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Serine 62 is a phosphorylation site in folliculin, the Birt–Hogg–Dubé gene product

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ABSTRACT

Recently, it was reported that the product of Birt–Hogg–Dubé syndrome gene (folliculin, FLCN) is directly phosphorylated by 5′-AMP-activated protein kinase (AMPK). In this study, we identified serine 62 (Ser62) as a phosphorylation site in FLCN and generated an anti-phospho-Ser62-FLCN antibody. Our analysis suggests that Ser62 phosphorylation is indirectly up-regulated by AMPK and that another residue is directly phosphorylated by AMPK. By binding with FLCN-interacting proteins (FNIP1 and FNIP2/FNIPL), Ser62 phosphorylation is increased. A phospho-mimic mutation at Ser62 enhanced the formation of the FLCN–AMPK complex. These results suggest that function(s) of FLCN–AMPK–FNIP complex is regulated by Ser62 phosphorylation.

Structured summary:
MINT-7298145, MINT-7298166: *Flcn* (uniprotkb:Q76JQ2) *physically interacts* (MI:0915) with *AMPK alpha 1* (uniprotkb:P54645) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7298267: *AMPK alpha 1* (uniprotkb:Q13131) *phosphorylates* (MI:0217) *tsc2* (uniprotkb:P49816) by *protein kinase assay* (MI:0424)
MINT-7298182: *FNIP1* (uniprotkb:Q8TF40) *physically interacts* (MI:0915) with *Flcn* (uniprotkb:Q76JQ2) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7298132: *AMPK alpha 1* (uniprotkb:Q13131) *phosphorylates* (MI:0217) *Flcn* (uniprotkb:Q76JQ2) by *protein kinase assay* (MI:0424)
MINT-7298229: *FNIPL* (uniprotkb:Q9P278) *physically interacts* (MI:0915) with *Flcn* (uniprotkb:Q76JQ2) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

Birt–Hogg–Dubé syndrome (BHDS) is an autosomal dominantly inherited syndrome and is characterized by the development of skin fibrofolliculomas and spontaneous pneumothorax, in addition to kidney cancers with chromophobic or oncocytic features [1]. The responsible gene for BHDS (*BHD*) is a tumor suppressor and has been identified by positional cloning [2]. We identified a germline mutation of the *BHD* homologue (*Bhd*) in the Nihon rat model of hereditary renal carcinoma [3].

To better understand the molecular mechanism of tumorigenesis caused by *BHD*/*Bhd*-deficiency, it is necessary to elucidate the function of BHD product (folliculin, FLCN). However, the physiological activity of FLCN has not yet been determined. Baba et al. identified an FLCN-binding protein, FNIP1, and demonstrated that FLCN is phosphorylated [4]. They also reported that FNIP1 binds 5′-AMP-activated protein kinase (AMPK), and that both FLCN and FNIP1 are phosphorylated by AMPK [4]. Phosphorylations of FLCN were influenced by treatment with an AMPK inhibitor, as well as rapamycin or by amino acid deprivation [4]. Our group and Hasumi et al. identified the second FLCN-binding protein, FNIP2/FNIPL, which is

Abbreviations: AMPK, 5′-AMP-activated protein kinase; FLCN, folliculin; FNIP, FLCN-interacting protein

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homologous to FNIP1 [5,6]. The level of FLCN, FNIP1 or FNIP2/FNIPL expression influenced the signaling pathway regulated by mammalian target of rapamycin kinase (mTOR), probably in a context-dependent manner [4–6]. From these observations, it has been suggested that FLCN is involved in energy and nutrient sensing through the AMPK and mTOR signaling pathways [4].

The rapamycin-sensitive function of mTOR is negatively regulated by AMPK and tuberous sclerosis tumor suppressor gene products (hamartin and tuberin) [7]. From the study of yeast mutants of the *BHD* homologue, opposite roles of FLCN and tuberin in TOR signaling have been suggested [8]. Thus, there may be a complex network of tumor suppressors involved in the regulation of mTOR. Detailed characterization of FLCN phosphorylation will provide important information that might allow us to clarify the function of FLCN in such a network.

In this study, we found that serine 62 (S62) is a major phosphorylation site on rat FLCN, and report on the production of an anti-phosphorylated FLCN antibody, which will be useful for the characterization of FLCN.

2. Materials and methods

2.1. General methods

For general methods (plasmid construction, cell culture, transfection, immunoblot analysis and immunoprecipitation), see Supplementary materials and methods.

2.2. Phosphatase treatment and metabolic labeling

Protein A/G beads (Calbiochem) with immunocomplex were washed three times with 0.5% Tween 20/Tris-buffered saline (TBST), then washed once with the buffer for calf intestinal alkaline phosphatase (CIAP, Takara). Beads were resuspended in 100 μl of CIAP buffer and treated with CIAP (80 units) for 20 min and then washed three times with TBST and subjected to further analysis. For metabolic labeling, Cos7 cells were transfected with plasmids for Flag-tagged proteins. After 48 h of culture, the medium was changed to phosphate-free DMEM (Gibco) supplemented with 10% dialyzed FBS (Gibco) and cells were incubated for 30 min. Then, ^{32}P-orthophosphate (GE Healthcare) was added to the medium (0.1 mCi/ml) and cells were labeled for 4 h and lysed for immunoprecipitation with anti-Flag antibody to detect ^{32}P incorporation.

2.3. Mass spectrometric analysis

Cos7 cells expressing FLCN-GST were lysed on ice in NP40 lysis buffer [6]. The lysate was mixed with Glutathione-Sepharose 4B beads (GE Healthcare) overnight at 4 °C and bound proteins were separated by a 10% SDS–PAGE and then visualized by silver staining. The FLCN bands were in-gel digested as previously described [9]. The tryptic peptides were extracted, the solvent was evaporated, and the peptides were redissolved in 10 μl of 1% formic acid. Mass spectrometry was performed using API-QSTAR pulsar *i* (Applied Biosystems) with a nanoliquid chromatograph (DiNa; KYA TECH Corporation) equipped with a 0.2 mm ID × 50 mm Magic C18 column. Amino acid sequences of the tryptic peptides were determined by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

2.4. Antibodies

Anti-phospho-FLCN (S62) was generated by immunizing rabbits with S62-phosphorylated peptide corresponding to aa 59–68 (Arg-Ala-His-Ser-Pro-Ala-Glu-Gly-Ala-Ser) of rat FLCN with amino-terminal cysteine for conjugation with haemocyanine (IBL). Antibodies were purified by antigen-affinity chromatography (IBL). For other antibodies, see the Supplementary materials and methods.

2.5. In vitro kinase assays

Cos7 cells expressing GST-tagged proteins were lysed on ice in NP40 lysis buffer. The proteins were purified with Glutathione-Sepharose 4B beads and treated with CIAP as described above and then the beads were incubated with or without active AMPKα1 subunit (Cell Signaling) in a solution containing 60 mM HEPES–NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, 0.5 μg/μl $PEG_{20.000}$, 100 μM ATP and 1 μCi/μl γ^{32}P-ATP (NEG002A; Perkin Elmer) at 30 °C for 20 min. The samples were subjected to SDS–PAGE and transferred to the nylon membrane, and then incorporation of ^{32}P was examined by autoradiography. In another system, in vitro kinase assay for AMPKα1 was performed using His-FLCN fragments as substrates. 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).

3. Results and discussion

3.1. Ser62 is a phosphorylation site in FLCN

During a previous study, which we conducted for the identification of the *Bhd* germline mutation, we observed that rat FLCN migrated as multiple bands in immunoblot analysis [4]. By using deletion mutants and metabolic labeling, it was suggested that the migration of FLCN was affected by phosphorylation in the amino-terminal region and that rat FLCN has multiple phosphorylation sites (Supplementary Fig. S1).

To identify the phosphorylation site in the amino-terminal region, we employed site-directed mutagenesis for candidate residues and examined its effects on the mobility of amino-terminal fragment (ΔC-ter) in immunoblots after transient expression in Cos7 cells. Among the mutations introduced, only the Ser62 (S62)-to-alanine (S62A) mutation completely eliminated slower migrated bands in ΔC-ter (Fig. 1A and data not shown). It did not completely abolish ^{32}P-incorporation in metabolic labeling, suggesting that other phosphorylation sites are present (Fig. 1A). Next, we searched for phosphorylation sites in the transiently expressed full-length FLCN–GST fusion protein by tandem mass spectrometry and a peptide phosphorylated at S62 was identified (Fig. 1B). In this analysis, no other phospho-peptide was detected.

To further analyze the FLCN phosphorylation at S62, we prepared a rabbit polyclonal antibody (BHD-P1) specific for the phospho-S62-containing FLCN peptide. We first found that BHD-P1 efficiently reacted with transiently expressed wild-type but not S62A mutant FLCN, in an upper band-specific manner (Fig. 1C). The reactivity of BHD-P1 was completely blocked by pre-treatment of antibody with phosphorylated antigen peptides, but not with control unphosphorylated peptides (Supplementary Fig. S2A). In addition, the reactivity of BHD-P1 was totally abolished by pre-treatment of FLCN with alkaline phosphatase (Supplementary Fig. S2B). These results suggested that BHD-P1 recognizes FLCN phosphorylated at S62. We tested and found the reactivity of BHD-P1 to endogenous FLCN from human, mouse and rat cell lines (Supplementary Fig. S2C and data not shown). Taking these results together, we concluded that FLCN is phosphorylated at S62 in vivo.

S62 and its surrounding region are conserved in terrestrial animal-specific manner (data not shown). As BHDS develops renal

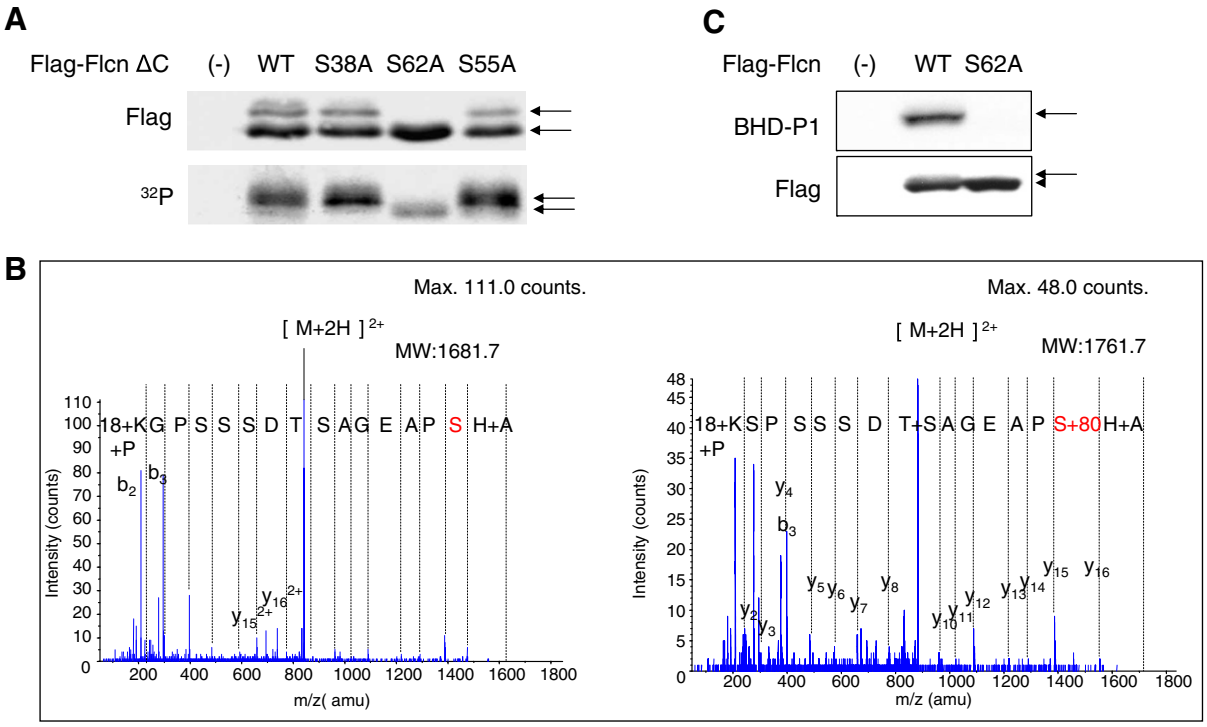

Fig. 1. Serine 62 (S62) is a phosphorylation site in FLCN. (A) Immunoblot and metabolic label analysis of site-directed mutants. Flag-tagged amino-terminal fragment of wild-type (WT) and mutant (S38A, S55A, and S62A) FLCNs, expressed in Cos7 cells, were detected by immunoblotting with anti-Flag antibody (upper panel). Arrows indicate two major bands of wild-type FLCN. ^{32}P-incorporation was detected by autoradiography after immunoprecipitation with anti-Flag antibody (lower panel). Note that the upper band was not detected and that the total intensity was diminished in the S62A mutant. (B) Mass spectrometry. Left and right panels show LC-ESI-MS/MS spectra of peptides containing non-phosphorylated and phosphorylated S62, respectively. (C) Immunoblot analysis with phospho-S62-specific antibody. Flag-tagged wild-type (WT) and S62A mutant (S62A) FLCN were immunoprecipitated with anti-Flag antibody from Cos7 cells and detected with phospho-S62-specific anti-FLCN (BHD-P1; upper panel) or anti-Flag (lower panel) antibody. Arrows and arrowheads indicate the position of the upper and lower of two major bands detected by anti-Flag. Note that BHD-P1 recognizes the upper band but not the lower band.

and/or pulmonary lesions, this conservation is interesting for us in terms of the species-specific as well as the tissue (respiratory or renal)-specific regulation of FLCN function.

3.2. Ser62 of FLCN is phosphorylated in the AMPK-related pathway, but is not a direct target of AMPK

We have detected an increase in the amount of slowly migrated bands of FLCN after co-expression with α1-subunit of AMPK, as well as several other kinases (Fig. 2A and B, and data not shown). Accordingly, the reactivity of FLCN protein with BHD-P1 was increased by co-expression of AMPKα1. This reactivity was partially reduced in FLCN from cells treated with an AMPK inhibitor (Compound C), but not with rapamycin, suggesting that the regulatory pathways for FLCN phosphorylation at S62 involve AMPK (Fig. 2A and B).

To determine whether S62 is directly phosphorylated by AMPK, we performed in vitro kinase assays in two different systems. Both wild-type and S62A mutant Flag–FLCN–GST fusion proteins, purified from Cos7 cells were labeled with ^{32}P by incubation with AMPK in vitro (Fig. 2D). Thus, the short amino-terminal and carboxy-terminal GST fusion may not perturb phosphorylation by AMPK. When deletion mutants were analyzed, ones covering residues from 293 to 356 were efficiently labeled (Fig. 2E). Consistent with these results, the bacterially-expressed fragments covering aa 293–356 of FLCN were phosphorylated whereas the amino-terminal region (aa 1–289) containing S62 was not (Supplementary Fig. S3). These data suggest that S62 of FLCN is not directly phosphorylated by AMPK.

As S62 is embedded in the consensus phosphorylation sequence for members of the AGC family kinase (R-X-R-X-X-S/T, where X means any residue) and is followed by a proline, some member(s) of the AGC family or proline-directed protein kinases might phosphorylate FLCN at S62 [10]. So far, we have not detected efficient kinase activities using S6K1, Akt, RSK1 or Sgk1 by in vitro kinase assay against FLCN [11]. Identification of the kinase responsible for S62 phosphorylation will clarify the complex relationship between AMPK-related pathways and the function of FLCN.

3.3. Ser62 phosphorylation of FLCN is induced by interaction with FNIP proteins and may affect the formation of a complex with AMPK

To elucidate the functional significance of S62 phosphorylation, we tested the activity of S62 mutant FLCN to form a complex with AMPK, FNIP1 and FNIP2/FNIPL [4–6]. When co-expressed with FNIP proteins, wild-type FLCN showed an increase in the amount of S62-phosphorylated form, consistent with previously reported results [4–6]. This suggests that phosphorylation at S62 is induced or stabilized by FNIP proteins. The increase in S62 phosphorylation by FNIP proteins was suppressed by Compound C, but not by rapamycin, suggesting that it is exerted by an AMPK-dependent mechanism (Fig. 3A). In the co-immunoprecipitation assay, both S62A and a phosphorylation-mimic serine-to-aspartic acid (S62D) mutants exhibited no significant change in the ability to bind FNIP proteins (Supplementary Fig. S4). Although the formation of a complex between FLCN and AMPKα1 was reported to be FNIP1-dependent, we detected their interaction in transient co-expression without FNIP1 expression (Fig. 3B). In the co-immunoprecipitation assay, the S62D mutant exhibited increased binding activity with AMPKα1 whereas the S62A mutant showed slightly reduced one compared with wild-type FLCN (Fig. 3B). These results suggest that FLCN phosphorylation at S62 may enhance or stabilize the formation of a complex in which AMPKα1 and FLCN are involved.

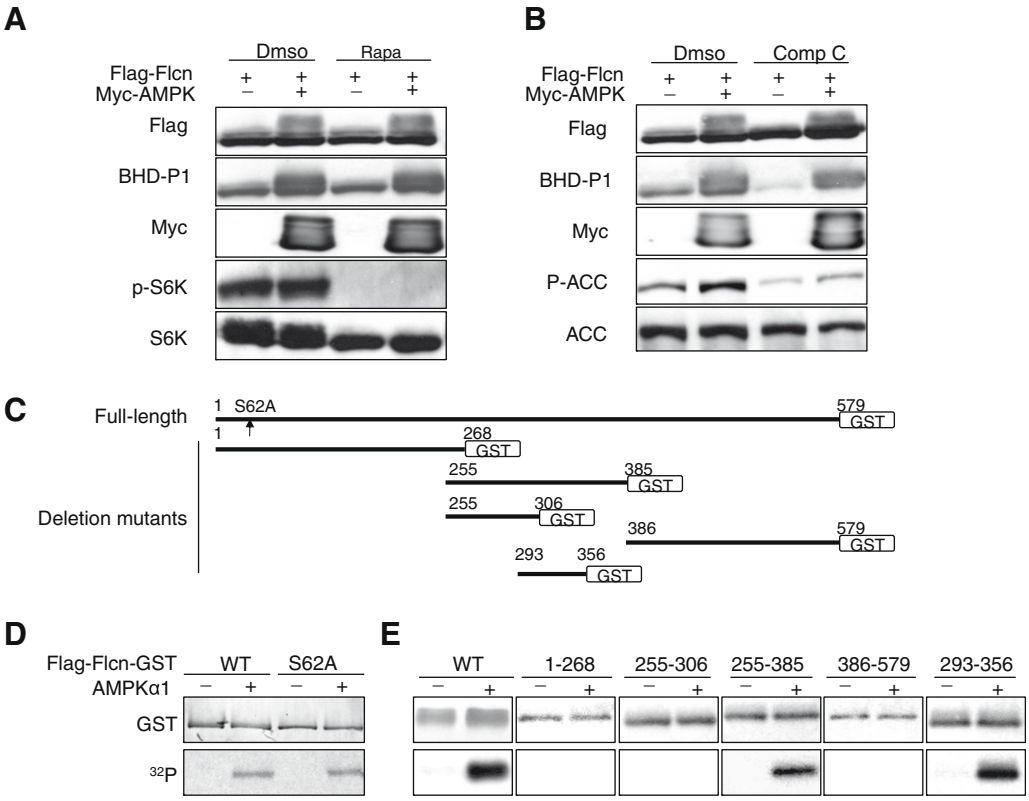

Fig. 2. S62 of FLCN is phosphorylated in the AMPK-related pathway, but may not be a direct target of AMPK. (A and B) Effects of rapamycin (A) and Compound C (B) on AMPK-induced S62 phosphorylation. Flag-FLCN was transiently expressed with or without Myc-AMPK in Cos7 cells. After 24 h, rapamycin (20 nM; Rapa in A), Compound C (30 μM; Comp C in B) or vehicle (DMSO) was added and cells were treated with drugs for 24 h. Immunoblottings were performed with the indicated antibodies: S6K, phospho-S6K; ACC, phospho-ACC. (C–E) In vitro kinase assay for AMPK using transiently expressed FLCN in mammalian cells. Recombinant proteins are schematically depicted in (C). Wild-type FLCN–GST (WT in D and E), full-length S62A mutant (S62A in D) and deletion mutants (aa numbers in E) were assayed. Upper panels show protein detection by anti-GST antibody and lower panels show ^{32}P incorporation.

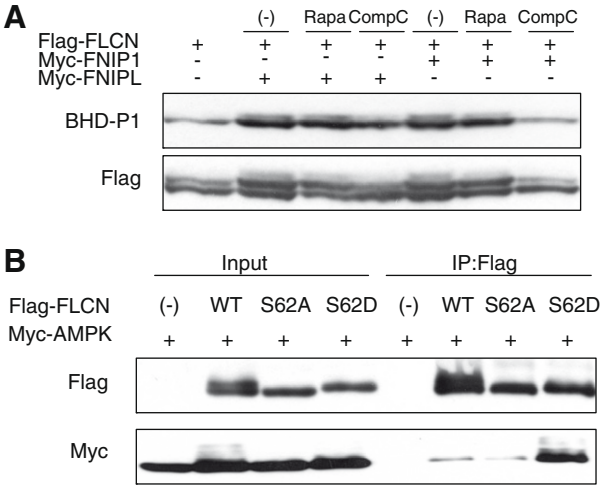

Fig. 3. Effects of S62A or S62D mutation of FLCN on the protein complex formation. (A) Effects of FNIP1 or FNIP2/FNIPL expression on S62 phosphorylation. Cos7 cells were transiently expressed with wild-type Flag-FLCN alone, or together with either Myc-FNIP1 (FNIP1) or Myc-FNIP2/FNIPL (FNIPL). Cells were treated with rapamycin (Rapa), Compound C (Comp C) or vehicle (−) for 24 h. The cell lysates 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), S62A or S62D mutant FLCN. 48 h later, cell lysates were subjected to immunoprecipitation with anti-Flag antibody. Total lysates (Input) and immunoprecipitates (IP) were analyzed by immunoblotting with indicated anti-tag antibodies.

Overall, the binding of FLCN with FNIP proteins may increase the FLCN–AMPKα1 complex via S62 phosphorylation. This may upregulate the direct phosphorylation of FLCN by AMPKα1 at residue(s) other than S62.

There may be complex mechanisms for FLCN phosphorylation in the AMPK and mTOR-related pathways. Phosphorylated FLCN-specific antibodies, such as BHD-P1, which were generated in this study, will be powerful tools for future studies. To unravel the molecular mechanism of tumorigenesis associated with *BHD* mutation, understanding of FLCN function is necessary. Further studies on the regulatory mechanism of FLCN phosphorylation will provide many important clues.

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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.febslet.2009.11.033.

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