Tumor Suppressor Folliculin Regulates mTORC1 through Primary Cilia*

Folliculin (FLCN) is the tumor suppressor associated with Birt-Hogg-Dubé (BHD) syndrome that predisposes patients to incident of hamartomas and cysts in multiple organs. Its inactivation causes deregulation in the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway. However, the underlying mechanism is poorly defined. In this study, we show that FLCN is a ciliary protein that functions through primary cilia to regulate mTORC1. In response to flow stress, FLCN associates with LKB1 and recruits the kinase to primary cilia for activation of AMPK residing at basal bodies, which causes mTORC1 down-regulation. In cells depleted of FLCN, LKB1 fails to accumulate in primary cilia and AMPK at the basal bodies remains inactive, thus nullifying the inhibitory effect of flow stress on mTORC1 activity. Our results demonstrate that FLCN is part of a flow sensory mechanism that regulates mTORC1 through primary cilia.

Birt-Hogg-Dubé (BHD) syndrome is a rare autosomal dominant genetic disorder characterized by development of hamartomas and cysts in multiple organs, including skin, lung, colon, and kidney (1, 2). The syndrome is caused by germ-line mutations in the BHD gene, which encodes the folliculin protein (FLCN), a 64-kDa polypeptide that shares little sequence similarity with any other known proteins (3). FLCN is found to complex with AMPK and FNIP1, although the significance of the complex for FLCN function remains unclear (4). In mouse models FLCN deficiency leads to development of polycystic kidneys and renal cell carcinoma that are characteristically similar to those found in BHD patients (5, 6). Inactivation of FNIP1, together with its homolog FNIP2, in mice also produces similar phenotypes as does by FLCN deficiency (7), suggesting that FNIP1 may be required for FLCN function. Analyses of tumors derived from BHD patients and FLCN deficient animals have revealed deregulation in mammalian target of rapamycin complex 1 (mTORC1) signaling, a key event in tumorigenesis (5, 8–12). This abnormality in mTORC1 signaling is believed to be a major contributor to the pathological conditions in BHD, as inhibition of mTORC1 with rapamycin has been found to reduce the BHD tumor growth in animal models (5, 6). However, the mechanism by which FLCN regulates mTORC1 remains poorly understood.

At non-cycling resting state, most eukaryotic cells possess a microtubule-based membranous protrusion from cell surface termed as primary cilium (13). This unique structure plays a critical role in maintaining tissue homeostasis by functioning as a sensor for extracellular fluidic shear stress and chemicals (14, 15). Many signaling pathways involved in cell growth and proliferation are regulated by this environmental sensor, among which is the mTORC1 pathway (16–18). Several upstream regulators of mTORC1 have been found to localize to primary cilia, including the tuberous sclerosis complex proteins, LKB1 and AMPK (19–21). A recent study has shown that primary cilia are able to act through a LKB1- and AMPK-dependent mechanism to down-regulate mTORC1 signaling activity and cell size in response to flow stress, demonstrating mTORC1 as a key signaling event mediated by primary cilia (19).

Primary cilium is formed by extension of axonemal microtubules from the centriole derived basal body. Its assembly and maintenance require the intraflagellar transport that is driven by kinesin-2 motor (22). The motor transports proteins toward the “plus” end of microtubule and is comprised of two motor subunits, KIF3A and KIF3B, and an associated protein, KAP3 (23, 24). In a yeast two hybrid screen aiming to isolate FLCN-interacting proteins, we identified one of the motor subunits, KIF3A, as a FLCN-interacting protein, which raised a possibility that FLCN was a ciliary protein. In the present study we show that tumor suppressor FLCN is part of a flow sensory mechanism that regulates mTORC1 through primary cilia.

Experimental Procedures

Cell Lines and Culture—HKC-8 cells were cultured in DMEM/F12 medium supplemented with 10% FBS and 100 units/ml penicillin/streptomycin. FLCN null line UOK257 and its corresponding FLCN restored line UOK257–2 were kind gifts from Laura Schmidt and Marston Linehan at NCI and...
have been described before (25). To induce cilium formation, cells were cultured for 3 additional days after cell density reaching confluence. Antibodies for FLCN (Cat. # 3697), AKT (Cat. # 9272), phospho-AKT(Thr-308) (Cat. # 9275), phospho-AKT(S473), LKB1 (Cat. # 3050), AMPKα (Cat. # 2793), phospho-AMPKα(T172) (Cat. # 2535), S6 (Cat. # 2317), phospho-S6 (S235/236) (Cat. # 2211), 4EBP1, phospho-4EBP1(T37/46) (Cat. # 2855), Tsc2 (Cat. # 4308), and phospho-Tsc2(S1387) (Cat. # 5584) were purchased from Cell Signaling Technology. Anti-KIF3A (Cat. # 376680) and KIF3B (Cat. # sc-50456) antibodies were from Santa Cruz Biotechnology. Anti-γ-tubulin (Cat. # T5326) and acetylated-tubulin (Cat. # T7451) antibodies were from Sigma and anti-IFT88 antibody (Cat. # 13967-1-AP) from Proteintech Group Inc.

shRNAs and Inducible Knockdown Cell Lines—The targeting sequences of the shRNAs are: FLCN (5'-GATGGGAGAGCT-CGCTGATT-3'), IFT88 (5'-GCTTGAGCCTATTACAT-TGA-3'), and LKB1 (5'-GCCAACGTGAAGAAGGAAT-GCTTGGAGCCTATTACAT-3'). The shRNAs were designed and cloned into Tet-pLKO-Puro vector (Addgene). The resulting plasmids were transfected into HKC-8 cells and selected against puromycin. Clones stably expressing the doxycycline-inducible shRNAs were isolated. The inducible cells were cultured in the presence of puromycin and the expression of shRNA was induced by 100 ng ml⁻¹ of doxycycline in the culture medium. Lentiviral vectors expressing FNIP1 specific shRNAs, pLKO-puro-FNIP1 shRNAs, were purchased from Sigma. Two of the shRNAs (TRCN0000239412 and TRCN0000239413) with similar knockdown efficiency were used. A vector expressing a scrambled shRNA from Addgene was used as the control. The vectors were packaged in lentiviral particles and used for infection of HKC-8 cells following the instruction from pLKO-puro vector supplier (Addgene). The two FNIP1-specific shRNAs produced comparable results. Only the data of two FNIP1-specific shRNAs and Inducible Knockdown Cell Lines—ShRNAs and Inducible Knockdown Cell Lines—

Results

FLCN Interacts with Kinesin-2 in a Cilium-dependent Manner—Because kinesin-2 functions mainly in primary cilia, we examined whether the interaction of FLCN with KIF3A was cilium-dependent. Using co-immunoprecipitation, we were able to detect the interaction between the endogenously expressed FLCN and KIF3A in cultured cells (Fig. 1). Importantly, the interaction was detectable only in ciliated resting cells but not in cycling cells, which do not contain primary cilia (Fig. 1, A and B). Removal of primary cilia by shRNA-mediated depletion of IFT88, a gene required for formation of primary cilia (Fig. 1C) (26), abrogated the interaction of FLCN with KIF3A (Fig. 1D), indicating that the interaction takes place in primary cilia. FLCN was also found to associate with the other motor subunit of kinesin-2, KIF3B. Again, the association was cilium-dependent (Fig. 1E). Using an in vitro binding assay, we demonstrated that purified bacterially expressed recombinant FLCN bound directly to the C-terminal globular domain of KIF3A (Fig. 1F), the region involved in interaction with the cargo proteins (23), indicating that FLCN may be a cargo protein for kinesin-2 motor.

FLCN Localizes to Primary Cilia—The finding that FLCN was able to associate with kinesin-2 indicated that the tumor suppressor might reside within primary cilia. Indeed, FLCN was detected in the primary cilia of UOK257–2 cells that expressed wild type FLCN but not those of FCLN-null UOK257 cells derived from renal tumors of a BHD patient (Fig. 2A) (25).
However, the reduction in the cilium length under flow condition, there was a small but statistically significant decrease in the length of the primary cilia (Fig. 2C). When UOK257 cells were grown under flow condition, there was a small but statistically significant decrease in the length of the primary cilium under no-flow condition (Fig. 2C). A similar result was seen in HKC-8 cells with FLCN down-regulated by stably expressed doxycycline inducible shRNA. However, the reduction in the cilium length under flow condition was not statistically significant (Fig. 2D). In addition, FLCN deficiency exhibited little effect on the frequency of the ciliated cells under flow and no-flow conditions (Fig. 2, E and F). These observations suggest that FLCN is not required for formation of primary cilia under no-flow and flow condition. Nevertheless, it may have a minor effect on the stability of the cilia under flow condition.

**FLCN Regulates mTORC1 Activity in Response to Flow Stress**—Primary cilia have been recently shown to play a critical role in repressing mTORC1 activity in noncycling cells in response to flow stress (19). The observation that FLCN is a ciliary protein hence raised a possibility that FLCN might act through primary cilia to control mTORC1 activity. To test this notion, we examined the effect of FLCN depletion on mTORC1 activity in cells grown under flow stress, which bends primary cilia. We found that flow stress effectively reduced mTORC1 activity in HKC-8 cells as measured by the mTORC1-directed phosphorylation of S6 and 4E-BP1 (Fig. 3A). However, the inhibitory effect of flow stress diminished when FLCN was depleted by doxycycline induced shRNA (Fig. 3, A and B). Similarly, flow stress inhibited phosphorylation of S6 and 4E-BP1 in FLCN-expressing UOK257–2 cells but was ineffective in FLCN-null UOK257 cells (Fig. 3C). When formation of primary cilia was blocked by shRNA-mediated knockdown of IFT88, the inhibitory effect of the flow stress on mTORC1 was largely attenuated (Fig. 3D), demonstrating that the effect of flow stress requires primary cilia. Depletion of FNIP1, which forms a complex with FLCN (4), also produced similar effect on mTORC1 activity as did by depletion of FLCN (Fig. 3E), indicating that FNIP1 is involved in the ciliary function of FLCN. Interestingly, when FNIP1 was reduced by shRNA, the level of FLCN also decreased, indicating that FNIP1 may stabilize FLCN. Under no-flow condition, however, neither knockdown nor absence of FLCN had obvious effect on mTORC1 activity (Fig. 3, A and C). Collectively, the above findings demonstrate that FLCN is essential for the flow stress-mediated down-regulation of mTORC1 in ciliated cells, and suggest that FLCN is part of a flow-sensing mechanism in primary cilia.

**FLCN Is Required for Flow Stress-induced AMPK Activation**—In response to flow stress, a reduction in AKT activity was observed in HKC-8 cells as measured by its phosphorylation levels at Thr-308 and Ser-473. However, FLCN did not appear to play a role in the process, since its depletion did not impact a difference in the phosphorylation (Fig. 3A). In the BHD tumor-derived UOK257–2 cells, AKT activity appeared to be insensitive to flow stress, yet mTORC1-dependent phosphorylation of S6 and 4E-BP1 remained responsive to the stress (Fig. 3C). These findings suggest that the flow-induced mTORC1 down-regulation is independent of AKT activity. On the other hand, in both HKC-8 and UOK257–2 cells grown under flow conditions, there was an increase in AMPK activity as indicated by the changes in the levels of activation-dependent phosphorylation of the kinase at Thr-172 (Fig. 3, A and C). This increase correlated with an increase in the AMPK-dependent phosphorylation of Tsc2 at serine 1387 (Fig. 3, A and C), as well as a decrease in mTORC1 activity (Fig. 3, B and F). When the flow-induced activation of AMPK was inhibited by an AMPK inhibitor, the Tsc2 phosphorylation and mTORC1
activity became insensitive to flow stress (Fig. 3), demonstrating that flow stress represses mTORC1 activity through activation of AMPK and phosphorylation of Tsc2. However, when FLCN was depleted by shRNA in HKC-8 cells or was absent as in UOK257 cells, flow stress was unable to activate AMPK, and correlative, the AMPK-dependent phosphorylation of Tsc2 was not increased and mTORC1 remained largely active (Fig. 3, A and C). This observation suggests that FLCN is required for flow-induced AMPK activation. In contrast, FLCN was not needed for ATP depletion-induced activation of AMPK, since the activation was not affected in FLCN-deficient cells treated with glycolysis inhibitor 2-deoxy-D-glucose (Fig. 3, H and I), which blocks ATP production.

**FLCN Recruits LKB1 for AMPK Activation under Flow Stress**—FLCN has been previously shown to associate with AMPK under normal growth conditions (4). We thus examined whether flow controls AMPK activation by modulating the association of FLCN with the kinase. We found that the amounts of AMPK co-immunoprecipitated with FLCN were similar under flow and no-flow conditions (Fig. 4, A), suggesting that flow does not affect the association. However, we found that the amount of phosphorylated AMPK associated with FLCN was greater under flow condition than that under no-flow condition (Fig. 4A). This observation indicates that FLCN is involved in recruiting an activator of AMPK for its activation in response to flow stress. We thus examined whether the AMPK activation kinase, LKB1, was required for FLCN-mediated AMPK activation. We found that reducing LKB1 levels by shRNA-mediated knockdown attenuated flow-induced AMPK activation (Fig. 4B). To determine whether FLCN is required for recruiting LKB1 to AMPK under flow condition, we examined the association of LKB1 with FLCN. We found that LKB1 was not in complex with FLCN in the absence of flow stress but became associated with it under flow condition (Fig. 4C). Flow also induced association of LKB1 with AMPK (Fig. 4C), which was reduced when FLCN expression was knocked down by shRNA (Fig. 4D), suggesting that the association requires FLCN. When formation of primary cilia was blocked by shRNA-mediated down-regulation of IFT88, the association of LKB1 with FLCN and AMPK also decreased (Fig. 4E), indicating that the association is mediated through primary cilia. Collectively, these results show that FLCN, in response to flow stress, recruits LKB1 to AMPK for its activation.

**FLCN Controls mTORC1**

activity became insensitive to flow stress (Fig. 3G), demonstrating that flow stress represses mTORC1 activity through activation of AMPK and phosphorylation of Tsc2. However, when FLCN was depleted by shRNA in HKC-8 cells or was absent as in UOK257 cells, flow stress was unable to activate AMPK, and correlative, the AMPK-dependent phosphorylation of Tsc2 was not increased and mTORC1 remained largely active (Fig. 3, A and C). This observation suggests that FLCN is required for flow-induced AMPK activation. In contrast, FLCN was not needed for ATP depletion-induced activation of AMPK, since the activation was not affected in FLCN-deficient cells treated with glycolysis inhibitor 2-deoxy-D-glucose (Fig. 3H), which blocks ATP production.

**FLCN Recruits LKB1 for AMPK Activation under Flow Stress**—FLCN has been previously shown to associate with AMPK under normal growth conditions (4). We thus examined whether flow controls AMPK activation by modulating the association of FLCN with the kinase. We found that the amounts of AMPK co-immunoprecipitated with FLCN were similar under flow and no-flow conditions (Fig. 4A), suggesting that flow does not affect the association. However, we found that the amount of phosphorylated AMPK associated with FLCN was greater under flow condition than that under no-flow condition (Fig. 4A). This observation indicates that FLCN is involved in recruiting an activator of AMPK for its activation in response to flow stress. We thus examined whether the AMPK activation kinase, LKB1, was required for FLCN-mediated AMPK activation. We found that reducing LKB1 levels by shRNA-mediated knockdown attenuated flow-induced AMPK activation (Fig. 4B). To determine whether FLCN is required for recruiting LKB1 to AMPK under flow condition, we examined the association of LKB1 with FLCN. We found that LKB1 was not in complex with FLCN in the absence of flow stress but became associated with it under flow condition (Fig. 4C). Flow also induced association of LKB1 with AMPK (Fig. 4C), which was reduced when FLCN expression was knocked down by shRNA (Fig. 4D), suggesting that the association requires FLCN. When formation of primary cilia was blocked by shRNA-mediated down-regulation of IFT88, the association of LKB1 with FLCN and AMPK also decreased (Fig. 4E), indicating that the association is mediated through primary cilia. Collectively, these results show that FLCN, in response to flow stress, recruits LKB1 to AMPK for its activation.

**FLCN Is Required for Accumulation of LKB1 in Primary Cilia under Flow Stress**—To determine whether FLCN recruits LKB1 through primary cilia, we examined the role of FLCN in ciliary localization of LKB1 and AMPK in response to flow. We found that in HKC-8 cells active AMPK accumulated at the basal bod-
ies after the cells were subjected to flow stress. However, in cells depleted of FLCN, the flow-induced accumulation of the active AMPK at the site was abolished (Fig. 5, A–C). In contrast to the active AMPK, LKB1 was undetectable in the primary cilia of a majority of the cells (Fig. 5D), with only ~5 ± 2% of ciliated cells showed a weak LKB1 staining in their cilia (Fig. 5E). Following flow treatment, LKB1 became visible in the cilia of a significant portion (~45 ± 9%) of the cells (Fig. 5, D and E). In cells depleted of FLCN by inducible shRNA knockdown, however, the flow-induced accumulation of LKB1 in primary cilia was blocked (Fig. 5, D and E). Similar results were obtained with FLCN-null UOK257 cells (data not shown). These observations suggest that FLCN is required for accumulation of LKB1 in primary cilia in response to flow stress. Because primary cilia are required for the association of LKB1 with AMPK under flow condition (Fig. 4E), it is conceivable that FLCN recruits LKB1 through primary cilia before targeting it to basal bodies for AMPK activation.

Discussion

Since its identification more than a decade ago, FLCN has been implicated in regulation of mTORC1. However, the underlying molecular mechanism remains unsolved (2).
Although FLCN has been found in complex with AMPK in cycling cells, the significance of the association is unclear, as FLCN does not appear to affect AMPK activity or the response of AMPK to changes in intracellular energy levels (4). A recent study demonstrates that FLCN affects cellular energy homeostasis through an AMPK-dependent manner. However, it is unclear how FLCN regulates AMPK (28). In animal studies, conflicting evidence exists showing opposite effects of FLCN on mTORC1 activity (5, 8–12), which has led to the suggestion that the function of FLCN may be cell-content dependent (12). In the present study, we show that FLCN is a ciliary protein acting in a cilium-dependent manner, which provides an explanation for its elusive action in mTORC1 regulation.

Our findings identify FLCN as an upstream regulator of LKB1 that is responsible for recruiting the kinase to primary cilia under flow stress. How FLCN recruits LKB1 to primary cilia remains unclear. It is possible that the recruitment involves other proteins. Alternatively, FLCN may act to retain LKB1 within primary cilia. While additional studies are needed to define the underlying mechanism, our findings demonstrate that the FLCN-mediated recruitment is responsible for bringing LKB1 to its downstream target, AMPK, localized at basal bodies. The association of LKB1 with AMPK in response to flow stress results in its activation and phosphorylation of Tsc2, a negative regulator of mTORC1 (Fig. 6). A previous study has shown that Tsc1 is localized to basal bodies in ciliated cells. It is likely that Tsc2, the binding and functional partner of Tsc1, is also associated with basal bodies, allowing phosphorylation by AMPK. In addition to Tsc2, AMPK has also been shown to phosphorylate Raptor, the regulatory subunit of mTORC1 (29, 30). However, we did not observe any obvious effect of FLCN deficiency on the AMPK-dependent phosphorylation of Raptor under flow and no-flow conditions (data not shown). This result is consistent with the observation that FLCN did not affect AMPK activation under metabolic stress in cycling cells (Fig. 3H), suggesting that FLCN controls mTORC1 through AMPK-dependent phosphorylation of Tsc2 but not of Raptor. Taken together, our findings unveil the action mechanism of the tumor suppressor in the cilium-dependent regulation of mTORC1.

**FIGURE 4. FLCN recruits LKB1 for AMPK activation in response to flow stress.**

A, extracts from HKC-8 cells grown under flow (Flow +) or no-flow (Flow −) condition were immunoprecipitated with anti-FLCN antibody (α-FLCN). The levels of the indicated proteins in the extracts (Ext) and precipitates (IP) were determined by Western blotting. B, HKC-8 cells stably expressing doxycycline inducible LKB1 shRNA were grown under flow or no-flow condition in the presence (Dox +) or absence (Dox −). The expression levels of LKB1, AMPK, actin, and phosphorylation of AMPK were analyzed by Western blotting. C, extracts from HKC-8 cells grown under flow (Flow +) or no-flow (Flow −) condition were immunoprecipitated with anti-LKB1 antibody (α-LKB1) and control IgG. D, extracts from HKC-8 cells grown under flow (Flow +) or no-flow (Flow −) condition were immunoprecipitated with anti-LKB1 antibody (α-LKB1) and control IgG. E, HKC-8 cells stably expressing doxycycline inducible IFT88 shRNA were grown under flow condition in the presence (Dox+) or absence (Dox−) of doxycycline. Extracts from the treated cells were immunoprecipitated with anti-LKB1 antibody (α-LKB1) and control IgG.
The ability of FLCN to interact directly with kinesin-2 motor suggests that this protein is involved in intraflagellar trafficking either as a transporter or as a cargo protein. However, the non-essential role of FLCN in formation of primary cilia distinguishes it from the intraflagellar transport proteins (IFT), such as IFT88, that supply materials for axonemal growth (31). The fact that the activity of FLCN is manifested only under flow condition further indicates FLCN as a part of the mechanosensory machinery than a structural component of primary cilia. It is likely that FLCN is involved in trafficking signaling molecules or maintaining their steady state levels within the organelle in response to flow and other environmental signals. In this regard, the FLCN-mediated accumulation of LKB1 in primary cilia represents a unique signaling mechanism for the function of primary cilia, as it suggests that flow stress signal controls cilium signaling through regulating the ciliary traffic of signaling molecules. A key question remained to be answered is how FLCN, a soluble intracellular protein, is able to sense flow stress. On this point, a potential link between FLCN and ciliary mechanosensory proteins, such as polycystin-1 and 2, warrants further studies.

The primary cilium is a signaling hub for noncycling resting cells and plays an important role in maintaining tissue homeostasis (32). Many tumor suppressors, including polycystin-1, LKB1, Ptch1, and pVHL, are found to function through this organelle (33). Defects in cilium formation and signaling have been associated with tumorigenesis in several organs, including kidney, skin, and lung (34, 35), which are manifested in BHD.

**FIGURE 5.** FLCN is required for ciliary localization of LKB1 and AMPK activation at basal bodies. HKC-8 cells stably expressing doxycycline inducible FLCN shRNA were grown in the absence (Ctrl) or presence (Dox) of doxycycline under flow or no-flow condition. A, cells were stained with anti-phospho-AMPK antibody (red), anti-acetylated tubulin (green) antibody that marked primary cilia, and DAPI (blue). B, cells were stained with anti-phospho-AMPK antibody (red), anti-γ tubulin antibody (green) that marked basal bodies, and DAPI (blue). C, quantitative presentation of phosphorylated AMPK shown in B. The density of the antibody staining at the basal bodies was analyzed with Image J (NIH) software. The relative levels of phosphorylated AMPK at the basal bodies were expressed as the ratio between the fluorescent intensities of phospho-AMPK and tubulin staining. A total of 100 pairs of basal bodies from three independent experiments performed under identical experimental settings was analyzed for each indicated condition. *, p < 0.01. D, cells were stained with anti-acetylated tubulin antibody (green), anti-LKB1 antibody (red), and DAPI (blue). The stained cells were imaged by confocal microscopy. Scale bar, 5 µm. E, percentage of the ciliated cells displayed detectable LKB1 under the indicated conditions was determined. A total of 200 ciliated cells from three independent experiments performed under identical experimental settings was examined for each condition. #, p < 0.001.

**FIGURE 6.** A schematic illustration of the ciliary action of FLCN in mTORC1 regulation (See text for detail).
syndrome. Our findings that FLCN controls LKB1 accumulation and mTORC1 activation through primary cilia suggest that FLCN, like many other tumor suppressors, may elicit its anti-tumor activity through this organelle in quiescent cells.

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Author Contributions—M. Z. conducted the imaging studies shown in Figs. 2 and 5 and part of biochemical analyses shown in Figs. 1 and 3. X. Z. did the biochemical analyses shown in Figs. 1, 3, and 4. J. L., G. Y., M. T., S. G., Y. Z., and Y. L. performed experiments. Yong J., Y. L., and Yu J. conceived and planned the experiments and interpreted data. Yu J. wrote the manuscript.

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