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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 γ-^{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 γ-^{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

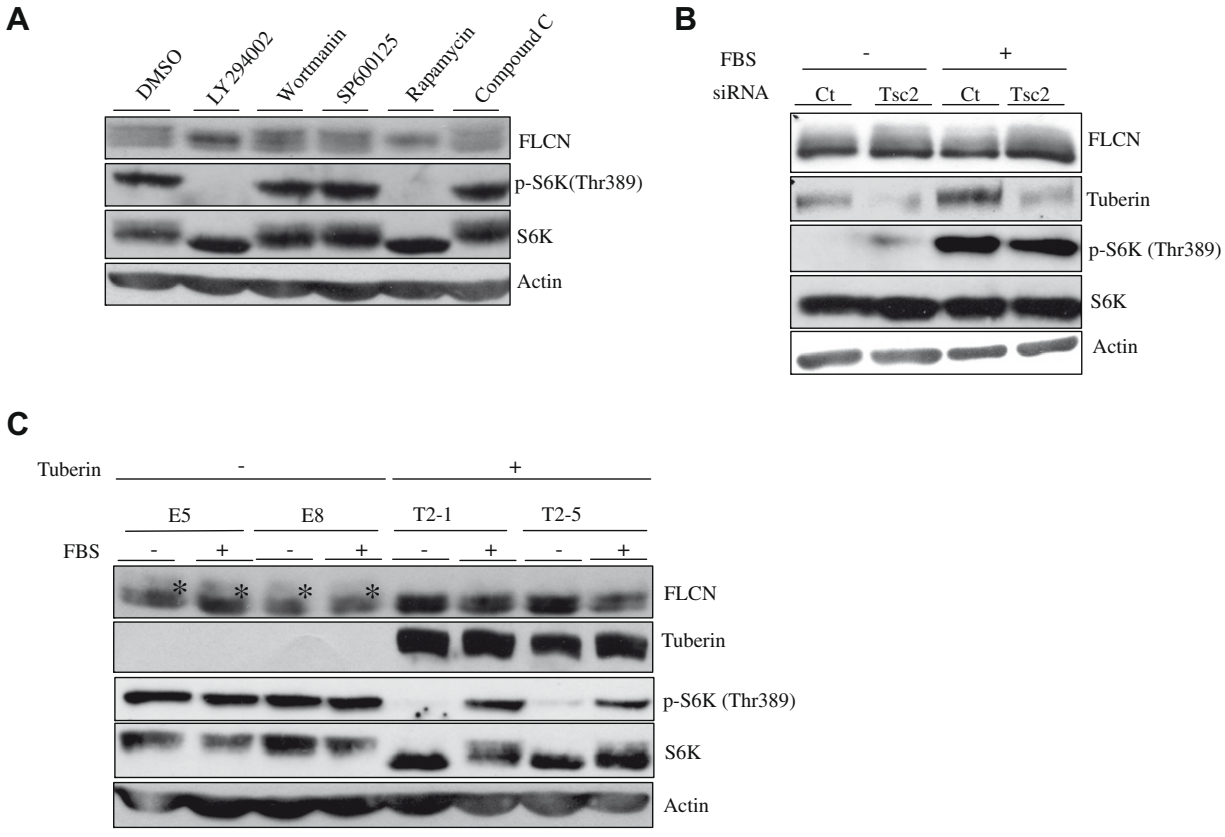

Fig. 1. Effects of drugs and tuberin expression on the migration of FLCN bands. (A) Drug treatments in ERC33 (*Tsc2*-deficient renal tumor cell line from the Eker rat) cells. ERC33 cells were treated with indicated drugs for 18 h. The cell lysates were analyzed by immunoblotting with indicated antibodies. (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).

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