Homozygous loss of BHD causes early embryonic lethality and kidney tumor development with activation of mTORC1 and mTORC2

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Germline mutations in the BHD/FLCN tumor suppressor gene predispose patients to develop renal tumors in the hamartoma syndrome, Birt-Hogg-Dubé (BHD). BHD encodes folliculin, a protein with unknown function that may interact with the energy- and nutrient-sensing AMPK-mTOR signaling pathways. To clarify BHD function in the mouse, we generated a BHD knockout mouse model. BHD homozygous null (BHD<sup>d/d</sup>) mice displayed early embryonic lethality at E5.5–E6.5, showing defects in the visceral endoderm. BHD heterozygous knockout (BHD<sup>d+</sup>) mice appeared normal at birth but developed kidney cysts and solid tumors as they aged (median kidney-lesion-free survival = 23 months, median tumor-free survival = 25 months). As observed in human BHD kidney tumors, three different histologic types of kidney tumors developed in BHD<sup>d+</sup> mice including oncocytic hybrid, oncocytoma, and clear cell with concomitant loss of heterozygosity (LOH), supporting a tumor suppressor function for BHD in the mouse. The PI3K-AKT pathway was activated in both human BHD renal tumors and kidney tumors in BHD<sup>d+</sup> mice. Interestingly, total AKT protein expression was elevated in kidney tumors compared to normal kidney tissue, but without increased levels of AKT mRNA, suggesting that AKT may be regulated by folliculin through post translational or post-transcriptional modification. Finally, BHD inactivation led to both mTORC1 and mTORC2 activation in kidney tumors from BHD<sup>d+</sup> mice and human BHD patients. These data support a role for PI3K-AKT pathway activation in kidney tumor formation caused by loss of BHD and suggest that inhibitors of both mTORC1 and mTORC2 may be effective as potential therapeutic agents for BHD-associated kidney cancer.

Birt-Hogg-Dubé syndrome | kidney cancer | mouse model | mTOR | tumor suppressor

Birt-Hogg-Dubé (BHD) syndrome is an inherited kidney cancer syndrome which predisposes patients to develop hair follicle tumors, lung cysts, spontaneous pneumothorax, and an increased risk of renal neoplasia (1–3). We previously identified germline mutations in the BHD (FLCN) gene in patients with BHD (4). About one-third of BHD patients develop bilateral multifocal renal tumors that are most frequently chromophobe renal tumors and renal oncocytic hybrid tumors with features of chromophobe renal carcinoma and renal oncocytoma (5). Somatic mutations in the wild-type copy of BHD and loss of heterozygosity at chromosome 17p11.2 have been identified in human BHD tumors, indicating that BHD is a classical tumor suppressor gene (6). The BHD protein folliculin (FLCN) is a 64-kDa protein with no known functional domains (4). We reported two FLCN binding proteins FNIP1 and FNIP2, which interact with 5′-AMP-activated protein kinase (AMPK), an important energy sensor in cells that negatively regulates mammalian target of rapamycin (mTOR), the master switch for cellular energy and nutrient sensing through interactions with the AMPK-mTOR signaling pathway. Mutations in several other tumor suppressor genes, including LKB1 (10), PTEN (11), and TSC1/2 (12), have been shown to lead to dysregulation of PI3K-AKT-mTOR signaling and to the development of other hamartoma syndromes. We and others previously reported the generation of a conditionally targeted BHD allele and kidney-directed BHD inactivation in the mouse using the cadherin16 (KSP)-Cre transgene (13, 14). Although BHD homozygous deletion in kidney epithelial cells was sufficient to cause uncontrolled cell proliferation and hyperplastic cell transformation, the kidney-targeted BHD-knockout mice lived only approximately 3 weeks and did not produce kidney tumors. A BHD heterozygous knockout mouse model that develops tumors with age will more accurately reflect tumor development in the human BHD patient and may be a better model for understanding how BHD inactivation leads to tumor initiation and progression. Here we report the analysis of an embryonic lethal phenotype that occurs in a BHD homozygous knockout mouse model and characterize and compare the kidney tumors that develop in a BHD heterozygous knockout mouse model with human BHD kidney tumors.

Results

Role of BHD during Early Embryogenesis. We have analyzed mouse BHD mRNA expression levels by qRT-PCR in wild-type embryos and adult tissues (Fig. S1). We detected consistent BHD mRNA expression from E8.5 to E12.5 with 4-fold elevation at E19 and high expression in adult heart, pancreas, and prostate with moderate expression in adult brain, kidney, liver, and lung. BHD mRNA expression was further analyzed during early embryogenesis by whole mount in situ hybridization (Fig. S2). BHD mRNA was expressed consistently throughout embryogenesis. At E5.5, BHD expression was restricted to extraembryonic tissues; however, by E6.5, BHD was expressed in both embryonic and extraembryonic tissues. We saw strong expression in certain tissues including neural ectoderm, headfold, and limb buds, but the signal was relatively weak in the surrounding endoderm and heart.

Next we evaluated BHD homozygous knockout (BHD<sup>d/d</sup>) embryos from intercrosses of BHD heterozygous knockout (BHD<sup>d+</sup>) mice. BHD<sup>d+</sup> mice appeared normal at birth and grew normally in utero, with no significant differences in growth by birth day 15 compared to wild-type controls. A high percentage (25%) of BHD<sup>d/d</sup> mice died at birth. A significant number of BHD<sup>d/d</sup> embryos survived to postnatal day (PND) 3, at which time survivors started to show macroscopic phenotypic differences compared to normal littersmates (Fig. 1A). BHD<sup>d/d</sup> mice had dilated lungs, a low birth weight, and died shortly after birth at a mean postnatal age of 6.5 days (PND 3–8; Fig. 1B).

The authors declare no conflict of interest.


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developed normally, but no BHD<sup>+</sup> mice were born in 75 neonates indicating embryonic lethality (Fig. 1A). No BHD<sup>+</sup> embryos were found at E9.5. BHD<sup>+</sup> embryos were found before E8.5 with lower frequency (9.3%, 15/161) than expected (25%).

There were many empty deciduas suggestive of early embryonic death and resorption (21.8%, 26/119 at E5.5 and E6.5). There were many isolated kidneys with occasional bleeding. There were no deaths and resorption (21.8%, 26/119 at E5.5 and E6.5). The median age of kidney-lesion-free survival for BHD<sup>+</sup> mice was 23 months (n = 65), compared with an undefined kidney-lesion-free survival for BHD<sup>+</sup> littermate controls (n = 28, P < 0.0001) (Fig. 2C).

The number of kidney lesions in BHD<sup>+</sup> mice between the ages of 20 and 25 months is shown in Fig. 2E (n = 35; no. cysts per animal, mean = 3.43, SD = 3.13; no. mixed lesions per animal, mean = 0.51, SD = 0.85; no. solid tumors per animal, mean = 0.51, SD = 0.82). Histological examination showed that kidney cysts that develop in BHD<sup>+</sup> mice were lined by hyperplastic cells with enlarged cytoplasm and nuclei (Fig. 3A). The cysts found in BHD<sup>+</sup> mice were lined by flat cyst cells characteristic of simple cysts, which are distinct from the hyperplastic cysts that develop in BHD<sup>+</sup> mice. Complex cysts, defined as cysts

![Figure 1](image1.png)

Fig. 1. Characterization of BHD<sup>+</sup> mouse embryo phenotype. (A) Embryos were isolated from BHD<sup>+</sup> intercrosses, observed under a dissection microscope and genotyped by PCR. Numbers in parentheses represent embryos with abnormal appearance, as shown in (B and D). Gross appearance of BHD<sup>+</sup> (B and D) or BHD<sup>+</sup> (C and E) embryos. H&E images of BHD<sup>+</sup> (F), BHD<sup>+</sup> (G), and BHD<sup>+</sup> (H and I) embryos at E6.5. Immunohistochemical staining of DAB2 was performed to see visceral endoderm of BHD<sup>+</sup> (L), and BHD<sup>+</sup> (K) embryos. Magnified images of visceral endoderm of BHD<sup>+</sup> (F’ and L) and BHD<sup>+</sup> (H’ and M) with H&E staining and DAB2 staining, respectively. Magnification: ×20 (F–I), ×40 (J and K), ×63 (F’ and H’), and ×100 (L and M)]. [Scale bar, 100 μm (F–I) and 50 μm (J and K).]
compared with adjacent analysis showed only very weak FLCN expression in the tumors. Loss of endogenous FLCN expression was also conferrent different histological features were observed in tumors, resembling clear cell (papillary projection. (Fig. 3). Loss of FLCN in mouse tumors, which developed in BHD mice, showed very low FLCN expression compared to normal kidney tissue. These results strongly support the premise that kidney tumors, which developed in the BHD mouse model, were produced as a consequence of loss of FLCN function.

Activation of the PI3K-AKT-mTOR Signaling Pathway in Kidney Tumors from BHDm/+ Mice and Human BHD Patients. Total AKT protein levels, AKT1, and AKT2 as well as total AKT, were dramatically elevated in kidney tumors compared with normal kidney tissue (Fig. 4A). Levels of AKT phosphorylation were also dramatically elevated at both the PKD1 phosphorylation site (Thr308) and the mTORC2 phosphorylation site (Ser473) (15) (Fig. 4A and B). AKT mRNA levels were measured by qRT-PCR in the same samples (Fig. 4C). Contrary to the Western blot results, mRNA levels of AKT1 and AKT2 were not significantly different between tumors and normal kidneys. Increased phosphorylation of downstream effectors of AKT signaling would support AKT activation in kidney tumors that developed in BHDm/+ mice. Indeed, p-GSK3, p-FOXO1, and p-FOXO3a were elevated in tumors compared with normal kidneys (Fig. 4 D–F). We also saw Cyclin D1 elevation, which might be a consequence of GSK3 phosphorylation (Fig. 4D). mTOR phosphorylation on serine 2448, which reflects mTORC1 activation (16), was higher in tumors than in normal kidneys. Furthermore, mTOR phosphorylation on serine 2481, indicating mTORC2 activation (17), was also higher in tumors compared with normal kidney tissue (Fig. 5A). Interestingly Rictor was more highly expressed in tumors from BHDm/+ mice, which may lead to mTORC2 activation and result in higher AKT phosphorylation on serine 473. Levels of phospho(p)-S6 kinase (Thr421/Ser424) and phospho S6 ribosomal protein (Ser240/244), readouts of mTORC1 activation, were higher in tumors than in normal kidney tissue, although total protein levels of S6K and S6R were also elevated (Fig. 5A). Consistent with the Western blot results, we were able to see stronger p-mTOR (Ser2448) and p-S6R (Ser240/244) staining in cells lining the cysts and in tumors from BHDm/+ mice when compared to adjacent normal kidney (Fig. 5 B–G).

Finally we evaluated these protein levels in human BHD tumors and normal kidneys. Western blotting and immunofluorescent staining showed higher levels of p-AKT(Ser473) in kidney tumors compared to normal kidney controls (Fig. 6 A, E, and F). We quantified p-AKT(Ser473), p-S6K(Thr421/Ser424), and p-S6R(Ser240/244) using a Meso Scale Discovery multiplex microtiter plate assay, a quantitative assay system using a combination of electrochemiluminescence detection and patterned arrays (Fig. 6 B–D). The protein levels of p-AKT (Ser473) were more than 30 times higher in human BHD tumors than in normal kidneys. Levels of p-S6K (Thr421/Ser424) protein and p-S6R protein were also higher in the BHD kidney tumors (Fig. 6 C and D). We compared the genotype and phenotype of the BHD patients whose kidney tumors were evaluated in this study (Fig. 6G). The kidney tumors were surgical specimens from three different female patients with different types of germline BHD mutations: missense, splicing defect and frameshift. All of the patients with different BHD mutations showed the classic triad of BHD phenotypic features—fibrofolliculomas, pulmonary cysts and renal tumors (Fig. 5 S). Importantly, the molecular phenotype was similar among the tumors derived from these three different types of BHD alterations, underscoring the importance of PI3K-AKT-mTOR activation for kidney tumorigenesis in BHD patients.

Discussion

The early embryonic lethality of BHD homozygous knockout mice supports an essential role for BHD in mouse development. Embryonic ectoderm-like cells of BHDf/f embryos did not form the proamniotic cavity or bilayered ectoderm structure. The visceral endoderm (VE) cell layer was disorganized with misaligned nuclei suggesting loss of polarity. Interestingly, VE cells displayed swollen cytoplasm with enlarged vacuoles. In addition
Fig. 4. Elevated total AKT and phosphorylated AKT protein in kidney tumors arising in BHD<sup>d</sup> mice. (A) Western blotting was performed on the protein lysates isolated from normal kidneys and kidney tumors. Both total AKT and phospho-AKT expressions were up-regulated in BHD<sup>d</sup> tumors. Isoforms of AKT, AKT1, and AKT2, were overexpressed in tumors. (B) Immunohistochemical staining showed highly expressed p-AKT (Ser-473) in kidney tumors consistent with Western blot results. (C) qRT-PCR was performed on total RNA isolated from frozen tissue samples corresponding to (A). mRNA expression for AKT1 and AKT2 was not significantly different between tumors and normal kidneys. (D) Western blotting on tissue lysates corresponding to (A). AKT activation resulted in phosphorylation of downstream effectors of the AKT pathway in tumors. (E and F) Immunofluorescent staining of p-GSK3α/β was consistent with Western blotting in (D). Magnification; ×63. (Scale bar, 20 μm.)

Kidney tumor development in BHD<sup>d</sup> mice mimics the kidney tumor phenotype found in humans with BHD. Previously we reported a kidney-targeted conditional BHD knockout mouse model, which produced enlarged highly cystic kidneys displaying profoundly increased cell proliferation and hyperplastic morphologic changes. However, the animals died of renal failure at 21 days of life and therefore did not live long enough to develop kidney tumors. Furthermore, additional genetic and/or epigenetic events may be required for tumor formation. BHD<sup>d</sup> mice developed hyperplastic kidney cysts, complex cysts and solid tumors at different frequencies and with different latency periods, providing evidence to support a multistep process in BHD-associated kidney carcinogenesis. Loss of both copies of BHD in all analyzed tumors arising in BHD<sup>d</sup> mice supports BHD inactivation as the initiating step for kidney tumorogenesis in BHD.

Although other naturally-occurring animal models for BHD have been described (20, 21), they may harbor additional genetic changes that could confound studies of the functional consequences of BHD inactivation. The genetically engineered mouse model in this report provides a “clean” system with which to pursue FLCN functional studies. Hartman et al. (22) has described another BHD heterozygously targeted mouse model in which a gene trap cassette inserted in intron 8 encodes a FLCN-βgeo fusion protein. These investigators reported a lower incidence of kidney tumors than in our BHD<sup>d</sup> model, (three tumors in 31 BHD<sup>d</sup/><sup>−/−</sup> mice and 0 tumors in 15 wild-type mice), which may possibly be due to a shorter observation period (17 vs. 30 months). Reduced phospho-S6R (Ser235/236) immunostaining of paraffin-embedded tumors led these investigators to conclude that mTOR activity was suppressed in kidney tumors that developed in Bhd<sup>d</sup/><sup>−/−</sup> mice. The inconsistencies between the results of Hartman et al. and our results may be due to differences in gene targeting strategy. In our model, mRNA transcribed from the BHD<sup>d</sup> allele creates a frameshift resulting in a premature termination codon at the beginning of exon 8 and will be degraded by nonsense mediated decay (NMD). In fact, no truncated forms of FLCN protein were detected by Western blotting in our BHD<sup>d</sup>/<sup>−/−</sup> mouse kidneys. However, although Hartman et al. did not evaluate the presence of the FLCN-βgeo fusion protein, successful selection of targeted embryonic stem cells by G418 screening would necessitate the expression of a FLCN-βgeo fusion protein that retains the N-terminal half of FLCN in the embryonic stem cells and, presumably, also in the Bhd<sup>d</sup> mouse tumors. Since Hartman et al. did not confirm LOH of BHD, it is not clear if the kidney tumors that developed in the Bhd<sup>d</sup> mice were caused by homozygous inactivation of
**BHD** or by another molecular mechanism. If the FLCN-βgeo fusion protein has partial function it could down-regulate mTOR and explain the discrepancy between the mTOR activation seen in tumors that developed in our **BHD** mice and the reduced mTOR activity reported by Hartman in tumors that developed in the gene trap mouse model for **BHD**. One additional difference between the two studies was the method of tissue preservation for immunostaining, which may affect the antigenicity of certain proteins. In this report freshly frozen tissues in OCT compound were analyzed, whereas paraffin embedded tissues were used in the Hartman’s study.

We found PI3K-AKT-mTOR pathway activation in both kidney tumors from **BHD** mice and human **BHD** tumors, consistent with the kidney-targeted **BHD** knockout kidney results, supporting a role for PI3K-AKT-mTOR pathway in both **BHD** null kidney tumorigenesis and in the development of hyperplastic kidney cysts. Interestingly, total AKT protein levels were elevated in those tumors without changes in AKT mRNA levels. Therefore FLCN may be involved in regulation of total AKT protein levels through post-translational or post transcriptional modification. It is possible that elevated total AKT could result in higher AKT activation as indicated by elevated AKT phosphorylation (Thr308/Ser473). AKT downstream target molecules were also highly phosphorylated in tumors from **BHD** mice, supporting the possibility that elevated total AKT may be driving the activation of the AKT pathway.

Downstream of AKT, we found mTORC1 was activated in tumors from **BHD** mice. The high levels of p-AKT (Ser473) and p-S6K (Thr421/Ser424) suggest mTORC2 activation. As expected, we saw elevated levels of p-mTOR on Ser2481, a readout of mTORC2 activity (17), in the mouse kidney tumors. Additionally we observed elevated Rictor expression in tumors from **BHD** mice. mTORC2 activity is regulated by Rictor expression level (23), and Facchinetti et al. reported that mTORC2 phosphorylated AKT on the turn motif and stabilized AKT (24). Taken together, the high expression level of Rictor and subsequent activation of mTORC2 may be the primary mechanism by which AKT activation occurs in **BHD** null tumors. Previously we found that FLCN phosphorylation was partially blocked by rapamycin (7), suggesting that FLCN function may be regulated by mTOR. This may support a hypothesis whereby FLCN is on a negative feedback loop suppressing PI3K-AKT-mTOR signaling (Fig. S4).

Each kidney tumor from human **BHD** patients that was analyzed showed PI3K-AKT-mTOR activation, regardless of type of **BHD** mutation. Most frameshift or splicing defect mutations found in **BHD** patients are predicted to produce aberrant mRNAs that would be degraded by nonsense mediated decay. Two different missense mutations have been reported to date; however, the physiological significance of these mutations has yet to be determined. Tumor 1 with the H255Y missense mutation (Fig. 6 A and G) showed the same molecular phenotype as the tumors from patients with germline frameshift or splicing mutations, suggesting loss of function of this mutant FLCN protein. Our data are consistent with a common consequence of **BHD** inactivation in mouse and man, regardless of **BHD** mutation type, and support activation of AKT signaling as an important mechanism driving kidney tumorigenesis in **BHD** syndrome. Rapamycin does not inhibit mTORC2 effectively, which may explain its partial effect on kidney-targeted **BHD** knockout mice (13). Taken together, data generated from our two **BHD** mouse models suggest that mTORC2 as well as mTORC1 inhibition may be needed for the development of an effective form of therapy for patients with **BHD**-associated kidney cancer.

**Materials and Methods**

**Development of **BHD** Knockout Mouse Model.** The **BHD** heterozygous knockout mice were generated as previously described (13). Details of the targeting strategy are described in the **SI Methods** (Fig. S5). All mice which were used in these experiments were housed in the National Cancer Institute (NCI)-Frederick animal facility according to the NCI-Frederick Animal Care and Use Committee guidelines.

**PCR-Based **BHD** Genotyping.** Mouse genomic DNA was isolated from tails (weaned neonates), yolk sacs (E8.5 or later), and whole embryos (E7.5 or earlier). Primers and details are in the **SI Methods**.

**Quantitative Real Time-PCR.** The qRT-PCR for **BHD**, AKT1, and AKT2 was performed as described in the **SI Methods**.
Whole Mount in Situ Hybridization. The embryos were collected from wild-type C57BL/6 mice intercrossed at different stages of gestation and processed for whole mount in situ hybridization as previously described (25). Negative staining was confirmed using a sense probe with wild-type embryos. Probe information is in the SI Methods.

Histological and Immunohistochemical Analysis. Embryo sections were stained with hematoxylin and eosin (H&E) or with DAB2 antibody (BD Bioscience) for immunohistochemical evaluation performed as previously described (26). The slides were read by at least three persons, including two pathologists (M.J.M. and D.C.H.).

Western Blotting and Antibodies. Immunoblotting was performed as described in the SI Methods.

Tissue Genotyping by Southern Blotting. Nonradioactive Southern blotting was performed with DIG OMNI System for PCR Probes according to the manufacturer's protocol (Roche). Probe information is in the SI Methods.

Endogenous FLCN Detection by Duolink System. Duolink in situ PLA was performed per manufacturer's instruction (Olink Biosciences). For further information, see the SI Methods.


**Human Sample Preparation and MSD Analysis.** Renal tumors were obtained from BHD patients surgically treated at the Urologic Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD with patient permission under a National Institutes of Health Institutional Review Board (IRB)-approved protocol #97-C-0147. All patients signed informed consent. MSD (Meso Scale Discovery) 96-well multipot AKT signaling pathway (phospho-AKT [Ser-473]/total GSK3β/Phospho-S6K [Thr-421/Ser-424]), and phospho-S6R (Ser-240/244) assays were carried out according to the manufacturer's protocol. For further information, see the SI Methods.

**Immunofluorescence Imaging of the AKT-mTOR Pathway.** Immunostaining for p-AKT, p-mTOR, and p-S6R was performed on frozen sections as described in the SI Methods.

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