The Birt–Hogg–Dubé tumor suppressor Folliculin negatively regulates ribosomal RNA synthesis

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Birt–Hogg–Dubé syndrome (BHD) is a human cancer disorder caused by mutations in the tumor suppressor gene *Folliculin* (*FLCN*) with unknown biological functions. Here, we show that the *Drosophila* homolog of FLCN, dFLCN (a.k.a. *dBHD*) localizes to the nucleolus and physically interacts with the 19S proteasomal ATPase, Rpt4, a nucleolar resident and known regulator of rRNA transcription. Downregulation of dFLCN resulted in an increase in nucleolar volume and upregulation of rRNA synthesis, whereas dFLCN overexpression reduced rRNA transcription and counteracted the effects of Rpt4 on rRNA production by preventing the association of Rpt4 with the rDNA locus. We further show that human FLCN exhibited evolutionarily conserved function and that Rpt4 knockdown inhibits the growth of FLCN-deficient human renal cancer cells in mouse xenografts. Our study suggests that FLCN functions as a tumor suppressor by negatively regulating rRNA synthesis.

INTRODUCTION

Birt–Hogg–Dubé syndrome (BHD) is an autosomally dominant human cancer disorder that predisposes patients to the development of benign fibrofolliculomas (neoplasms of the hair follicle), lung cysts that may cause pneumothorax and various subtypes of renal carcinoma (1,2). The disease is associated with germline frameshift and nonsense mutations in the *Folliculin* (*FLCN*; a.k.a. *BHD*) gene that comprises 14 exons (3–5) and is predicted to encode a 579 amino acid protein, FLCN (5). Although it displays no significant homology to any known human proteins, FLCN is widely conserved across species, suggesting a crucial biological function (6). In normal human tissues, *FLCN* mRNA expression was observed in various organs and cell types, including the stromal cells and type 1 pneumocytes of the lung, the distal nephron of the kidney, the macrophages and lymphocytes in the tonsils and spleen and the skin and its appendages (7). Renal tumors from BHD patients, however, showed reduced expression of *FLCN* (7), consistent with reports of somatic inactivation of the wild-type *FLCN* allele or loss-of-heterozygosity

that occurs at a high frequency in BHD syndrome patients, suggesting that FLCN functions as a tumor suppressor (6).

Recently, several genetic and biochemical studies have attempted to understand the molecular function of FLCN. *In vitro* studies in mammalian cell culture have demonstrated an interaction between FLCN and adenosine monophosphate-activated protein kinase (5'AMPK), mediated by two novel proteins, FLCN-interacting protein-1 and -2 (FNIP1 and FNIP2) (8–10). Because AMPK is a negative regulator of the mammalian target of rapamycin (mTOR) and a key energy-sensing molecule in the cell (11–14), the interaction between FLCN with FNIP1/2 and AMPK is indicative of FLCN's role in nutrient/energy sensing through the AMPK/mTOR signal transduction pathways. However, some other studies have reported opposing effects of *FLCN*'s downregulation on phosphorylated ribosomal protein S6 (p-S6) signaling cascade (15). Induction of siRNA in human cell lines resulting in transient downregulation of *FLCN* produced a reduction in p-S6 (10,15). Renal cysts in mice heterozygous for *FLCN* also showed reduced p-S6 (15). In contrast, generation of kidney-specific homozygous knockout of *FLCN* mice, however, led

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to the development of polycystic kidneys and increased p-S6 (16,17). On the other hand, deletion of the yeast homolog of *FLCN* in *Schizosaccharomyces pombe* demonstrated that *FLCN*, believed to act in parallel with AMPK, had a completely opposite biochemical function to the AMPK target tuberous sclerosis complex (TSC2) in amino acid homeostasis (18). These findings were especially surprising because both BHD syndrome and TSC disorder have highly similar disease symptomatology, i.e. skin lesions, cysts of the lung and kidney and renal neoplasia. Finally, a recent genetic study has shown that *Drosophila* *FLCN* (*dFLCN*) plays a role in regulating male germline stem cell renewal in the fly testis and functions downstream of the JAK/STAT and Dpp signaling pathways (19). These studies suggest complicated and cell-type- or tissue-specific functions for *FLCN* in different biological contexts.

The Ras oncoproteins are membrane-associated molecular switches that function to transduce extracellular signals to a variety of intracellular response mechanisms. Activating mutations in *ras* genes are present in 15% of all cancers and almost 30% of metastatic human cancers (20). The Ras proto-oncoproteins are encoded by three genes—*Ki-ras*, *Ha-ras* and *N-Ras* (20). Activation of the *Ki-ras* oncogene has been implicated in colorectal cancer carcinogenesis (21). Although colorectal neoplasia was not initially reported to be part of the BHD syndrome phenotype, some recent studies suggest that *FLCN* inactivation may also contribute to colorectal tumorigenesis (22). Ras effectors such as the Raf Ser/Thr protein kinases (23,24) function as mitogen-activated protein kinases (MAPK) (MAP3K) directly activating MAPK kinases that lie upstream of the ERK group of MAPKs (25). Interestingly, increases in Raf, MEK and ERK activities have been observed in cell lines deficient in *FLCN* (16). In addition, *FLCN* and TSC disorders share similar skin lesion symptoms. These observations raise the possibility that *FLCN* may interact with the Ras/Raf signal transduction pathway in causing human diseases.

Changes in cell growth and proliferation depend heavily on the rapid and efficient transcription of rRNA genes (rDNA) in the nucleolus by RNA polymerase I (Pol I) and associated transcription factors (26). Recent studies have provided compelling biochemical evidence indicating that numerous tumor suppressors and proto-oncogenes can regulate rRNA synthesis, the first and rate-limiting step in ribosome biogenesis (27). Because the misexpression and deregulation of components of the protein synthesis machinery in cancer cells have been extensively documented (28), together with the knowledge that ribosome biogenesis is tightly coupled to proper cell-cycle progression (29,30), it is now becoming evident that disruption of key steps in the process of ribosome production can contribute to cellular transformation.

To understand the molecular function of *FLCN*, we studied its *Drosophila* homolog *dFLCN* (19). We found that *dFLCN* genetically interacts with oncogenic Ras using the *Drosophila* eye system. Furthermore, we identified Rpt4 as a novel interactor of *dFLCN*. Recent studies have established that Rpt4 is present at the rDNA promoter and coding regions and plays an essential role in rRNA gene transcription (31). Here, we examine the consequences of *FLCN* overexpression or knock-down on pre-rRNA synthesis and ribosome production and

find that *FLCN* controls rRNA synthesis by modulating Rpt4 binding to the rDNA locus. Finally, we characterize the interaction between *FLCN* and Rpt4 in an *in vitro* mammalian system using a cell line (UOK-257) derived from the kidney tumor of a 46-year-old BHD patient. We demonstrate that knocking down Rpt4 in these cells inhibits tumor growth in a mouse xenograft model. Our results suggest that the tumor suppressor *FLCN* might normally limit rRNA synthesis by antagonizing Rpt4 function.

RESULTS

FLCN is involved in *Ras*^{V12}-induced oncogenic growth

In the process of studying tumorigenesis caused by oncogenic Ras in *Drosophila*, we identified the fly homolog of human *FLCN*, a tumor suppressor gene mutated in renal carcinoma. In this study, we utilized a constitutively active form of the *Drosophila* *Ras1* gene (*Ras*^{V12}). *Ras1* regulates cell growth and cell proliferation by modulating the MAPK signaling cascade during eye development (32,33). Expression of *Ras1*^{V12} with an eye-specific driver *eyeless-Gal4* during *Drosophila* imaginal disk development induces cellular overproliferation and hyperplastic growth of the eye and causes lethality (34,35). Utilizing a set of chromosomal deficiency lines (FlyBase), we sought to identify genes that in half dose would enhance or suppress the *Ras1*^{V12}-induced eye phenotype (see Materials and Methods). A chromosomal deficiency, *Df(3L)Exel6111*, that deletes *dFLCN*; a.k.a. *BHD* dominantly enhanced Ras-induced tumorigenic phenotypes (Supplementary Material, Table S1). We found that expressing *dFLCN-RNAi* enhanced and overexpressing *dFLCN* suppressed the *Ras1*^{V12}-induced eye overgrowth phenotype.

Identification of a novel *FLCN*-interacting partner, Rpt4

Although widely conserved among species, *FLCN* does not contain any known functional domains (5), and thus its molecular function has remained unclear. Other than a chromosomal deficiency, which deletes *dFLCN* and a few other genes (FlyBase), and the RNAi lines, mutations in *Drosophila* *dFLCN* are not available. To understand *FLCN*'s molecular role, we sought to identify its interacting partners. To this end, we used the full-length *dFLCN* as a bait to isolate binding partners expressed from a *Drosophila* cDNA library using the Cytotrap^{TM} Yeast Two-Hybrid System (36,37). This system detects protein–protein interactions that occur in the cytoplasm (Fig. 1A), significantly reducing the number of false positives associated with the conventional yeast 2-hybrid assays that rely on nuclear interaction ((38); see Materials and Methods). This screen identified four putative interactors of *dFLCN*, one of which was the 26S proteasome component and ATPase, Rpt4 (Fig. 1B; Supplementary Material, Table S2).

We confirmed the physical interaction between *dFLCN* and Rpt4 by coimmunoprecipitation experiments using protein extracts from HEK-293T cells cotransfected with Rpt4-Flag and *dFLCN*-HA. Using an anti-HA antibody to immunoprecipitate *dFLCN*-HA-associated proteins, we detected Rpt4-Flag

Figure 1. dFLCN and Rpt4 directly interact and associate with each other (A) Two-hybrid assay. The Stratagene Cytotrap system was used for two-hybrid (Y2H) screening of interacting partners of dFLCN expressed from a *Drosophila* cDNA library. Interaction with this 'prey' library, targeted and anchored to the plasma membrane via a myristoylation moiety, recruits the 'bait' to the plasma membrane, restoring a deficient SOS/cdc25 pathway and allowing survival of the yeast at elevated temperatures. (B) dFLCN and Rpt4 interact in a two-hybrid system. To identify interacting partners of dFLCN, the full-length hSos-dFLCN (bait) plasmid was cotransformed with a (prey) *Drosophila* cDNA library into temperature-sensitive yeast and positive Y2H interactions were detected by growth on galactose media at a permissive temperature of 37°C. Representative screening in the cytotrap system shows restoration of growth at elevated temperature (37°C) on galactose media via positive protein–protein interaction. This screen identified a putative interaction between dFLCN and Rpt4. (C) dFLCN interacts with Rpt4 *in vitro*. dFLCN-HA and Rpt4-Flag were transfected into HEK-293T cells and detected by anti-Flag (Panel 2) and anti-HA (Panel 3) immunoblotting. Cell lysates were immunoprecipitated using an anti-HA antibody (Panel 1), and Rpt4 bound to dFLCN was detected by immunoblotting with an anti-Flag antibody (Panel 4). Total protein loading was analyzed using anti-tubulin (Panel 4). (D) dFLCN and Rpt4 colocalize in the nucleus. dFLCN-HA and Rpt4-Flag were cotransfected into S2 cells, followed by fixation and immunostaining using indicated anti-sera anti-HA (left panel) and anti-Flag (right panel). Visualization performed using confocal microscopy showed overlapping staining pattern of both proteins (Supplementary Material, Fig. S1A). (E) dFLCN-HA localizes to the nucleolus. HA-tagged dFLCN was transfected into S2 cells. Immunostaining was performed using antibodies against HA and Fibrillarin, the nucleolar marker. Note that the epitope-tagged dFLCN colocalizes with Fibrillarin (see Supplementary Material, Fig. S1B for overlay in color). (F) Repression of dFLCN increases nucleolar size. The *tubulin80-Gal4* driver was utilized to induce dFLCN-RNAi in two independent lines and a *white-RNAi* (targeting the *white* gene) as control RNAi. The *w¹¹¹⁸* strain was used as wild-type control. Salivary glands from these animals were immunostained using anti-Fibrillarin and were visualized using confocal microscopy. Representative images are shown. Note that tissue sections from dFLCN-RNAi larvae showed a multilobed nucleolus (middle) when compared with wild-type control (left), and that this phenotype was neutralized when dFLCN was overexpressed (right). (G) Repression of dFLCN produces an increase in nucleolar volume. Induction of dFLCN-RNAi in salivary gland cells resulted in increased nucleolar volume when compared with wild-type and dFLCN-HA cells. An average of 10 cells in 5 different salivary glands were counted for each genotype. Average nucleolar volume was calculated by taking the ratio of the area stained with fibrillarin, to the total nuclear area. Student's *t*-test was used to calculate *P*-values when compared with wild-type control (*w¹¹¹⁸*). dFLCNi-1 and dFLCNi-2 denote two independent RNAi lines. Significant values are indicated by *P*-values.

in the immunocomplex (Fig. 1C). Moreover, we immunostained *Drosophila Schneider* (S2) cells expressing transgenes and observed that Rpt4-Flag and dFLCN-HA were colocalized

in the nucleus (Fig. 1D; Supplementary Material, Fig. S1A). Collectively, these findings demonstrate that dFLCN and Rpt4 interact and physically associate with each other.

Figure 2. Changes in *dFLCN* and *Rpt4* expression alter pre-rRNA levels and affect animal growth. (A) Schematic of a typical *Drosophila* rRNA gene locus. The 5.8S rRNA is separated from the 18S and 28S rRNA genes by internal transcribed spacers 1 and 2 (ITS1, ITS2) respectively. The 5' ETS is included in the first rRNA precursor. (B) *Rpt4* expression directly affects rRNA synthesis. Total RNA was isolated from equal number of third-instar larvae of wild type, *Rpt4*^{+/-} and *UAS-Rpt4*/+ genotypes. QPCR, using primers spanning the 5'ETS of the *Drosophila* rDNA locus, was used to measure pre-rRNA synthesis. Pre-rRNA levels were decreased in *Rpt4*^{+/-} flies, whereas flies overexpressing *Rpt4* animals had 3-fold higher levels of pre-rRNA when compared with wild-type flies. (C) Reduced *dFLCN* expression promotes rRNA synthesis. *dFLCN*-RNAi and *dFLCN*-HA expression were driven using the *tubulin80-Gal4* promoter. Total RNA was isolated from equal number of third-instar larvae of wild type, *dFLCN*-RNAi (1 & 2) and *dFLCN*-HA genotypes. Pre-rRNA levels were 4–7-fold higher in both *dFLCN*-RNAi strains when compared with wild type, whereas *dFLCN*-HA overexpression produced no significant change in the amount of pre-rRNA. (D) *Rpt4*-regulated changes in rRNA synthesis result in a concomitant decrease in body size of *Rpt4*^{+/-} and an increase in *tubulin80-Gal4*-driven *UAS-Rpt4*/+ animals. Student's *t*-test was used to calculate *P*-values compared to *w[1118]* used as control. (E) Greater rRNA synthesis detected in the *dFLCN*-RNAi lines was phenotypically manifested in higher body weight in flies raised at 29°C. Student's *t*-test was used to calculate *P*-values compared to *w[1118]* used as control. (F) *dFLCN* inhibits *Rpt4* binding to the rDNA locus. S2 cells were transfected with an insect cell-specific green fluorescent

dFLCN localizes to the nucleolus and is important for nucleolar size

Because epitope-tagged dFLCN and Rpt4 were colocalized in a region of the nucleus that was reminiscent of the nucleolus (Fig. 1D; Supplementary Material, Fig. S1A), we further investigated the subcellular localization of dFLCN. By coimmunostaining with the nucleolar marker Fibrillarin, we demonstrated that dFLCN localizes to the nucleolar subcompartment (Fig. 1E; Supplementary Material, Fig. S1B). Although dFLCN has been reported to localize in the cytoplasm (8,19) or in both nuclear and cytoplasmic compartments (9), our finding is consistent with reports of yeast Rpt4 residing almost exclusively in the nucleus, throughout the cell cycle (39) and the presence of proteasomes in the nucleolus (40,41).

Next, we investigated the functional significance of dFLCN's localization by examining nucleolar morphology in larval salivary glands that contain large polyploid cells with easily identifiable nucleoli. In contrast to wild-type polytene cells and cells expressing control RNAi, which contained a single round nucleolus (Fig. 1F), we observed that salivary glands from larvae expressing any of the two independent *dFLCN-RNAi* lines had multiple-lobed nucleoli (Fig. 1F, middle), with increased total nucleolar volume (Fig. 1G). The efficiency of our respective RNAi fly strains was confirmed by real-time PCR (RT-PCR) and western blot analysis (Supplementary Material, Fig. S2). The effects of the *dFLCN RNAi* lines on nucleolar volume were neutralized by overexpressing dFLCN-HA (Fig. 1F and G), suggesting that the RNAi-induced defects were not due to off-target effects.

Modulation of pre-rRNA levels and animal growth

Although Rpt4 is an essential component of the 26S proteasome responsible for protein degradation, a direct role for Rpt4 in rRNA transcription has been reported (31,42). And, because dFLCN localizes to the fibrillar centers—the sites of transcription and processing of rRNA (43), we next explored the possibility that dFLCN may be involved in regulating aspects of rRNA synthesis or ribosome processing.

Because Rpt4 directly regulating rRNA transcription was previously shown in mammalian cells (31), we first examined whether changes in *Rpt4* expression could alter pre-rRNA levels in *Drosophila* as well, using quantitative RT-PCR (qPCR) with probes to the *Drosophila* 5' external transcribed spacer (ETS) region (Fig. 2A). Indeed, flies heterozygous for an *Rpt4* loss-of-function allele (*Rpt4*^{G0345}) exhibited an almost 2-fold decrease, whereas overexpression of an *Rpt4* transgene resulted in a 6-fold increase in pre-rRNA levels (Fig. 2B). An increase in pre-rRNA levels was confirmed by *in situ* hybridization experiment (Supplementary Material, Fig. S3). These findings were consistent with results from a previous study of Rpt4, where treatment with proteasomal

inhibitor MG132 suppressed pre-rRNA production in yeast, human and mouse cells and diminished Rpt4 occupancy at the rDNA promoter and coding regions in NIH3T3 cells (31). Consistent with the changes in pre-rRNA levels, flies overexpressing *Rpt4* exceeded their wild-type counterparts in both body weight and wing size, whereas *Rpt4*^{+/-} animals weighed less (Fig. 2D) and had smaller wings than control animals (Supplementary Material, Fig. S4A).

To investigate if dFLCN plays a role in controlling rRNA transcription, we examined pre-rRNA synthesis in flies overexpressing *dFLCN-RNAi* or *dFLCN-HA* and compared with control flies. Ubiquitous expression of *dFLCN-RNAi* and *dFLCN-HA* using the *tubulin80-Gal4* driver resulted in a 3–5-fold increase in pre-rRNA levels in both *dFLCN-RNAi* lines, whereas the amount of pre-rRNA in animals overexpressing *dFLCN-HA* was marginally decreased when compared with wild-type flies, as measured by qPCR (Fig. 2C) and *in situ* hybridization (Supplementary Material, Fig. S3). *dFLCN* transgene-induced changes in pre-rRNA synthesis were also manifested in the growth of the animals. Transgenic flies expressing *dFLCN-RNAi* weighed more (Fig. 2E) and had larger wings than wild-type animals (Supplementary Material, Fig. S4A), although *dFLCN* overexpression did not show significant changes in body weight or wing size (Fig. 2E, Supplementary Material, Fig. S4B), consistent with their rRNA levels. These results are consistent with a role for dFLCN in regulating rRNA transcription.

Overexpression of dFLCN can inhibit Rpt4 binding to the rDNA locus

It has been reported that Rpt4 specifically regulates rRNA transcription via direct binding to the rDNA promoter and coding regions and recruiting the transcription machinery (31). Because loss of *dFLCN* had similar effects to *Rpt4* overexpression on rRNA levels and because dFLCN and Rpt4 physically interact, we tested the possibility that dFLCN might affect pre-rRNA levels through antagonizing Rpt4 function. To test this idea, we conducted chromatin immunoprecipitation (ChIP) analysis using *Drosophila* S2 cells that were transfected with *Rpt4-Flag*, both *Rpt4-Flag* and *dFLCN-HA*, or the control vector. Because a previous study reported binding of Rpt4 to both the promoter and coding regions of the rDNA locus in NIH3T3 cells (31) without underscoring any specific binding sites, we used several primer pairs that spanned the *Drosophila* rDNA coding and promoter regions (Fig. 2A). We found that, in the presence of overexpressed dFLCN-HA, Rpt4 occupancy at the promoter and coding regions was dramatically reduced (Fig. 2F). As a negative control, Rpt4 showed minimal binding to the *rp49* promoter, and this binding was not affected by expression of dFLCN-HA and or its lack thereof (Fig. 2F).

To investigate the effects of *dFLCN* expression on Rpt4-stimulated rRNA transcription, we measured pre-rRNA

protein (GFP)-expressing vector, Rpt4-FLAG alone or cotransfected with both Rpt4-FLAG and dFLCN-HA plasmids. ChIP assay using a FLAG antibody demonstrates reduced *Rpt4* transcript levels at the rDNA promoter and coding regions when coexpressed with dFLCN, as measured by qPCR. (G) dFLCN regulates rRNA synthesis by inhibiting *Rpt4*. Total RNA was isolated from S2 cells, expressing GFP or Rpt4-FLAG alone or both Rpt4-FLAG and dFLCN-HA. Pre-rRNA levels were lower in cells expressing both *dFLCN* and *Rpt4* when compared with control cells with GFP only or Rpt4-FLAG, as measured by qPCR using primers to the *Drosophila* ETS region. *P*-values were calculated using Student's *t*-Test for paired samples.

levels in cells transfected with *Rpt4-Flag* in the presence or absence of *dFLCN-HA*. We observed an 1.5-fold decrease in pre-rRNA levels in cells expressing both Rpt4-FLAG and dFLCN-HA when compared with cells that only expressed Rpt4-Flag (Fig. 2G). To ascertain whether this finding could be attributed to differential protein expression, we verified the protein levels in cells expressing either Rpt4-Flag or dFLCN-HA or both by western blot analysis (Supplementary Material, Fig. S5A and B) and observed relatively equal levels of both species in the doubly transfected cells, thus confirming the validity of pre-rRNA suppression that was observed on induction of dFLCN-HA. Taken together, these results suggest that dFLCN plays a role in regulating rRNA transcription by inhibiting Rpt4 association with the rDNA locus.

Ribosomal RNA synthesis is important for *Ras*^{V12}-induced oncogenic growth

We identified *dFLCN* in a genetic survey of modifiers of *Ras*^{V12}-induced overproliferation phenotype (Supplementary Material, Table S1). To confirm the genetic interaction between *dFLCN* and *Ras*^{V12}, we further examined whether loss or overexpression of *dFLCN* could suppress or enhance *Ras*^{V12}-induced tumor phenotype. Indeed, expression of *dFLCN-RNAi* enhanced *Ras*^{V12}-induced tumorigenicity as manifested by the severity of hyperplasticity of the eye (Fig. 3A) and decreased fly survival rate (Fig. 3B) in flies heterozygous for *dFLCN-RNAi* and *Ras*^{V12}. Conversely, the induction of *dFLCN-HA* expression was able to partially rescue the *Ras*^{V12}-induced overgrowth phenotype (Fig. 3A), albeit unable to significantly promote animal survival (Fig. 3B). These results suggest that dFLCN counteracts *Ras*^{V12}-induced hyperplastic growth, although does not mediate all the effects of *Ras*^{V12} (such as lethality).

To further understand the effects of dFLCN on *Ras*^{V12}-induced overgrowth, we investigated the role of dFLCN in *Ras*^{V12}-mediated cell overproliferation in the developing eye. As demonstrated by the number of cells positively stained for the mitotic marker phospho-histone H3 (pH3) in the eye imaginal disks, *Ras*^{V12} expression drives cellular proliferation that was exacerbated by the loss of *dFLCN* and restored to almost wild-type levels when *dFLCN* was overexpressed (Fig. 3C and D), suggesting that dFLCN is essential for mediating cell proliferation in the context of oncogenic *Ras*^{V12}.

Because increased rRNA synthesis and ribosome biogenesis are widely recognized features of transformed cells (27,44), we suspected that changes in the levels of Rpt4, which is known to directly bind to the rDNA locus and regulate rRNA synthesis, could influence the *Ras*^{V12}-dependent eye phenotype. Indeed, heterozygosity of *Rpt4* significantly improved the viability of flies expressing *Ras*^{V12} (Fig. 4A). Consistent with this finding, overexpression of *Rpt4*, which elevates rRNA synthesis in adult flies (Fig. 2B), adversely affected fly survival in the *Ras*^{V12} genetic background (Fig. 4A).

To confirm that rRNA transcription is important for *Ras*^{V12}-mediated tumorigenesis, we examined a mutant allele of Rpl135 (Rpl135^{k16513}) that has reduced levels of rRNA (45) in the *Ras*^{V12} genetic background. Rpl135 encodes a large RNA Pol I subunit essential for rRNA transcription (46). We found that heterozygosity for Rpl135 (Rpl135^{k16513/+}) was able to suppress the lethality induced by oncogenic *Ras*^{V12} in the developing *Drosophila* eye and improve fly survival as well (Fig. 4B). Thus, rRNA synthesis indeed affects oncogenic growth, consistent with previous findings (27,44).

Collectively, these results indicate that dFLCN plays a role in negatively regulating rRNA synthesis and that loss of *dFLCN* promotes cell growth in both normal and neoplastic tissues, likely via increasing rRNA synthesis.

Human FLCN is localized in the nucleolus and interacts with Rpt4

We next investigated whether the function of FLCN as shown in *Drosophila* is conserved in mammals. We first carried out immunostaining in human cells, using antibodies raised against human FLCN (see Materials and Methods; Supplementary Material, Fig. S6). We found that, similar to dFLCN, human FLCN is enriched in the nucleolus in HeLa cells (Fig. 5A–D) and also in normal human skin and kidney tissue samples (Fig. 5E and F; Supplementary Material, Fig. S7). To further substantiate these results, first we examined subcellular distribution of FLCN in HeLa cells by fractionation studies. We found that FLCN was indeed mainly detected in the nuclear fraction (Fig. 5G). Further fractionation of the HeLa cell nucleus lysates showed that human FLCN was predominantly detected in the nucleolus fraction, although a small amount of FLCN was detected in the nucleoplasm (Fig. 5H). Second, we examined FLCN and Rpt4 interaction by coimmunoprecipitation. Using anti-FLCN to immunoprecipitate FLCN from HEK293T cell lysates (Fig. 5I),

Figure 3. Effects of dFLCN and Rpt4-directed rRNA synthesis on *Ras*^{V12}-induced tumorigenicity and fly viability (A and B). dFLCN acts as a tumor suppressor in the *Ras*^{V12} background. *Ras*^{V12} denotes expression of the *UAS-Ras*^{V12} transgene using the *eyeless-Gal4* driver at room temperature (21–22°C), which results in animal lethality and in hyperplastic or abnormal eye phenotypes in animals that eclosed. Transgene expression was induced by the same *eyeless-Gal4* driver in single copy. ‘+/+’ denotes wild-type control. (A) The left panel shows images of eyes of adult flies of the indicated genetic backgrounds, showing the range of phenotypes. The right panel shows percentage of each indicated phenotype in the indicated genetic backgrounds. Note that coexpression of *dFLCN-HA* resulted in a partial rescue of the overgrowth and eye deformation phenotypes, and that *dFLCN-RNAi* expression exacerbated the characteristic *Ras*^{V12} eye phenotype. (B) Changes in *dFLCN* expression affect *Ras*^{V12} induced lethality. Note that *dFLCN* knockdown significantly worsened *Ras*^{V12}-induced lethality. Overexpression of *dFLCN-HA* was unable to improve the dramatic lethality caused by *Ras*^{V12} expression. *** indicates *P* < 0.001 by Student’s *t*-Test. (C and D) Eye imaginal disks from transgenic animals under the control of the *eyelessGal4* driver were immunostained using pH3 antibodies and photographed with a confocal microscope. Note that induction of *dFLCN-RNAi* in an oncogenic *Ras*^{V12} background promoted cell proliferation resulting in massive overgrowth of the eye disk, whereas overexpression of *dFLCN-HA* restores mitotic cell population to almost wild-type levels, rescuing the hyperplasticity of the eye tissue. The number of pH3-positive (pH3+) cells in at least 10 imaginal eye disks was used to quantify the average number of pH3+ cells per imaginal disk for each genotype. *** indicates *P* < 0.001 by Student’s *t*-Test.

Figure 4. Changes in rRNA synthesis contribute to tumorigenicity. (A and B) Percentage of expected viability of the indicated genotypes was calculated by counting the number of flies eclosed and normalizing to control siblings in the same food vial. *P*-values indicate Student's *t*-Test. (A) Rpt4-regulated rRNA synthesis can affect fly viability. Note that coexpression of *Ras*^{V12} together with either half dose of *Rpt4* or UAS-*Rpt4* overexpression, previously shown to downregulate and upregulate rRNA synthesis respectively, concomitantly improved and worsened the viability of *eyeless-Gal4*-driven *Ras*^{V12} animals. (B) Reduced rRNA synthesis can improve *Ras*^{V12}-induced lethality. A mutant line, *Rpl135*^{K16513}, of the *Drosophila* Pol I complex, known to cause a marked decrease in rRNA synthesis, was crossed with recombinants expressing *Ras*^{V12} under the control of the eye-specific *eyeless-Gal4* promoter. Induction of the oncogenic *Ras*^{V12} produces significant fly lethality, which is partially suppressed in the *Rpl135*^{K16513/+} background.

we detected Rpt4 in the immunoprecipitates, suggesting that human FLCN and Rpt4 occur in a complex in cell culture. These results suggest that FLCN and Rpt4 interaction may be evolutionarily conserved between flies and humans.

Reexpressing *FLCN* in BHD patient-derived UOK-257 cells reduces rRNA synthesis

To determine if FLCN-dependent regulation of rRNA synthesis is also conserved in mammalian cells, we examined a cell line (UOK-257) derived from the renal tumors of a BHD patient who is deficient in *FLCN* expression (47). We infected UOK-257 with a human *FLCN*-expressing lentiviral vector to create an *FLCN*-supplemented cell line (UOK-257-*FLCN*) (Fig. 6B) and measured levels of pre-rRNA in both the *FLCN*-null (UOK-257) and *FLCN*-expressing cells (Fig. 6C). We observed a 2-fold decrease in pre-rRNA levels in *FLCN*-expressing cells when compared with *FLCN*-deficient (UOK-257) and mock infected cells (UOK-257-empty) (Fig. 6C). We also compared the ribosome distribution profiles of these particular cell lines and found that consistent with our measurement of pre-rRNA levels, *FLCN*-deficient cells had significantly higher amounts of the 40S, 60S ribosomal precursor subunits and greater levels of mature 80S ribosome when compared with the *FLCN*-supplemented and mock infected cells (Fig. 6D–G). Normal levels of the immature and mature ribosomal subunits in HEK-293T cells (Fig. 6G) confirmed that increased ribosomal biogenesis is a specific feature of UOK-257 cells and not a general characteristic of transformed cells. We also conducted FACS analysis on each of the respective cell lines to see if reexpressing *FLCN* in UOK-257 cells could alter the cell size (Supplementary Material, Fig. S8). Although the UOK-257-*FLCN* cells showed greater granularity, likely due to more cell debris, in the dot plot (Supplementary Material, Fig. S8A), the cell size remained

unchanged in all three UOK-257 cell lines. HEK293T cells, however, differed from the UOK-257 groups as expected. Analysis of cell count versus fluorescence intensity (Supplementary Material, Fig. S8B) also showed relatively similar cell sizes in all three UOK-257 cell lines (UOK-257, UOK-257-*FLCN* and UOK-257-Empty) when compared with HEK-293T cells. These findings are consistent with other published reports, where the expression of wild-type *FLCN* in UOK-257 cells was insufficient in restoring normal cell growth *in vitro*, but suppressed tumor formation when implanted in nude mice (48). On the whole, these results indicate that the mechanistic control of rRNA synthesis and ribosome production exerted by FLCN may also be conserved between flies and humans.

Knocking down Rpt4 expression in UOK-257 cells inhibits tumor growth in nude mice

Our results in flies and in cell culture systems suggest that inhibiting Rpt4 activity or its binding to the rDNA locus can suppress rRNA synthesis, which may contribute to tumor suppression. To determine whether this is also true *in vivo*, we established UOK-257 cell lines stably transfected with constructs expressing small hairpin RNA (shRNA) targeting Rpt4 or expressing a control shRNA (see Materials and Methods; Supplementary Material, Fig. S9). Interestingly, we found that knocking down Rpt4 had no effect on cell growth *in vitro* (Supplementary Material, Fig. S10A) and had small effects on anchorage-independent growth, as assay by colony formation in soft agar (Supplementary Material, Fig. S10B). We next injected UOK-257 cells expressing shRNA-Rpt4 constructs, scrambled-green fluorescent protein (GFP) vector and untransfected cells subcutaneously into flanks of 3-week-old nude/severe combined immunodeficiency mice and monitored the tumor growth for 2–3 weeks. We

Figure 5. Human FLCN is predominantly localized in the nucleolus. Immunofluorescence staining of HeLa cells with polyclonal antisera against human FLCN (A), anti-fibrillarin (B) and 4',6-diamidino-2-phenylindole, dihydrochloride (D). C is a merged image of A and B. No mutations were known in the FLCN coding region in the HeLa cell line. Immuno-histochemical staining on normal human tissues including skin (E) and kidney (F) using anti-FLCN. Magnification bars represent 10 μ m. Western blot of subcellular fractions of HeLa cell lysates shows that human FLCN was mainly detected in the nucleus (G). Further fractionation of the HeLa cell nucleus lysates showed that FLCN was predominantly localized in the nucleolus, although a small amount of FLCN was detected in the nucleoplasm (H). (I) Immunoprecipitation experiments were done with antisera against human FLCN or control IgG from HEK293T cell lysates. The immunoprecipitates were subjected to SDS-PAGE, along with cell lysates (input), followed by immunostaining with anti-Rpt4 (human). Note that Rpt4 is detected in anti-FLCN, not control, immunoprecipitates.

found that downregulation of Rpt4 expression significantly inhibited tumor growth *in vivo* when compared with the scrambled-GFP and untransfected control (Fig. 7A–C). Mice were sacrificed after 3 weeks, their tumors excised and subjected to RT-PCR to assess Rpt4 mRNA levels (Supplementary Material, Fig. S11). In the tumor cells expressing shRpt4 that did grow in mice, we detected Rpt4 mRNA expression (Supplementary Material, Fig. S11), suggesting that shRpt4 expression had been selected against these cells. These results indicate that downregulation of Rpt4 can effectively counteract tumor growth resulting from loss of FLCN and suggest that Rpt4 inhibition may present a novel methodology to inhibit tumor development in BHD patients.

DISCUSSION

We have identified a novel role for the tumor suppressor gene FLCN in negatively regulating rRNA synthesis by inhibiting binding of the proteasomal ATPase, Rpt4, to the ribosomal DNA locus (rDNA). Rpt4 is known to associate with components of the rRNA transcriptional machinery (31) and promote rRNA synthesis, a key step in the progression of cancer (27). Our analysis also uncovered an interaction between FLCN and the Ras/Raf pathway. Additionally, our study demonstrated that improper regulation of rRNA gene synthesis contributes to the pathology of eye hyperplasia caused by expression of an oncogenic allele of Ras (Ras^{V12}) in *Drosophila*. We have

Figure 6. Analysis of FLCN function in a patient-derived UOK-257 cell line. (A) Schematic of a typical human rRNA gene locus. The 18S, 5.8S and 28S genes are separated by various external and internal spacers. The 5' ETS is retained in the transcribed precursor molecule before being cleaved to produce the mature transcript. (B) UOK-257 cells were stably infected with a control or full-length human *FLCN*-expressing lentiviral vector. *FLCN* expression was restored in the UOK-257-*FLCN* cell line as shown by RT-PCR, whereas the control vector did not alter *FLCN* levels in the UOK-257-Empty cell line. GAPDH was used as a loading control. (C) Pre-rRNA synthesis is elevated in UOK-257 and UOK-257-Empty cells, whereas stable induction of *FLCN* in UOK-257-*FLCN* suppressed pre-rRNA levels. HEK-293T cells also exhibited lower pre-rRNA production when compared with UOK-257 and UOK-257-Empty, suggesting that deregulation of rRNA synthesis is unique to UOK-257 cells. Total RNA was extracted from each cell line, followed by qPCR to measure pre-rRNA levels, using primers spanning the 5' ETS of the human rDNA locus, which persists in the rRNA precursor. Normalization was conducted using primers to the mRNA encoding acidic ribosomal phosphoprotein P0 (ARPP P0). (D, E, F, G and H) Sucrose sedimentation profiles of all cell lines were generated using a 10–40% gradient. Centrifugation at 31 000 rpm for 3.5 h at 4°C was performed and the gradient measured at A₂₅₄. UOK-257 and UOK-257-Empty cell lines have equal levels of the 40S, 60S and the mature 80S subunits, whereas reconstitution of *FLCN* in the UOK-257-*FLCN* cells downregulated the production of the mature and immature subunits. HEK-293T cells were used to demonstrate that elevated levels of ribosomal subunits are a feature endemic to the UOK-257 cell line, and not a general characteristic of transformed cells. (H) Individual heights of each peak corresponding to the 40S, 60S and 80S subunits were calculated and plotted as bar graph. The average height was computed from three runs. * indicates *P* < 0.05 by Student's *t*-Test for paired samples. Note that although the empty vector transfection seems to reduce the 80S peak, the difference was not statistically significant.

further examined a cell line (UOK-257) derived from the renal tumor of a BHD patient and found that these cells, indeed, exhibit increased production of rRNA and ribosomes. Thus, the function of the tumor suppressor FLCN in controlling rRNA transcription may be conserved across species.

Consistent with a role of rRNA transcription in tumorigenesis, upregulation of rRNA synthesis by overexpression of *Rpt4* increased *Ras*^{V12}-induced lethality, whereas reducing rRNA synthesis by heterozygosity for *Rpt4* or *Rpl135*, encoding the large RNA Pol I subunit (46), decreased *Ras*^{V12}-induced lethality (Fig. 4A). In addition, regulation of rRNA gene expression is also important for organism survival

and development (41). We found that the disruption of rRNA synthesis by altering *dFLCN* or *Rpt4* levels indeed affected animal growth (Fig. 2D and E). Biochemical analysis of a BHD-patient-derived cell line also revealed a defect in rRNA synthesis (Fig. 6B–H), thus suggesting that the molecular function of FLCN in inhibiting rRNA transcription may be conserved in humans.

FLCN may have functions in addition to regulating rRNA transcription. For instance, whereas alterations in FLCN levels affected both growth and ectopia of the eye tissue (Fig. 3A and B), changes in *Rpl135* and *Rpt4* dosage affected *Ras*^{V12}-induced lethality, but not the eye phenotypes (Figs 3

Figure 7. Knocking down Rpt4 expression in UOK-257 cells inhibits tumor growth in nude mice (A) Mice injected with UOK-257 cells expressing sh-Rpt4-C12 and sh-Rpt4-D2 inhibit development of tumors when compared with mice injected with either sh-GFP or untransfected UOK-257 cells. (B) Deceleration of tumor growth in mice injected with sh-Rpt4-C12 and sh-Rpt4-D2 constructs when compared with sh-GFP or UOK-257 cells. (C) Mice injected with sh-Rpt4-C12 have smaller tumors, whereas those injected with sh-Rpt4-D2 have none, as evidenced by the average tumor volume when compared with mice injected with either sh-GFP or UOK-257 cells.

and 4; not shown). This may be because Rpl135 and Rpt4 can only modulate rRNA production, whereas FLCN influences both cell cycle and rRNA transcription, as it has previously been reported that cell lines deficient in *FLCN* show upregulation of checkpoint cyclins D and E (8). It is believed that the coordination of key checkpoints during cell cycle progression with proper rRNA production is crucial in neoplastic transformation, and the cooperative deregulation of both processes by FLCN could be the stimulus for cancer development. Consistent with this idea, we found significantly large populations of mitotic cells in eye imaginal disks, where *dFLCN* had been knocked down and the oncogenic *Ras*^{V12} allele was overexpressed (Fig. 3D and E). The interaction between FLCN and Ras signaling is consistent with a previous report showing an increase in Raf/MEK/Erk activation in FLCN conditional knockout mouse model (16).

In addition to the Ras pathway, it has also been shown that FLCN interacts with the 5'AMPK-mTOR pathway, which functions in energy and nutrient sensing, and that kidney-specific deletion of *FLCN* in mice led to the development of enlarged polycystic kidneys resulting in animal death from renal failure at 3 weeks of age (16). On treatment of *FLCN* knockout mice with the mTOR inhibitor Rapamycin, the cystic kidney and cell overproliferation defects were partially rescued but not completely reversed, with the mice eventually dying (16). The data provided in this paper with regard to FLCN-Rpt4 interaction do not rule out the involvement of mTOR in the defects associated with the loss of FLCN. Indeed, mTOR has been shown to regulate rRNA synthesis (49), which could explain the elevated rRNA levels in

FLCN-deficient animals. Moreover, although overexpression of FLCN effectively inhibits recruitment of Rpt4 to rDNA (Fig. 2F), it has little effects on the steady state pre-rRNA level or body weight (Fig. 2C, E, G), consistent with the idea that other pathways are involved in regulating rRNA synthesis. Thus, multiple pathways could contribute to renal cell overgrowth and kidney dysfunction caused by the loss of *FLCN*.

The emergence of Rpt4 as a novel interacting partner of FLCN might provide new opportunities for therapeutic intervention. By uncovering the connection between FLCN and the Ras/Raf signaling module, we could exploit rational drug design and cooperation to improve efficacy and specificity or to reduce the administering dosage. For example, use of Raf kinase inhibitor together with an rRNA transcription inhibitor and/or Rapamycin, mTOR inhibitor, might be a more effective treatment of *FLCN*-associated cancers than either drug alone. Because cellular signaling pathways have been extensively characterized in *Drosophila* and because many genes are conserved from flies to humans, the results of our study could offer additional targets for the treatment of neoplasias found in BHD syndrome and also provide a genetically tractable whole organismal model for searching novel drug targets.

MATERIALS AND METHODS

Drosophila stocks and genetics

UAS-dFLCN-HA and *UAS-Rpt4* transgenic lines were established using P-element-mediated transformation (50), following cloning of full-length *Rpt4* and haemagglutinin-tagged

dFLCN (*dFLCN-HA*) cDNA, into pUAST and pUAST-HA vectors, respectively (51,52). *UAS-dFLCN RNAi* (line 1) flies were kindly provided by S.X. Hou (19) and *UAS-dFLCN RNAi* (line 2) by the Vienna *Drosophila* RNAi Center (Vienna, Austria). The following stocks were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN, USA): *UAS-Ras^{V12}* (on II), *eyeless(ey)-Gal4,tubulin80-Gal4/CyO*, *Rpt4^{G0345}/FM7c* and *Rpl135^{k16513}/CyO*. Fly cultures were maintained at 25°C on standard media unless otherwise indicated.

Genetic screening using chromosomal deficiency strains was as previously described (53,54) with the following specifics. To identify enhancers or suppressors of *Ras^{V12}*, a recombinant chromosome *ey-Gal4, UAS-Ras^{V12}* was generated. *ey-Gal4, UAS-Ras^{V12}/CyO* virgin females were crossed with chromosomal deficiency (*Df*) lines, obtained from the Bloomington *Drosophila* Stock Center, and were kept at room temperature (21–22°C). The F1 adult flies of the genotype *ey-Gal4, UAS-Ras^{V12}/+, Df/+* (identified by the lack of balancer chromosomes) were scored for eye morphology and were given a score corresponding to the percentage of flies with normal or nearly normal-sized eyes. Typically, about 10% of *ey-Gal4, UAS-Ras^{V12}/+* flies exhibit normal to nearly normal eye morphology at room temperature (21–22°C), with the rest of the flies having either hyperplastic or deformed eyes, or lacking one or both eyes (see Fig. 3A).

Two-hybrid assay

The Stratagene Cytotrap system was used for two-hybrid (Y2H) screening (38) of interacting partners for *dFLCN* in a *Drosophila* cDNA library. *dFLCN* was cloned into the pSOS bait plasmid (Stratagene, La Jolla, CA, USA) and in-frame insertion was confirmed by DNA sequencing. ‘Bait’ plasmid (*hSOS-dFLCN*) and ‘prey’ *Drosophila* cDNA library were cotransformed into temperature-sensitive yeast (strain *cde25H*) using a modified lithium acetate transformation protocol as previously described (36,37). Interaction with this ‘prey’ library, targeted and anchored to the plasma membrane via a myristoylation moiety, recruits the *dFLCN* ‘bait’ to the plasma membrane, restoring a deficient *SOS/cde25* pathway and allowing survival of the yeast at elevated temperatures. Each identified candidate was replated for growth selection, and DNA was recovered using yeast lysis digestion and plasmid isolation (Promega, Madison, WI, USA). Purified interactors were then rescreened in the yeast-2 hybrid assay by direct cotransformation of the isolated prey plasmid and the *hSOS-dFLCN* bait. All prey plasmids that passed this rigorous screening process were sequenced for identification.

Cell lines, cell culture and transfections

A human renal cell carcinoma line, UOK-257, deficient in FLCN, derived from a patient afflicted with BHD syndrome, was a generous gift from Dr L. Schmidt (47). To generate a stable FLCN-supplemented cell line (FLCN+), UOK-257 was infected with a *FLCN*-expressing vector using the Vir-Power Lentiviral Expression System (Invitrogen, Carlsbad, CA, USA). Cells with stably integrated viral DNA were selected using puromycin and pooled. UOK-257 cells, infected

with an empty viral vector, served as the control. Dr Schmidt generously provided both the *FLCN*-expressing and control lentiviral vectors. We developed stable *Rpt4*-knockdown UOK cell lines by infecting UOK-257 cells with two different small hairpin (sh) RNA-expressing lentiviruses (Open Biosystems) according to the instructions of the manufacturer. All cell lines were maintained in DMEM, containing high glucose (4.5 g/l), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 2.5 units/ml penicillin and 2.5 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. S2 cells (Invitrogen, Carlsbad, CA, USA) were grown in Schneider’s *Drosophila* Medium containing 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin, at 26°C. HEK-293T cells were transfected using Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. S2 cells were transfected with 1–2 µg of input plasmid using the Calcium Phosphate method (55). Expression of *pUAST-dFLCN-HA* and *pUAST-Rpt4-FLAG* was induced by *pWA-Gal4* that drives *Gal4* under the control of the *actin5C* promoter. Cells transfected with vector *pUAST* were used as the control.

Immunostaining and microscopy

Immunostaining of *Drosophila* tissues and cells is as previously described (56,57) with the following modifications. *Drosophila* eye imaginal disks from third-instar larvae were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, blocked in PBS 0.3% Triton X-100, 0.3% bovine serum albumin (BSA), 1% normal goat serum for 30 min and incubated overnight in primary antibody at 4°C. Tissues were washed four times in PBS 0.3% Triton X-100 and subsequently immunostained with tetramethyl rhodamine isothiocyanate (TRITC) 546-, FITC 488- or Cy5 660- conjugated (1:250; Molecular Probes) secondary antibody at 4°C overnight. S2 cells cultured on 22 × 22-1 glass cover slips (Fisher Scientific) were washed twice with ice-cold PBS, 48 h post transfection, fixed in 3.7% formaldehyde, blocked in PBS 0.1% Triton X-100, 0.3% BSA and incubated with primary antibody and fluorophore-conjugated secondary antibody overnight at 4°C. The following antibodies were used: rabbit antifibrillarin (diluted 1:250; Abcam), mouse phosphohistone 3 (diluted 1:250; Cell Signaling), rat anti-HA (diluted 1:250; Roche) and rabbit anti-FLAG (diluted 1:250; Rockland).

Hela cells were seeded on chamber slides coated with poly-L-lysine, were fixed in 4% paraformaldehyde and permeabilized in methanol. The cells were sequentially incubated at room temperature in anti-FLCN antibody solution (1:100) for 2 h, washed and then with anti-rabbit antibody (1:200) conjugated with FITC for 1 h, followed by incubation with mouse anti-Fibrillarin antibody solution (1:500) for 2 h and then with anti-mouse antibody (1:200) conjugated with TRITC (red fluorescence) for 1 h. The slides were then counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride to show the position of the nuclei. Fluorescence microscopy was used to observe the staining. Immunostaining of human kidney and skin were done at the Pathology Lab Services at University of Rochester Medical Center.

Coimmunoprecipitation and western blotting

HEK-293T cells were harvested 48 h post-transfection in lysis buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 5% Glycerol) supplemented with complete protease inhibitor cocktail and 2 mM phenylmethylsulfonyl fluoride (PMSF). Lysate containing equal amounts of protein (200 µg) was then immunoprecipitated overnight at 4°C with protein G-Agarose (Roche)-bound antibodies, and the beads were washed six times with lysis buffer. Associated protein complexes were recovered in SDS sample buffer and subjected to immunoblot analysis. Protein samples were resolved on a 10% SDS-polyacrylamide gel and transferred onto pure nitrocellulose blotting membrane (Pall). Rabbit anti-FLAG (1:500; Rockland) and goat anti-HA (1:500; VWR) primary antibodies were used to detect Rpt4 and FLCN, respectively, followed by incubation with HRP-conjugated secondary antibodies and visualization by enhanced chemiluminescence kit (Pierce). Mouse anti-γ-tubulin (1:3000; Sigma) was used to show equal loading.

Chromatin immunoprecipitation

ChIP assays were done using the cross-linking immunoprecipitation protocol (X-ChIP) from Abcam. Briefly, 1×10^7 cells were harvested, cross-linked using formaldehyde and sonicated to shear chromatin. Approximately, 25 µg of chromatin, used for every IP sample, was precleared with protein G-Agarose beads (Roche) saturated with salmon sperm DNA and then precipitated by an anti-FLAG antibody (Rockland). Immunoprecipitated-chromatin fragments were subsequently used for qPCR using a set of primers that spanned the promoter and coding regions of the *Drosophila* ribosomal DNA (rDNA) locus:

Promoter forward 5'-TGTATATAGGGGAAAAATAATCATATAAT-3',
 Promoter reverse 5'-ACCAATATAATCATTATACGAAATATATATTC-3',
 5'18S forward 5'-TCTGGTTGATCCTGCCAGTAG,
 5'18S reverse 5'-GATCTTGCGATCGCTTGG-3',
 Mid 28S forward 5'-TGGCGTAAGCCTGCATGGAG-3',
 Mid 28S reverse 5'-CGCCTTGAAGCTTAGGACCGA-3'.

Reverse transcription and qPCR

Total RNA was extracted from whole flies or infected cells using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by agarose gel electrophoresis and ethidium bromide staining. Then, 1.5–3 µg of RNA was used as the template to perform reverse transcription with SuperScript III first strand synthesis system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Q PCR analysis was done using the iCycler™ Optical Module (Bio Rad). Reactions were set up in a volume of 25 µl containing 12.5 µl of 2× SYBR Green PCR Master Mix (Bio Rad), 4 µl of 10× diluted cDNA and 25 µM of primer mix. Amplification conditions consisted of a 2 min hot start at 94°C, followed by 40 cycles each of 30 s at 95°C, 30 s at 58°C and 50 s at 72°C. All quantitative PCR reactions were set up in triplicates. Data were analyzed using iCycler software (Bio Rad).

Xenograft tumor assay

The experiment was conducted as previously described (48). Briefly, stably infected UOK-257 cells (1×10^6) were suspended in 300 µl of medium (Sigma-Aldrich) and injected subcutaneously into both flanks of nude mice. Tumor growth was measured once a week and mice health was monitored daily. Mice bearing tumors larger than 2 cm, or showing severe health problems, were sacrificed and examined. Otherwise, tumor growth was monitored for 3 weeks after injection. At the end of Week 3, the mice were sacrificed, and tissue sections excised and flash frozen in liquid nitrogen for RNA extraction. All animal work was performed in accordance with the guidelines provided by the Animal Care and Use Committee of the University of Rochester Medical Center.

Cell proliferation assay

1×10^4 cells of each individual cell line were seeded in 6-well plates, in triplicates, trypsinized and counted every 24 h for 6 days.

Sucrose gradient analysis of ribosomes

Sucrose sedimentation profiles were generated as described (58). Briefly, 1×10^7 cells were collected in polysome extraction buffer (50 mM MOPS, pH 7.4, 15 mM MgCl₂, 150 mM NaCl, 0.5% Triton X-100, 100 µg/µl Cyclohexamide, 1 mg/ml heparin, 2 mM PMSF, 1× protease inhibitor cocktail), lysed by freeze thawing twice in dry ice and centrifuged at 13 000 rpm for 10 min at 4°C. Standardization of lysate was done by measuring A₂₅₄ and equal A₂₅₄, and then loaded onto a 10–40% sucrose gradient containing 20 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 100 mM NH₄Cl. Gradients were spun in an SW41 rotor at 31 000 rpm, for 3.5 h at 4°C. The Biocomp Piston Gradient Fractionator with a BIORAD Econo UV Monitor was used to measure gradients at A₂₅₄. Data were analyzed using the DataQ DI-158-UP data acquisition software.

In situ hybridization

Digoxigenin (DIG)-labeled RNA probe was generated according to the manufacturer's protocol (Roche Applied Science). Tissue sections or embryos were incubated for 30 min in equal parts PBST (1× PBS, 0.1% Tween 20) and fixative (5% paraformaldehyde in PEM), followed by washing with PBST and rinsing with 1:1 solution of PBST and Hybridization solution B (Hybe B, pH 6.5, 50% Deionized formamide, 5× SSC, 10 mg/ml sonicated salmon testis DNA, 20 mg/ml tRNA, 100 mg/ml heparin). Tissues and embryos were preincubated for 1 h in Hybe B at 55°C, followed by overnight hybridization at 55°C with DIG-labeled probe diluted in Hybe B (1:100). On the next day, excess probe was washed off using Hybe B, followed by preincubation for 1 h, washing with PBST, incubation with anti-DIG antibody (1:2000) for 2 h at R.T, washing with PBST and rinsing with staining buffer (0.1 M Tris pH 9.5, 1 M MgCl₂, 0.1% Tween, 5 M NaCl, 1 M Levamisol). Colorimetric detection was carried out by incubating tissues and embryos in staining buffer, NBT (75 mg/ml) and

BCIP (50 mg/ml) until staining was detectable. The reaction was paused by rinsing twice and washing with PBST for 20 min and mounting in 70% glycerol/PBS.

FACS analysis

BHD patient-derived kidney tumor cells, UOK-257, were compared with stably transfected *FLCN*-supplemented (UOK-257-*FLCN*), control (UOK-257-Empty) and HEK-293T cells. Approximately, 2×10^6 cells were collected and resuspended in chilled PBS and subjected to FACS analysis using the Accuri C6 (2 laser, 4 color) flow cytometer. The data were analyzed using FlowJo 5.4+.

Plasmids

Drosophila Rpt4 was amplified from cDNA clone (RE23388) and inserted into the vector pGEM-T, then excised and cloned into plasmid pUAST-FLAG. The correct sequence was verified by sequencing using the T7 sequencing primer and commercial service provided by GENEWIZ (South Plainfield, NJ, USA). *FLCN*-expressing and empty viral vectors were generous gifts from Dr Laura Schmidt (NIH, MD, USA).

Antibody production

A synthetic polypeptide, KVDSRPKEDTQKLLS, was chosen from the C-terminal region (amino acid 523–537) of the full-length human *FLCN* protein (NCBI accession number: NP-659434) based on hydrophilic profiles and was used as an antigen to raise polyclonal antisera in rabbits by a commercial source (Invitrogen, Carlsbad, CA, USA). The antisera were further affinity purified using the same peptide and validated using ELISA (performed by Invitrogen, Carlsbad, CA, USA). Western blot analyses for recombinant full-length *FLCN* and fragments of *FLCN* as well as endogenous and overexpressed *FLCN*, and antigen absorption assay, were done to confirm the specificity of the sera (see Supplementary Material, Fig. S6).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

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Conflict of Interest statement. The authors declare that they have no competing financial interests.

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