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# Regulation of folliculin (the *BHD* gene product) phosphorylation by *Tsc2*-mTOR pathway

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ABSTRACT

The Birt–Hogg–Dubé gene (*BHD*) encodes the tumor suppressor protein folliculin (FLCN). The function of FLCN has recently been implicated in the regulation of rapamycin-sensitive mTOR complex (mTORC1). Reciprocally, the mTORC1-dependent phosphorylation of FLCN was reported. However, precise mechanism of FLCN phosphorylation and functional interaction of FLCN with tuberin, the product of tuberous sclerosis 2 gene (*TSC2*) which is a negative regulator of mTORC1, are unclear. Here we report that multiple phosphorylation in FLCN are evoked by downregulation of tuberin as well as by Rheb expression. We found that phosphorylation at Ser62 and Ser302 are differently regulated by mTORC1-dependent pathway. Some unknown kinases downstream of tuberin-mTORC1 are thought to directly phosphorylate FLCN. Interestingly, our results also suggest that the complex formation of FLCN with AMPK is modulated by FLCN phosphorylation. These results suggest that FLCN is involved in a novel mechanism of signal transduction downstream of tuberin.

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## Introduction

The mammalian target of rapamycin kinase (mTOR) exerts a pivotal role in regulation of various cellular activities. There are two complexes in which mTOR is involved: mTORC1 consists of raptor and mLST8, and mTORC2 consists of rictor, mSIN1 and mLST8, in addition to mTOR [1,2]. mTORC1 phosphorylates p70 S6 kinase 1 (S6K1) and 4E-BP1 in a rapamycin-sensitive manner and regulates translation, whereas mTORC2 phosphorylates Akt kinase and regulates cytoskeletal organization, which is not directly affected by rapamycin [1,2]. It appears that a number of tumor suppressor gene products, including PTEN and LKB1, are implicated in the control mechanism of mTOR [3]. Products of two causative genes of tuberous sclerosis (TSC), *TSC1* (hamartin) and *TSC2* (tuberin), form a complex and inhibit the small GTP-binding protein Rheb by acting as its GTPase activating protein (GAP), thereby downregulating the downstream mTORC1 [4].

We have studied multi-step tumorigenesis by using the Eker rat model of hereditary renal carcinoma (RC), which has a mutation in the *TSC2* homolog (*Tsc2*) [5]. In recent years, we have studied the Nihon rat model of hereditary renal cancer and identified a germline mutation in the homolog of the human Birt–Hogg–Dubé gene (*Bhd*) [6]. Birt–Hogg–Dubé syndrome (BHDS) is an autosomal dominantly inherited disease and predisposes patients to develop fibrofolliculomas, lung cysts and renal neoplasia [7]. *BHD/Bhd* is a tumor suppressor and encodes folliculin (FLCN), an evolutionarily conserved protein (67 kDa), with no functional motif [8,9]. Although detailed molecular mechanism of the pathogenesis in BHDS as well as the function of FLCN have not been fully elucidated, recent findings suggest that FLCN is involved in mTORC1-related pathways [10].

Two FLCN-interacting proteins, FLCN-interacting protein 1 (FNIP1) and its homolog FLCN-interacting protein 2 (FNIP2/FNIPL), have been reported [10–12]. Baba et al. demonstrated that AMPK-activated protein kinase (AMPK) interacts with FNIP1 and phophorylates both FNIP1 and FLCN [10]. They also reported that FLCN phosphorylation was diminished by rapamycin treatment and amino acid starvation. On the other hand, in particular conditions, modulation of FLCN expression induces change in the phosphorylation status of mTORC1 substrates in *BHD*-deficient renal cancer cells [10]. Moreover, we have reported that the suppression of *BHD, FNIP2/FNIPL* or *FNIP1* expression by RNA interference reduced S6K1 phosphorylation in HeLa cells [12]. These results

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suggest that the mTORC1-related pathways are regulated by FLCN in a context-dependent manner. Thus, there is a complex relationship between FLCN and mTORC1. Unravelment of this complex relationship is necessary not only to better understand the pathogenic mechanism of BHDS but also to reveal the network of tumor suppressors that may include therapeutic target points for many diseases.

In this study, we further explore the mechanism of FLCN phosphorylation.

## Materials and methods

*Antibodies.* Anti-FLCN C1 and 223 antibodies were described previously [12]. Anti-phospho-S302 FLCN (295P) was generated by immunizing rabbits with S302-phosphorylated peptide corresponding to aa 295–306 (Glu-Ser-Glu-Ser-Trp-Asp-Asn-Ser-Glu-Ala-Glu-Glu) of rat FLCN and purified by antigen-affinity chromatography followed by absorption with non-phosphorylated peptides (IBL). Anti-phospho-S62 antibody was generated as described elsewhere (L. Wang et al., submitted). Other antibodies are described in Supplementary Materials and methods.

*Protein expression, purification, and in vitro kinase assay.* GST-S6K1, FLCN-GST, and GST were transiently expressed in Cos7 cells. Cells were lysed in NP-40 lysis buffer and proteins were affinity purified by glutathione–Sepharose 4B (GE Healthcare Bioscience) [12]. Amino-terminal His-tagged proteins were expressed in *Escherichia coli* M15 [pREP4] strain (Qiagen) by induction with isopropyl-b-D(−)-thiogalactopyranoside and were affinity purified through Ni–NTA resin using Purelumn System His-tag Purification Kit (GE Healthcare Bioscience).

*In vitro* kinase assay for mTORC1 or mTORC2 was performed using the immunoprecipitated kinase complex. Semi-confluent HeLa cells in a 100 mm dish were lysed with CHAPS lysis buffer [20 mMTris–HCl (pH 7.4), 120 mM NaCl, 1 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 50 mM NaF, 0.3% CHAPS, 1 mM DTT, 4 μg/ml aprotinin and 4 μg/ml leupeptin] and the lysate was subjected to immunoprecipitation with anti-raptor or anti-rictor antibody. In the case of mTORC1, the resin was washed twice with CHAPS lysis buffer and then twice with buffer A [10 mM Hepes–NaOH (pH 7.4), 50 mM NaCl, 50 mM β-glycerophosphate]. In the case of mTORC2, the resin was washed four times with CHAPS lysis buffer and then once with buffer B [25 mM Hepes–NaOH (pH 7.5), 100 mM potassium acetate and 1 mM $MgCl_2$]. In the assay for mTORC1, GST-S6K1 or FLCN-GST was incubated with the immunoprecipitated complex in a solution consisting of 10 mM Hepes–NaOH (pH 7.4), 50 mM NaCl, 50 mM β-glycerophosphate, 10 mM $MnCl_2$, 100 μM ATP, and 15 μCi/tube $\gamma$-$^{32}$P-ATP incubated for 30 min at 30 °C [13]. In the assay for mTORC2, Akt (Upstate) or FLCN-GST was incubated with the immunoprecipitated complex in a solution consisting of 25 mM Hepes–NaOH (pH 7.5), 100 mM potassium acetate, 1 mM $MgCl_2$, 500 μM ATP, 15 μCi/tube $\gamma$-$^{32}$P-ATP for 30 min at 37 °C [2]. *In vitro* kinase assay for p70 S6 kinase was performed using His-S6 and His-FLCN fragments as substrates, and recombinant S6K1 (Upstate) according to the manufacturer's instructions. Samples were separated by SDS–PAGE and either visualized by (CBB) staining or transferred onto nylon membrane

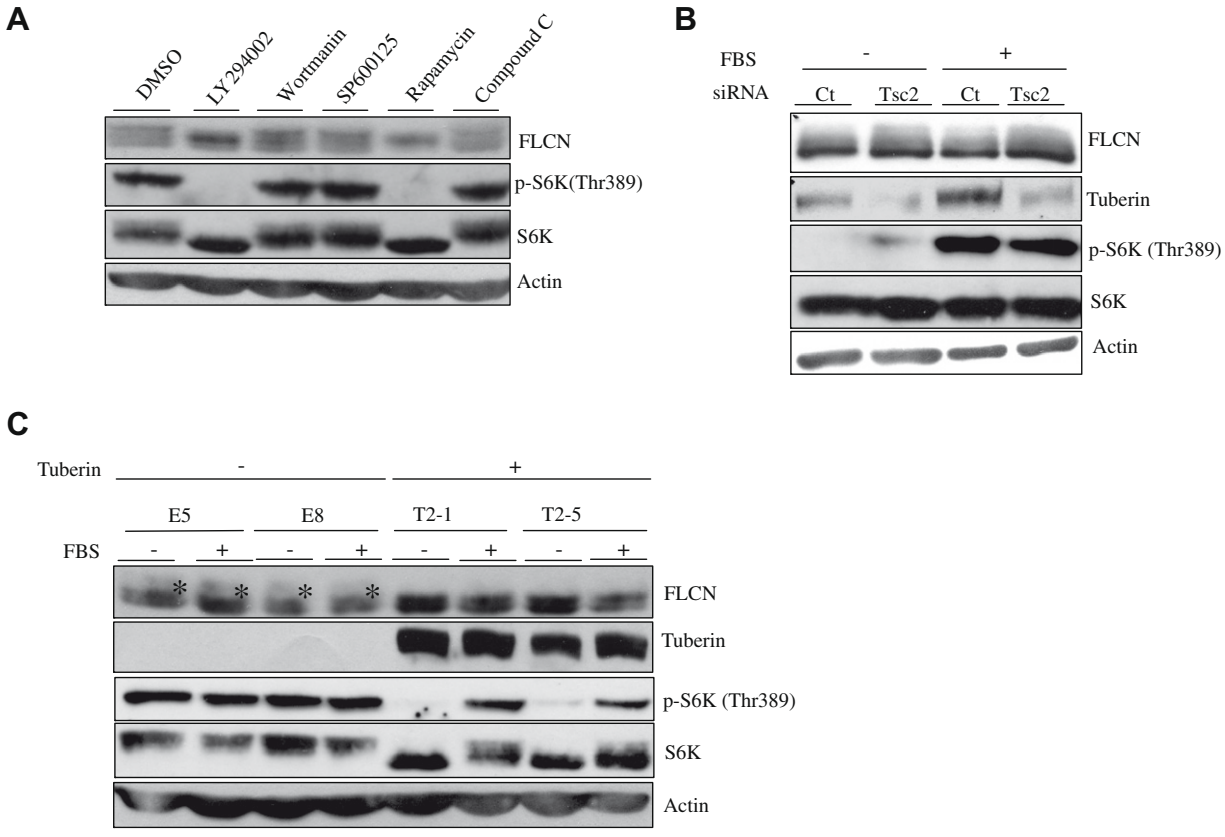


**Fig. 1.** Effects of drugs and tuberin expression on the migration of FLCN bands. (A) Drug treatments in ERC33 (*Tsc2*-deficient renal tumor cell line from the Eker rat) cells. ERC33 cells were treated with indicated drugs for 18 h. The cell lysates were analyzed by immunoblotting with indicated antibodies. (B) Suppression of tuberin expression by RNA interference. 1B1 (*Bhd*-restored renal tumor cell line from the Nihon rat) cells were treated with control (Ct) or *Tsc2* siRNA (Tsc2) for 48 h. During the last 24 h, cells were serum-starved and then re-stimulated (+) or not stimulated (−) with 10% serum (FBS) for 2 h. Total cell lysates were analyzed by immunoblotting with indicated antibodies. (C) Expression of FLCN in *Tsc2*-deficient (E5 and E8) and *Tsc2*-restored (T2-1 and T2-5) cells. Serum-starvation and stimulation were performed as in (B) and cell lysates were analyzed by immunoblotting with indicated antibodies. Asterisks show that slower migrating bands increased in E5 and E8 cells. Note that phospho-S6K was increased in *Tsc2*-suppressed or -deficient cells in (B) and (C).

(Millipore). Then, autoradiography was performed. Proteins on nylon membrane were visualized by immunoblot with alkaline phosphatase conjugated secondary antibodies using the substrate, nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (WAKO).

*RNA interference.* Transfection of siRNA (25 nM, final) was performed with Lipofectamine 2000 (Invitrogen) and OPTI-MEM (Gibco) according to the manufacturer's protocols. Forty-eight hours after transfection, cells were lysed for further analysis. Sequences of siRNAs (sense strands without 3′-overhang) were: *Tsc2*, 5′-GCCCU CACAGACAAUGGAA-3′; *raptor*, 5′-GCCUGAGUCUGUGAAUGUA-3′; *rictor*, 5′-CCUCCUCAGUUAGGUCUAU-3′; *S6K1*, 5′-CUCAGCGUUCGU AAGGAUU-3′; *S6K2*, 5′-GCCAGUAGAUAGUCCAGAU-3′ control, 5′-G CUGCAAUCGAUUGAUAGC-3′.

*Other methods.* For other methods (cell lines, plasmid construction, plasmid transfection, preparation of cell lysate, immunoblot analysis, immunoprecipitation, phosphatase treatment, and metabolic labeling) are described in Supplementary Materials and methods.

## Results and discussion

### *Stimulation of FLCN phosphorylation by Tsc2-deficiency*

In the *Tsc2*-deficeint ERC33 cells [5], FLCN migrates predominantly as three bands in immunoblots (Fig. 1A). As reported by Baba et al., the slowest migrating band disappeared when ERC33 cells were treated with LY294002 or rapamycin (Fig. 1A). The concentration of LY294002 used was high enough to inhibit mTOR [14]. In contrast, the uppermost band did not disappear by treatment with Wortmanin (PI3-kinase inhibitor) or Compound C (AMPK inhibitor), suggesting that *Tsc2*-deficiency conferred insensitivity to these drugs (Fig. 1A). When the expression of tuberin was suppressed with siRNA in 1B1 cells (FLCN reconstituted renal tumor cells from Nihon rat), slower migrating FLCN bands appeared in both serum-starved and serum-added conditions (Fig. 1B). There was also a slight increase in the amount of faster migrating bands by *Tsc2*-deficiency in both conditions (Fig. 1B). Next, we tested the effect of tuberin expression on FLCN phosphorylation by using *Tsc2*-deficient mouse renal tumor cell lines in which an expression vector for tuberin or a control empty vector was introduced (T2-1 and T2-5 cells, or E5 and E8 cells, respectively). Compared with *Tsc2*-deficient cells, *Tsc2*-restored cells showed less shifted bands in both serum-starved and serum-added conditions, suggesting that FLCN phosphorylation was downregulated by tuberin (Fig. 1C). Interestingly, an increase in the amount of total FLCN expression by serum starvation was specifically seen in *Tsc2*-restored cells (Fig. 1C). These data suggest that phosphorylation of FLCN is regulated by tuberin signaling.

### *Identification of a phosphorylation site (Ser302) in the carboxy-terminal region of rat FLCN*

We have identified a major phosphorylation site (serine 62; S62) in the amino-terminal half of rat FLCN by mass spectrometry and generated a phospho-S62-FLCN-specific antibody (L. Wang et al., submitted). The carboxy-terminal half of FLCN, without Ser62, also showed incorporation of $^{32}P$ by metabolic labeling (L. Wang et al., submitted). Therefore, we searched phosphorylation sites by site-directed mutagenesis at candidate residues (Fig. 2A and data not shown.). A mutant with a substitution for Ser 302

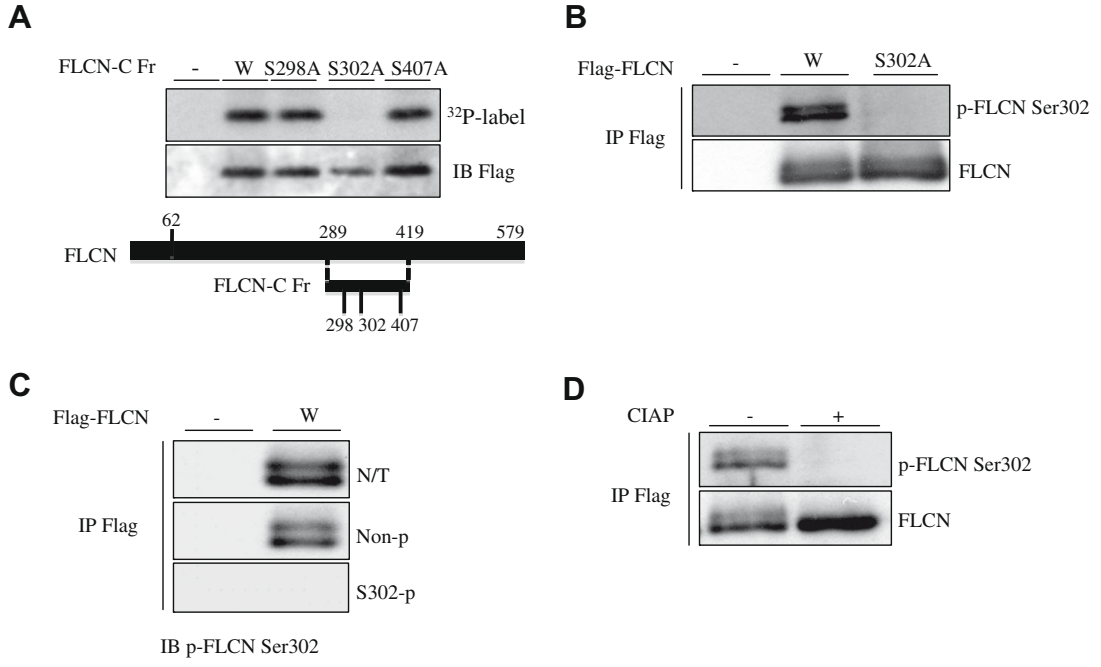


**Fig. 2.** Identification of a phosphorylation site at serine 302 (S302) in FLCN and generation of a phospho-S302-specific antibody. (A) Site-directed mutagenesis in the carboxy-terminal half of FLCN. Flag-tagged amino-terminal deletion mutants of FLCN with indicated aa substitution (lower scheme) were expressed in Cos7 cells. Cells were metabolically labeled with $^{32}P$-orthophosphate and proteins immunoprecipitated with anti-Flag antibody. $^{32}P$-label was analyzed by autoradiography after SDS–PAGE and membrane transfer. Proteins were detected by immunoblotting with anti-Flag antibody. (B) Immunoblotting of full-length FLCN with phosho-S302-specific antibody (BHD295P). Cos7 cells were transfected with the expression plasmid for Flag-tagged, full-length wild-type (W) or A mutant (S302A) FLCN or empty vector (−). Forty-eight hours later, immunoprecipitates with anti-Flag antibody were analyzed by immunoblotting with indicated antibodies. (C) Pre-absorption with peptides. BHD295P was pre-absorbed with S302-phosphorylated (S302-P) or non-phosphorylated (non-P) peptide and used for detection of Flag-FLCN. Panel N/T shows a control blot with non-treated BHD295P. All panels show results with the same exposure time. Lane -; immunoprecipitates from the cells transfected with empty vector. (D) Phosphatase treatment of FLCN. Flag-FLCN was immunoprecipitated with anti-Flag and the beads with immunocomplex were treated with (+) or without (−) CIAP. Proteins were analyzed by immunoblotting with indicated antibodies.

(S302) to Ala (S302A) showed reduced radioactivity of $^{32}P$ by metabolic labeling. To further characterize the FLCN phosphorylation at S302, we prepared a rabbit anti-phospho-S302 antibody. Specificity of this antibody was examined by immunoblot analysis of transiently expressed wild-type and S302A-substituted mutant FLCN proteins (Fig. 2B). Wild-type but not S302A FLCN reacted with this antibody. This reaction was inhibited by pre-absorption with antigen phospho-peptide or with phosphatase treatment (Fig. 2C and D). From these observations, we concluded that S302 of FLCN is phosphorylated and anti-phospho-S302 can be utilized for monitoring of FLCN phosphorylation at this site.

*Analysis of S62 and S302 phosphorylation*

To further clarify an involvement of tuberin in the regulatory mechanism for FLCN phosphorylation including at S302 as well as S62, we analyzed 1B1 cells with knockdown of *Tsc2*. This system was employed because the expression level of FLCN in 1B1 is high enough to detect with phospho-specific antibodies. Knockdown of *Tsc2* led to slower migrating FLCN bands as seen by immunoblot and phosphorylation at S302 was increased in normal growth-stimulating conditions, although S6K phosphorylation showed no obvious difference (Fig. 3A; rapamycin −, FCS +). The phosphorylation at S62 showed minimal increase. However, in serum-starved conditions, a significant increase in phosphorylation at S62 as well as S302 was observed by *Tsc2* knockdown (Fig. 3A; rapamycin −, FCS −). Under this condition, the phosphorylation of S6K was maintained in *Tsc2*-suppressed cells, indicating that the suppression of *Tsc2* was effective to downstream pathways. These data indicate that phosphorylation at S62 and S302 was regulated by the downstream pathway of tuberin in different manners. When Rheb was expressed under serum-starved conditions, the phosphorylation of S6K (Thr389) was stimulated as compared with mock-control (Fig. 3B). At the same time, increased phosphorylation of FLCN at S62 as well as S302, was observed (Fig. 3B). Whereas S6K phosphorylation was almost completely inhibited by rapamycin treatment, FLCN phosphorylation remained at the background level, suggesting that phosphorylation at S62 and S302 was regulated, at least in part, by mTORC1-independent pathway (Fig. 3A and B; rapamycin +).

When raptor was knocked-down in ERC33 cells, an apparent downward shift of the slowest migrating FLCN band to the

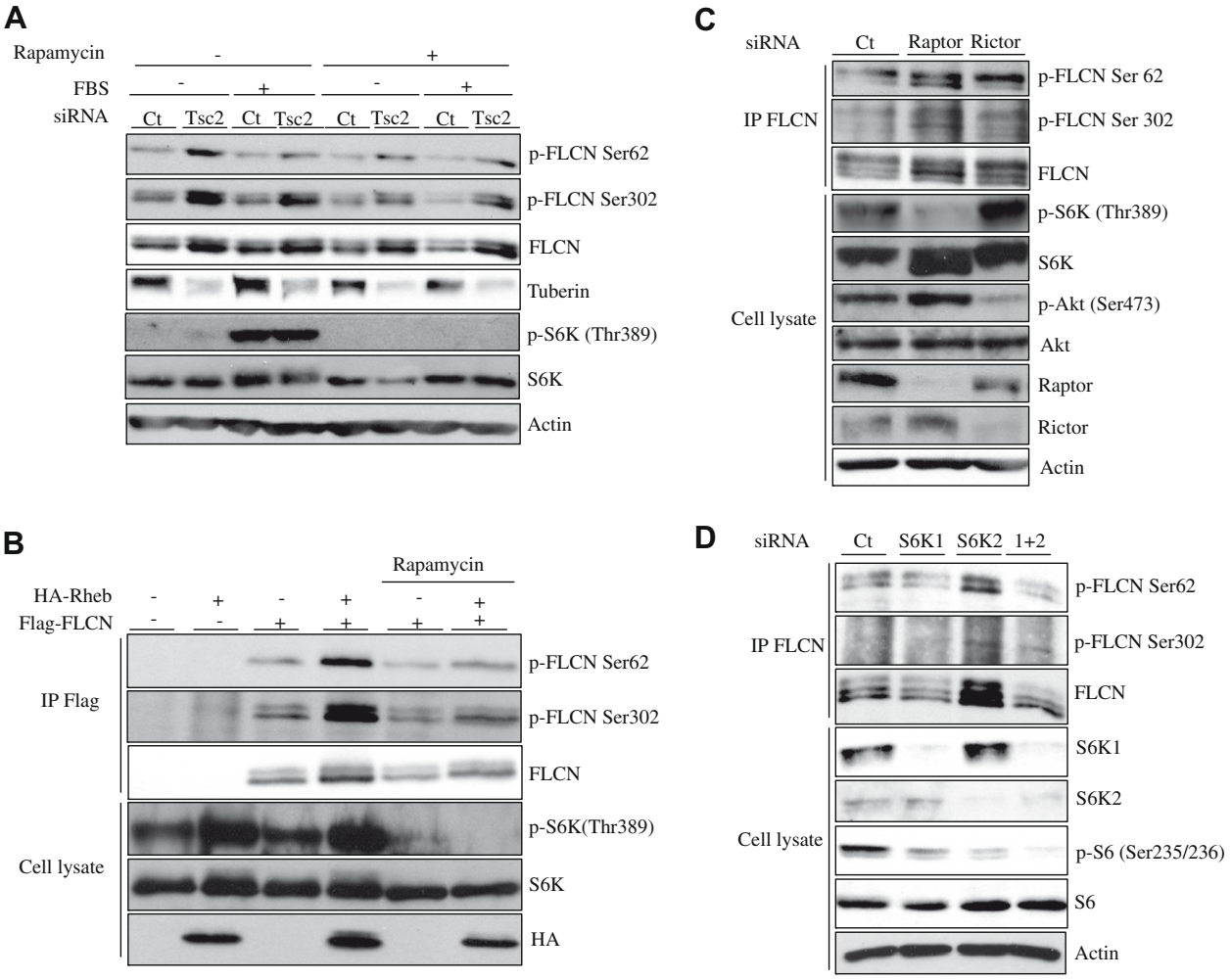


**Fig. 3.** mTORC1-dependent phosphorylation of FLCN analyzed with phospho-specific antibodies. (A) Examination of FLCN phosphorylation induced by *Tsc2* suppression. 1B1 cells were treated as described in Fig. 1B in the presence (Rapamycin +) or absence (Rapamycin −) of rapamycin (20 nM) during last 24 h. Cell lysates were analyzed by immunoblotting with indicated antibodies. (B) Rapamycin-sensitive FLCN phosphorylation induced by Rheb. HEK293 cells were transiently expressed with Flag-FLCN alone or together with HA-Rheb. Twenty-four hours after plasmid transfection, cells were serum-starved for 24 h, in the presence or absence of rapamycin (20 nM). Then cell lysates were subjected to immunoprecipitation with anti-Flag antibody and immunoblotting with indicated antibodies. (C) Changes in FLCN phosphorylation induced by downregulation of mTORC1 (raptor), but not mTORC2 (rictor). ERC33 cells were transfected with scramble (Ct), *raptor* or *rictor* siRNA. Forty-eight hours later, the cell lysates were subjected to immunoprecipitation with FLCN antibody and immunoblotting with indicated antibodies. (D) Effect of S6K1, S6K2 suppression on FLCN phosphorylation. ERC33 cells were transfected with scramble (Ct), *S6K1*, *S6K2* or both *S6K1* and *S6K2* siRNA and analyzed as in (C), with indicated antibodies.

middle one was observed, which is similar to that caused by rapamycin treatment (Figs. 1A and 3C). However, there seemed to be no obvious change in the total level of phosphorylation at S62 and S302. In contrast to raptor, the knockdown of rictor did not induce apparent changes in the pattern of FLCN bands nor in the level of S62 or S302 phosphorylation (Fig. 3C). These results suggest that mTORC1, but not mTORC2, participates in the rapamycin-sensitive mechanisms of FLCN phosphorylation. The reason why no apparent reduction in the level of S62 and S302 phosphorylation was observed by raptor knockdown is not clear. We speculate that the hyper-active state of mTORC1 in *Tsc2*-deficient ERC33 cells was not completely blocked by raptor siRNA treatment and therefore, still influenced S62 and S302 phosphorylation. Thus, the phosphorylation site which influences mobility in a rapamycin-sensitive manner may be different from S62 and S302.

To determine whether phosphorylation of FLCN is regulated by S6K1 or S6K2, we used knockdown of S6K1 and S6K2 in ERC33 cells. When S6K1 or S6K2 alone, or both S6K1 and S6K2 were suppressed, the pattern of FLCN bands did not significantly change (Fig. 3D). In the case of S6K2 knockdown, the amount of total FLCN was slightly increased (Fig. 3D). The amount of phosphorylated S62 or S302 in FLCN was increased in parallel with total FLCN. These results suggest that although S6K1 and S6K2 may have some role in the regulation of FLCN, they are not the major kinases responsible for phosphorylation at S62, S302 or at the site which contributed to mTORC1-dependent band shift.

### *Possibility of direct phosphorylation of FLCN by unknown kinase(s)*

To determine whether FLCN is directly phosphorylated by mTORC1 or mTORC2, we performed *in vitro* kinase assays using complex-specific immunoprecipitates prepared by anti-raptor or anti-rictor antibody from HeLa cells (Supplementary Fig. S1A). As a positive control, GST-S6K1 was phosphorylated when incubated with mTORC1 immunoprecipitates. However, in the same condition, FLCN-GST could not be phosphorylated (Supplementary Fig. S1B). Similarly, whereas Akt was phosphorylated by mTORC2 immunoprecipitates *in vitro*, FLCN-GST was not (Supplementary Fig. S1C). These results indicate that neither mTORC1 nor mTORC2 directly phosphorylate FLCN. In addition, we have not detected direct phosphorylation of FLCN by S6K1 (Supplementary Fig. S1D). Other unidentified kinase(s) downstream of mTORC1 may be responsible for rapamycin-sensitive FLCN phosphorylation at S62 and S302.

### *Enhancement of FLCN–AMPK complex formation by S302A substitution*

Previously, we found that substitution of S62 to aspartic acid enhanced complex formation of FLCN with α1 subunit of AMPK (AMPKα1) (L. Wang et al., submitted). In this study, we performed co-immunoprecipitation analyses to ascertain whether modifications at S302 influence complex formation of FLCN with FNIP1, FNIP2/FNIPL or AMPKα1 [10–12]. When full-length FLCNs with S302A or S302D (serine-to-aspartic acid substitution mimicking phosphorylation) mutations were tested, they did not exhibit a significant decrease in the binding activity with FNIP1 or FNIP2/FNIPL, compared with wild-type FLCN (Fig. 4A). We have found that the complex formation of FLCN with AMPKα1 could be detected by co-transfection/co-immunoprecipitation analyses in the absence of overexpressed FNIP proteins (Fig. 4B). Interestingly, S302A mutant, but not S302D mutant, formed a complex with AMPKα1 more efficiently than wild-type FLCN (Fig. 4B). These results suggest that S302 phosphorylation inhibits FLCN–AMPKα1 complex formation, independently from binding affinity between FLCN and FNIP proteins. Affinity of FLCN to AMPK as a substrate may be changed by S62 or S302 phosphorylation. Since AMPK is known to indirectly downregulate mTORC1 by phosphorylating and activating tuberin, FLCN phosphorylations analyzed in this study may be associated with unknown feedback mechanisms regulating mTOR-related pathways [10].

Relationship of mTORC1-dependent pathways with FLCN function and phosphorylation suggests that dysregulation of those pathways may play a role in the development of BHDS. We and others reported the possibility of mTOR activation by FLCN [12,15]. In recent reports using *Bhd* knockout mice, development of cysts and/or tumors in the kidney and activation of signal transduction systems including mTORC1-related pathway in those lesions were reported [16,17]. It is expected that FLCN could be both positive and negative regulator of mTORC1 in a context-dependent manner. With regard to FLCN phosphorylation at S62 and S302 sites, identification of responsible kinases and exploration of the effects on mTORC1- and/or AMPK-dependent pathways as well as other signaling pathways will facilitate the understanding of *BHD* mutation-related pathogenesis.

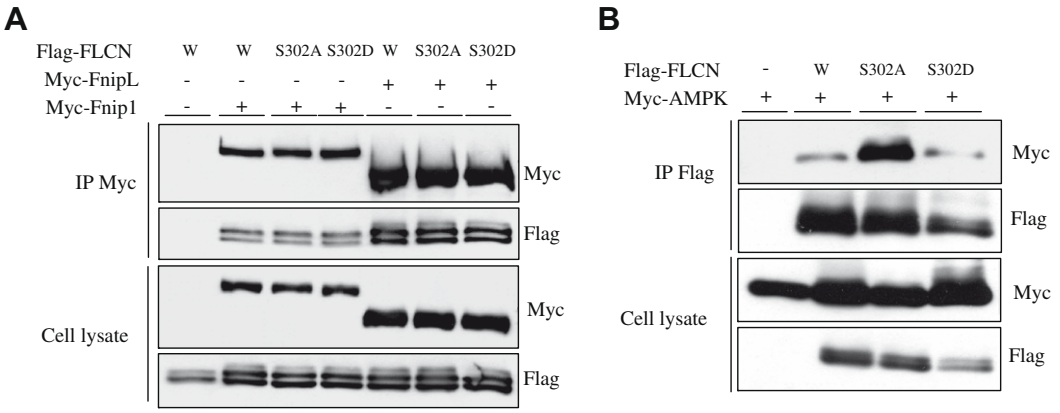


**Fig. 4.** Effects of S302A or S302D mutation in FLCN on the complex formation. (A) Binding of mutant FLCNs with FNIP1 or FNIP2/FNIPL. Cos7 cells were transfected with the expression plasmid for Flag-tagged wild-type (WT), S302A or S302D mutant FLCN together with that for Myc-tagged FNIP1 or FNIP2/FNIPL (+), or with empty vector (−). Cell lysates and immunoprecipitates with anti-Myc antibody (IP Myc) were analyzed by immunoblotting with indicated antibodies. (B) Binding of mutant FLCNs with AMPKα1 subunit. Myc-AMPKα1 was transiently expressed in Cos7 cells with or without (−) Flag-tagged wild-type (WT), S302A or S302D mutant FLCN. Assay by immunoprecipitation with anti-FLAG antibody was performed as in (A).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.070.

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