syndrome.

If the effect of aberrant FLCN on tumour formation is elucidated and key pathways are identified, further studies can focus on targets to stop, reverse or even prevent malignancies in BHD syndrome. Furthermore, BHD syndrome may be a good model for renal tumours in general, as the disorder can cause all types of renal cell carcinoma (RCC). Uncovering the function of FLCN may also give better understanding of the pathobiology of other RCCs. As an example, such discoveries have facilitated treatment in von Hippel Lindau (VHL) disease, a disorder that strongly predisposes to clear cell RCC. The *VHL* gene was found to be mutated or lost in most sporadic clear-cell RCCs as well. Further research showed *VHL* to be involved in hypoxic inducible factor mediated RCC growth, which can be antagonised by multi-targeted tyrosine kinase inhibitors. These drugs proved effective and are now used to treat metastatic renal cancer (as reviewed[97]). Similarly, the research presented in this thesis and the method established in **chapter 5** could contribute to a cascade of discoveries, eventually leading to treatment options for BHD syndrome and possibly for a broader range of renal tumours. Therefore, not only BHD syndrome patients, but also patients suffering from other types of kidney tumours may benefit from research into BHD syndrome.

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