**INTRODUCTION**

Extracellular stimuli, including growth factors (e.g. EGF), cytokines, and environmental stresses (e.g. ultraviolet), activate the receptor tyrosine kinase at the cytoplasmic membrane by dimerization-mediated autophosphorylation at the cytoplasmic domain [1]. The phosphorylated tyrosine residues of EGF receptor act as an initiation signal to recruit the Shc- Growth factor receptor-bound protein 2 (Grb2)-Ras complex and phospholipase C\(_\gamma\) in the cytoplasm, resulting in Son of sevenless-mediated Ras activation and intracellular Ca\(^{2+}\)-mediated protein kinase C activation [2]. Activated Ras induced activation of the signaling axis of Rafs, mitogen-activated protein kinase kinases (MEKs), extracellular signal-regulated kinases (ERKs), and p90 ribosomal S6 kinase (RSKs), including RSK2 which regulates cell proliferation, carcinogenesis, adaptation, apoptosis [3], and protein stability regulation [4]. ERK1 and ERK2 are downstream kinases of MEKs and approximately share an 85% amino acid similarity. ERKs activated by MEKs-mediated direct phosphorylation at Thr and Tyr residues in the activation loop phosphorylate or interact with a large number of cytosolic and nuclear substrates specifically involved in cellular signaling of transcription factors responsible for particular cellular processes [5]. Thus, dysregulation of the aforementioned cascade frequently leads to the development of diverse diseases, including > 90% of all cancers [6]. Due to the importance of ERK as an intermediary signaling molecules, its activity must be precisely regulated. Our previous study demonstrated that ERK 1 or 2 take part in forming the SCF\(_{\beta\text{TrCP}}\) complex that plays a key role in regulating ubiquitination-mediated protein stability, with Kruppel-like factor 4 (Klf4) [4].
cellular signaling pathways have been studied mainly with focus on phosphorylation-mediated transient activity regulation, the ERK 1 and 2 protein stability regulation has been poorly understood.

βTrCP 1 and 2 are members of F-box proteins, which confer substrate selectivity for ubiquitination. F-box proteins are currently classified according to their potential roles, such as tumor suppressors (FBXW7, FBXO11, FBXW8, FBXL3, FBXO1, FBXO4, and FBXO18), oncogenes (SKP2, FBXO5, and FBXO9), and context-dependent different functions (βTrCP1 and βTrCP2) [7,8]. Accumulating data suggest that the roles of βTrCP 1 and 2 in cellular phenotypes are context dependent [7]. Ectopic expression of βTrCP1 controlled under the mouse mammary tumor virus promoter developed tumors such as mammary, ovarian, and uterine carcinoma in about 38% transgenic mice [9]. Moreover, high tumors such as mammary, ovarian, and uterine carcinoma developed through the D, F-box, and linker domains of βTrCP1, Cullines, ERK2, and truncated and serial deleted βTrCP1 open reading frame was incorporated into multicloning sites of the pACT-VP16 mammalian two-hybrid vector. The open reading frame of kinases was amplified from the human kinase open reading frame kit, containing 556 distinct human kinases and kinase-related protein open reading frames in pDONR-223 Gateway® Entry vectors (Adgene, Watertown, MA, USA), and inserted into the pBIND-Gal4 mammalian two hybrid vector. For transient transfection of βTrCP1, Cullines, ERK2, and truncated and serial deleted βTrCP, mammalian expression vectors pcDNA3-HA, pcDNA3-Myc, and pcDNA4-HisMax-Awere utilized. All recombined expression vectors were confirmed by DNA sequencing.

Mammalian two-hybrid assay
To screen for protein-binding partners, we conducted mammalian two-hybrid assays in accordance with the Promega Checkmate mammalian two-hybrid system protocols (Promega, Madison, WI, USA). HEK293T cells (2 × 10⁶ cells/well) were seeded into 48-well plates and maintained with 10% FBS-DMEM for 18 hours before conducting transfection. The vectors pACT-VP16-βTrCP1, pBIND-Gal4-kinases, and pG5-luciferase were mixed at the same molar ratios (1:1:1) and the total amount of DNA was no more than 100 ng per well. Transfection was done utilizing jetPEI according to the manufacturer’s recommendations. For the luciferase assay, the cells were disrupted by directly adding a cell lysis buffer and gently shaking for 30 minutes at room temperature. Then, 60 μL aliquot was added to each-well luminescence plate. Luminescence activity was automatically measured through a VICTOR X3 plate reader (PerkinElmer, Waltham, MA, USA). To evaluate transfection efficiency, relative lucifere...
erase activity was calculated based on the pG5-luciferase basal control and was normalized against Renilla luciferase activity, which was included in the pBIND vector.

Gene silencing

To silence βTrCP1 or ERK2 in HEK293T, lentiviral expression plasmids of pLenti-sh-ERK2 (Dharmacon, Lafayette, CO, USA) were co-transfected into HEK293T cells with pSPAX2 and pMD2.G (Addgene, Cambridge, MA, USA) as indicated by the manufacturer’s recommended protocols. At 24 hours and 48 hours after transfection, we obtained a Lenti-sh-ERK2 medium containing viral particles from the HEK293T cells. The medium was filtered with a 0.45 µm filter (Cat. #: 723-2545, Thermo Fisher Scientific, Waltham, MA, USA) and used, with 1 to 2 µg/mL of polybrene, to infect HEK293T. After a maximum of 16 hours, the cell medium was exchanged with fresh complete medium. After 48 hours of maintenance, non-infected control cells were killed over a period of 3 days by treatment with 2 µg/mL of puromycin (Cat. #: A111308, Thermo Fisher Scientific). Surviving cells were immediately examined to determine protein levels by Immunoprecipitation (IP) and Western blotting.

Western blot analysis

Samples containing equal amounts of proteins (30 to 50 µg) were resolved by 8% to 10% SDS PAGE and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 5% skim milk and hybridized with specific primary and HRP-conjugated secondary Abs as indicated. The membranes were washed, and target proteins

**Figure 1. ERK2 and βTrCP1 are components of the Cullin 1 containing SCF βTrCP1 complex.** (A) Mammalian two-hybrid assay screening. Mammalian two-hybrid recombinant plasmids including pACT-VP16-βTrCP1, pG5-luciferase reporters, and each of pBIND-Gal4-kinases (as indicated) were transfected into HEK293T cells with a molar ratio 1:1:1. Relative luciferase activity was converted by comparison to luciferase activity obtained from pACT-VP16-βTrCP1/pBIND-Gal4-mock/pG5-luc. Equal transfection was normalized by Renilla luciferase activity obtained from each of pBIND-Gal4-kinase expression vectors. (B) Confirmation of βTrCP1 and ERK2 interaction obtained through IP, pBIND-Gal4-MAPKs (as indicated) and pcDNA3-HA-βTrCP1 were co-transfected into HEK293T cells. The interaction of ERK2 and βTrCP1 was visualized by IP/Western blotting as indicated. (C) Confirmation of ERK2 and Cullin 1 interaction, pcDNA3-Myc-Cullins and pcDNA4-HisMAX-ERK2 were co-transfected into HEK293T cells. The interaction of ERK2 and each of the Cullins was visualized by IP/Western blotting as indicated. CDK, cyclin-dependent kinase; DAPK, death-associated protein kinase; GRK5, G protein-coupled receptor kinase 5; LCK, lymphocyte-specific protein-tyrosine kinase; NEK6, NIMA-related kinase 6; CSK, C-terminal SRC kinase; STK16, serine/threonine-protein kinase 16; TOPK, lymphpokine-activated killer T-cell-originated protein kinase; PAK2, p21 protein-activated kinase 2; SGK, serum/glucocorticoid-regulated kinase; ERK, extracellular signal-activated kinase; JNK2, c-Jun N-terminal kinase 2; IP, immunoprecipitation; HA, hemagglutinin; WCL, whole cell lysates.
were visualized by an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) using a Chemidoc XRS* imager (Bio-Rad Laboratories, Hercules, CA, USA).

**IP**

HEK293T cells (2 × 10^6) were seeded into 100-mm dishes and incubated overnight. Individual expression vectors were transfected into HEK293T with jetPEI as indicated, and the cells were incubated for 24 hours. Protein samples from the cells were extracted by using a NP-40 cell lysis buffer. IP was conducted with the same amount of each sample and the Ab specific to the vector-transfected sample. The protein extracts were combined with protein G beads (50% slurry) (Cat. #: 17-0618-02, Protein G Sepharose 4 Fast Flow, GE Healthcare, Little Chalfont, UK) by rocking at 4°C for at least 5 hours or overnight. The protein G beads were washed and mixed with 6× SDS sample buffer and boiled. The precipitated proteins were resolved by 10% to 15% SDS PAGE and detected by Western blotting using specific Abs as indicated.

**Statistical analysis and figure panels**

Mammalian two-hybrid screening data was obtained from a triplicated experiment. Data are expressed as mean ± SEM values. Student’s t-test using the Microsoft Excel program (Microsoft, Redmond, WA, USA) was used to compare values between two groups. P-values < 0.05 (two-tailed) were considered significant. The Western blotting and IP experiments were conducted at least twice. The figure panels are representative photographs for Western blotting.

**RESULTS**

**ERK2 and βTrCP1 are components of Cullin 1 SCF complex**

To identify the new binding partners to βTrCP1, we conducted a mammalian two-hybrid assay using kinases which were constructed in our laboratories. We found that mitogen-activated protein kinases (MAPK), including ERK2, ERK3, p38α, p38δ, and JNK2, showed a relatively high interaction strength (about 3-5.4 folds) compared to other kinases, including CDK1 (cyclin-dependent kinase 1), CDK10, CHK1 (cell cycle

![Figure 2. ERK2 binds to F and linker domains of βTrCP1.](http://www.jcpjournal.org)

(A) Amino acid alignment of putative ERKs docking sites in βTrCP1. MAPK docking sequence was noted in footnote. (B) Construction strategies for βTrCP1 truncated mutants. The mutants were recombined into pcDNA3-HA. (C) The constructs of (B) were co-transfected as indicated. (D) Construction strategies for βTrCP1 intradomain deletion mutants. The mutants were recombined into pcDNA3-HA. (E) Interaction domain deciphering of βTrCP1 to ERK2. The constructs in (D) were co-transfected as indicated. (C, E) The interaction of ERK2 and each of βTrCP1 mutants were visualized by IP/Western blotting as indicated. MKP3, mitogen-activated protein kinase phosphatase 3; ERK, extracellular signal-regulated kinase; PTPN5, protein-tyrosine phosphatase nonreceptor type 5; DUSP8, dual-specificity phosphatase 8; MEK1, MAP kinase kinase 1; RSK1, ribosomal S6 kinase 1; N. D., not determined; MAPK, mitogen-activated protein kinases; D, D domain; F, F-box domain; FL, full length; IP, immunoprecipitation; HA, Hemagglutinin; WCL, whole cell lysates, Xp, Xpress.

http://www.jcpjournal.org
check kinase 1), DAPK2 (death-associated protein kinase 2), DAPK3, GRK5 (G protein-coupled receptor kinase 5), LCK (lymphocyte-specific protein-tyrosine kinase), NEK6 (NI-
MA-related kinase 6), CSK (C-terminal SRC kinase), STK16 (serine/threonine-protein kinase 16), TOPK (lymphokine-activated killer T-cell-originated protein kinase), PAK2 (p21 protein-activated kinase 2), SGK (serum/glucocorticoid-regu-
lated kinase) (Fig. 1A). Since \( \beta \)TrCP1 was a member of Cullin 1 containing SCF complex [13], ERK2 showed a band in IP with Cullin 1 when IP was conducted with Myc Ab (Fig. 1C, top panel). Additionally, the His-ERK2 protein levels in WCL were similar to other lanes in which interaction with the MAPK members, IP was utilized to confirm binding to the following groups: ERK1, ERK2, ERK3, JNK1, JNK2, JNK3, p38\(\alpha\), p38\(\beta\), p38\(\gamma\), and p38\(\delta\) (Fig. 1B). The IP of \( \beta \)TrCP1, using the HA tag Ab, showed strong coprecipitation with ERK2 (Fig. 1B, upper panel of IP). ERK3, p38\(\beta\), and p38\(\delta\) bands coprecipitated with \( \beta \)TrCP1 were weak compared to ERK2, which was attributable to the lower expression levels of these proteins in the whole cell lysates (WCL) (Fig. 1B, upper panel). Notably, since \( \beta \)TrCP1 has been classified as a member of the Cullin 2, 3, 4A, 4B, and 7 (Fig. 1C, 3rd panel from top). These results indicate that ERK2 is a new interacting partner with \( \beta \)TrCP1.

ERK2 binds to F-box and linker domains of \( \beta \)TrCP1

After the discovery of the interaction between \( \beta \)TrCP1 and ERK2, the ERK2-binding domains of \( \beta \)TrCP1 were then determined. Since ERK2 is a member of MAPK family proteins, and ERKs docking consensus sequences are published as (R/K)(R/K)xxxxx(L/V/I) [14], we searched whether \( \beta \)TrCP1 contained the amino acid sequences or not. Surprisingly, \( \beta \)TrCP1 harbored two consensus sequences in the F-box domain and the linker domain between the F-box and WD40 domains (Fig. 2A). The conserved KK-----V (referred to as PEDS1) and RR-----L (referred to as PEDS2) were located at amino acid 190 to 197 and 209 to 216 (Fig. 2A). To decipher more detail, we constructed \( \beta \)TrCP1 deletion mutant expression vectors using a pcDNA3-HA tag expression vector (Fig. 2B). The IP experiment using cell lysates expressing His-ERK2 and each of HA-mock, \( \beta \)TrCP1-FL, \( \beta \)TrCP1-1-228, and \( \beta \)TrCP1-229-569 showed that ERK2 was co-immuno-
pcipitated with \( \beta \)TrCP1-FL and \( \beta \)TrCP1-1-228 but not HA-mock and \( \beta \)TrCP1-229-569 (Fig. 2C). To verify the interaction between aa 190-216 of \( \beta \)TrCP1 and ERK2, we constructed \( \beta \)TrCP1 truncated mutant expression vectors: \( \beta \)TrCP1-dD-F-box and -dD-L (Fig. 2D). We confirmed that none of \( \beta \)TrCP1 truncated proteins co-immunoprecipitated with ERK2, while HA-\( \beta \)TrCP1-FL presented a strong co-immunoprecipitated band for ERK2 (Fig. 2E). Since the WD40 domain of \( \beta \)TrCP1 is located in \( \beta \)TrCP1-229-569, these results suggested that ERK2 and \( \beta \)TrCP1 binding may not trigger the ERK2 protein stability, but rather ERK2-mediated \( \beta \)TrCP1 phosphorylation.

**Determination of \( \beta \)TrCP1 binding sites to ERK2**

Our previous results indicated that PEDS1 and PEDS2 of \( \beta \)TrCP1 located in the F and linker domains played a key role in the interaction between \( \beta \)TrCP1 and ERK2. Since ERK 1 and 2 play a pivotal role in intracellular signal transduction activated by diverse mitogenic stimuli that include growth factors [15,16], we hypothesized that PEDS1 and PEDS2 of \( \beta \)TrCP1 might have been highly conserved in different spe-
cies. Thus, we conducted comparative analysis of \( \beta \)TrCP1 in various species including mouse, rat, cow, monkey, snake, salmon, ant, and xenopus (Fig. 3A). Despite varying numbers of amino acids from the N-terminus, it was found that amino acid sequences for the ERK docking sites were perfectly conserved across species.

**Figure 3. Determination of ERK2 binding sites of \( \beta \)TrCP1.** (A) Amino acid homology of putative ERK2 docking sites (PEDS). Amino acids for two putative ERK2 docking sites were compared to different animal species as indicated. The numbers at the top indicate the position of amino acids. The red color indicates amino acids for putative ERK2 docking sites. (B) Mutation strategies to construct mutant PEDS1 and PEDS2. (C) Confirmation of \( \beta \)TrCP1 PEDS 1 and 2 for ERK2 binding. The pcDNA4-HisMAX-ERK2 and each of the constructs in (B) were co-transfected as indicated. The interaction between ERK2 and each of \( \beta \)TrCP1 mutants was visualized by IP/Western blotting as indicated. (D) The constructs in (B) were transfected into HEK293T cells. Endogenous Cullin 1 binding to \( \beta \)TrCP1-mtPEDS1 and -mtPEDS2 were visualized by IP/Western blotting as indicated. WT, wildtype; D, D domain; F, F-box domain; mtPEDS, mutant putative ERK docking site; Xp, Xpress tag; ERK2, extracellular signal-regulated kinase 2; IP, immunoprecipitation; HA, hemagglutinin; WCL, whole cell lysates.
conserved in these animal species (Fig. 3A). To confirm whether the PEDS1 and PEDS2 played an essential role in the interaction with ERK2, we constructed two different mutants of βTrCP1: βTrCP1-mtPEDS1 and βTrCP1-mtPEDS2. βTrCP1-mtPEDS1 was constructed by replacing the lysines at aa 190 and 191 and valine at aa 197 to alanine and βTrCP1-mtPEDS2 by replacing the arginines at aa 209 and 210 and leucine at aa 216 to alanine (Fig. 3B). The essential role of PEDS 1 and 2 of βTrCP1 in the interaction with ERK2 was proved by the IP using Xp-ERK2 and each of HA-βTrCP1-PEDS1 or -PEDS2. We found that ERK2 IP with Xp-tag Ab showed a dramatic decrease of both HA-βTrCP1-PEDS1 or -PEDS2 (Fig. 3C). Importantly, we further found that the HA-βTrCP1-PEDS1 and -PEDS2 decreased the co-immunoprecipitated Cullin 1 (Fig. 3D). These results indicated that the amino acids, such as lysines at 190 and 191, valine 197, arginines at 209 and 210 and leucine 216, play a role in not only ERK2 docking, but also Cullin 1-mediated SCF complex formation.

**ERK2-mediated βTrCP1 phosphorylation affects βTrCP1 stability**

Our previous results suggested that ERK2 and βTrCP1 interaction may involve ERK2-mediated βTrCP1 phosphorylation. Since EGF-mediated signaling pathways induce ERK 1 and 2 phosphorylation and activation, we examined EGF-induced phosphate and time-dependent change of βTrCP1 (Fig. 3E). We found that EGF stimulation induced βTrCP1 phosphorylation (Fig. 3E). To confirm that the IP of βTrCP1 phosphorylation, HEK293T cells transiently expressing HA-βTrCP1 were utilized. (E) ERK2 reduces the half-life of βTrCP1. The pcDNA3-HA-βTrCP1 was co-transfected with pBIND-Gal4-mock or -ERK2 into HEK293T cells. The cells were treated with cycloheximide (10 µg/mL) and harvested at the indicated time points. The βTrCP1 protein levels were visualized by Western blotting as indicated. (F) ERK2 knockdown attenuates βTrCP1 destabilization. HEK293T cells stably expressing sh-ERK2 cells were harvested at the indicated time point after CHX (10 µg/mL) treatment. The proteins were visualized by Western blotting using specific antibodies as indicated. (G) IP:HA-βTrCP1, resulting in βTrCP1 inter-

---

Figure 4. ERK2-mediated βTrCP1 phosphorylation affects βTrCP1 stability. (A) EGF stimulation induces βTrCP1 phosphorylation. The HEK293T cells transiently expressing HA-βTrCP1 were starved, stimulated with EGF, and harvested as described in Materials and Methods. (B) ERKs Inhibition suppresses βTrCP1 phosphorylation. The HEK293T cells transiently expressing HA-βTrCP1 were starved, stimulated with EGF or the EGF/MEK inhibitor U0126 as indicated, and harvested after 30 minutes. (C) ERK2 knockdown suppresses βTrCP1 phosphorylation. HEK293T cells stably expressing sh-ERK2 cells were transfected with pcDNA3-HA-βTrCP1. (D) Disruption of βTrCP1 and ERK2 interaction prevents βTrCP1 phosphorylation. Cell lysates transiently expressing βTrCP1 in HEK293T cells were utilized. (E) ERK2 reduces the half-life of βTrCP1. The pcDNA3-HA-βTrCP1 was co-transfected with pBIND-Gal4-mock or -ERK2 into HEK293T cells. The cells were treated with cycloheximide (10 µg/mL) and harvested at the indicated time points. The βTrCP1 protein levels were visualized by Western blotting as indicated. (F) ERK2 knockdown attenuates βTrCP1 destabilization. HEK293T cells stably expressing sh-ERK2 cells were harvested at the indicated time point after CHX (10 µg/mL) treatment. The proteins were visualized by Western blotting using specific antibodies as indicated. (G) IP:HA-βTrCP1, resulting in βTrCP1 inter-
βTrCP1 phosphorylation. To verify βTrCP1 phosphorylation, we conducted IP using HA-tag Ab by combining the cell lysates transiently expressing HA-βTrCP1 and stimulated with EGF. The phosphorylation levels of HA-βTrCP1 were observed by Western blotting using phospho-serine/threonine Ab. The results indicated that the HA-βTrCP1 phosphorylation level by EGF stimulation was increased at 15 minutes, sustained to 60 minutes, and decreased at 120 minutes (Fig. 4A). The induction pattern of HA-βTrCP1 phosphorylation was similar to ERK 1 and 2 phosphorylation by EGF stimulation (Fig. 4A). Interestingly, HA-βTrCP1 total protein levels in whole cell lysates had an inverse correlation with ERK 1/2 phospho- and total-protein levels (Fig. 4A). Importantly, the MEKs inhibitor U0126 treatment completely blocked HA-βTrCP1 phosphorylation induced by EGF stimulation (Fig. 4B). By Western blotting using the whole cell lysates, EGF-induced phosphorylation of ERK 1 and 2 disappeared with MEKs inhibitor U0126 treatment (Fig. 4B). Notably, genetic knockdown of ERK2 using pLenti-sh-ERK2 (Fig. 4C, 3rd panel from top) suppressed phosphorylation of HA-βTrCP1 (Fig. 4C, top panel). The evidence indicating that ERK2 is an upstream kinase of βTrCP1 was provided by IP/Western blotting using cell lysates transiently expressing HA-βTrCP1-WT, HA-βTrCP1-mtPEDS1 and HA-βTrCP1-mtPEDS2 (Fig. 4D). The results demonstrated that disruption of ERK2 and βTrCP1 interaction blocked HA-βTrCP1 phosphorylation (Fig. 4D). Surprisingly, ectopic co-expression of ERK2 and βTrCP1 reduced total protein levels and the half-life of HA-βTrCP1 protein after cycloheximide treatment (Fig. 4E). In contrast, ERK2 knockdown using sh-ERK2 attenuated the βTrCP1 protein reduction, but not strong, by cycloheximide treatment (Fig. 4F). Taken together, these results indicate that ERK2 is an upstream kinase of βTrCP1 and growth factor-induced βTrCP1 phosphorylation by ERK2 reduces the half-life of βTrCP1 (Fig. 4G).

**DISCUSSION**

EGF-mediated signaling pathways are well-known oncogenic signaling pathways regulating cell proliferation, cell transformation, metastasis, and apoptosis [3,15]. Stimulation at the cytoplasmic membrane evokes activation signals and transduces the activation signal to the nucleus via a phosphate-delivery system. Since the members are stimulated by diverse mitogenic factors, including growth factors, serum, cytokines, hormones and environmental stresses, the members are referred to as MAPK, which consists of ERKs, p38 kinases, and JNK [16]. Although Ras proteins, upstream signaling molecules of ERKs, often show constitutively active mutations with high percentage in many human solid cancers [17], we were curious about why ERKs mutations have not been reported in human solid cancer. We hypothesized that 1) since ERKs play a pivotal role in cell survival, ERKs mutation may be lethal to the cells, resulting in spontaneous elimination 2) since ERKs’ roles are backed up by other ERK isozymes, apparent phenotypes are not observable; and 3) since ERKs roles are trivial in biological processes, ERKs mutations may have not affected the manifestation of phenotypes. However, for the last several decades, research has emphasized the importance of ERK as a prime signaling molecule [16]. Recently, we found that ERK1 and 2 induce Klf4 phosphorylation, resulting in the formation of a SCF^{Klfl} complex and Klf4 degradation [4]. Although there is no evidence that the SCF^{Klfl}-Klf4 complex contains ERK 1 or 2, it was found that ERK2 is a new binding partner of βTrCP1 (Fig. 1). Thus, ERK2 might affect the biological processes depending on not only the protein stability regulation, but also gene transcription.

The role of βTrCP 1 and 2 are controversial. Since βTrCPs mRNA and protein levels are increased in 56% of colorectal cancers and showed poor prognosis [10]. Moreover, hepatoblastoma [11] and some breast cancers [18] showed high expression of βTrCP1 and βTrCP2. NF-κB activation was observed in hepatocellular carcinoma [19]. Since the constitutively active NF-κB was associated with βTrCP-mediated IkB degradation [20], the signaling pathways produced by various cytokines, growth factors, and diverse stresses might be regulated by βTrCP. In contrast, somatic mutations of βTrCP1 and βTrCP2 that abolish E3 ligase activity were detected in human gastric cancer [12,21]. These mutations increase β-catenin stabilization in gastric cancer tissues [21]. Thus, the role of βTrCPs is context-dependent as a tumor suppressor or oncogene. Since our research has demonstrated that RSK2, downstream of ERK 1 and 2, enhances NF-κB trans-activation activity [22], ERK 1 and 2-mediated tumorigenesis might have a connection with βTrCP-mediated protein stability regulation.

In this study, we found that βTrCP1 and ERK2 interaction is mediated via F and linker domains of βTrCP1, but not the WD40 domain (Fig. 2 and 3). Since substrates of F-box proteins generally interact with the WD40 domain, this point was crucial in hypothesizing that the interaction between βTrCP1 and ERK2 was not aimed to degrade ERK2 proteins. However, since ERK2 phosphorylates βTrCP1 (Fig. 4), the ERK2 and βTrCP1 interaction might affect βTrCP1 enzymatic activity although the detailed mechanisms are unknown. We have considered a possible mechanism. Phosphorylation plays a key role in protein-protein interaction as well as protein degradation, especially in ubiquitin-proteasome systems [23]. In fact, many proteins such as c-Myc, cyclin E, c-Jun, Notch1 and androgen receptor are degraded by E3 ubiquitin ligase after phosphorylation by specific kinases, respectively [24-27]. Although we did not identify specific E3 ligase that regulates the protein stability of βTrCP1, we found that ERK2 could phosphorylate βTrCP1 and that the mutants of βTrCP1 (mtPEDS1 and mtPEDS2) decreased the interaction with ERK2 (Fig. 3C). Furthermore, overexpression of ERK2 reduced βTrCP1 protein half-life under cycloheximide treatment
ERK2 is still unknown. In independent PEDSs; however, the phosphorylation site(s) by processes [28]. Since at least 200 ERK substrates have been ERK1 and ERK2 which play essential roles in diverse cellular processes [29]. In this study, we found that substrates occurs at Pro-X-Ser/Thr-Pro consensus sequenc- as being a proline-directed kinase, the phosphorylation of ERKs activation by protein-protein interaction [16,28]. As ERK2 and their substrates and stimulus-dependent signaling axis might be dependent on the subcellular distribution of ERKs and their substrates and stimulus-dependent signaling axis activation by protein-protein interaction [16,28]. As ERK2 being a proline-directed kinase, the phosphorylation of ERKs substrates occurs at Pro-X-Ser/Thr-Pro consensus sequenc- es [29]. In this study, we found that βTrCP1 contained two independent PEDSs; however, the phosphorylation site(s) by ERK2 is still unknown.

ACKNOWLEDGMENTS

This research was funded by the Ministry of Science, ICT and Future Planning (NRF-2020R1A2B5B02001804 and NRF- 2020R1A4A2002894), and the Ministry of Education (BK21- 4th-sponsored Advanced Program for SmartPharma Leaders 4299990814607).

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

ORCID

Cheol-Jung Lee, https://orcid.org/0000-0002-0322-2018
Ga-Eun Lee, https://orcid.org/0000-0001-8700-209X
Hyun-Jung An, https://orcid.org/0000-0002-3189-7944
Eun Suh Cho, https://orcid.org/0000-0001-5373-2916
Weidong Chen, https://orcid.org/0000-0002-4015-6005
Joo Young Lee, https://orcid.org/0000-0002-6020-3040
Han Chang Kang, https://orcid.org/0000-0003-0696-1155
Hye Suk Lee, https://orcid.org/0000-0003-1055-9628
Yong-Yeon Cho, https://orcid.org/0000-0003-1107-2651

REFERENCES

1. Hubbard SR, Miller WT. Receptor tyrosine kinases: mechanisms of activation and signaling. Curr Opin Cell Biol 2007;19:117-23.
2. Cullen PJ, Lockyer PJ. Integration of calcium and Ras signalling. Nat Rev Mol Cell Biol 2002;3:339-48.
3. Arul N, Cho YY. A rising cancer prevention target of RSK2 in human skin cancer. Front Oncol 2013;3:201.
4. Kim MO, Kim SH, Cho YY, Nadas J, Jeong CH, Yao K, et al. ERK1 and ERK2 regulate embryonic stem cell self-renewal through phosphorylation of Klf4. Nat Struct Mol Biol 2012;19:283-90.
5. Yoon S, Seger R. The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. Growth Factors 2006;24:21-44.
6. Maik-Rachline G, Seger R. The ERK cascade inhibitors: towards overcoming resistance. Drug Resist Updat 2016;25:1-12.
7. Wang Z, Liu P, Inuzuka H, Wei W. Roles of F-box proteins in cancer. Nat Rev Cancer 2014;14:233-47.
8. Lee CJ, An HJ, Kim SM, Yoo SM, Park J, Lee GE, et al. FBXW7-mediated stability regulation of signal transducer and activator of transcription 2 in melanoma formation. Proc Natl Acad Sci USA 2020;117:584-94.
9. Kudo Y, Guardavaccaro D, Santamaría PG, Koyama-Nasu R, Latres E, Bronson R, et al. Role of F-box protein βTrcp1 in mammary gland development and tumorigenesis. Mol Cell Biol 2004;24:8184-94.
10. Ougolkov A, Zhang B, Yamashita K, Bilim V, Mai M, Fuchs SY, et al. Associations among β-Trcp, an E3 ubiquitin ligase receptor, β-catenin, and NF-κB in colorectal cancer. J Natl Cancer Inst 2004;96:1161-70.
11. Koch A, Waha A, Hartmann W, Hrychyk A, Schüller U, Waha A, et al. Elevated expression of Wnt antagonists is a common event in hepatoblastomas. Clin Cancer Res 2005;11:4295-304.
12. Kim CJ, Song JH, Cho YG, Kim YS, Kim SY, Nam SW, et al. Somatic mutations of the β-Trcp gene in gastric cancer. APMIS 2007;115:127-33.
13. Jin J, Cardozo T, Lovering RC, Elledge SJ, Pagano M, Harper JW. Systematic analysis and nomenclature of mammalian F-box proteins. Genes Dev 2004;18:2573-80.
14. Smith JA, Poteet-Smith CE, Malarkey K, Sturgill TW. Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo. J Biol Chem 1999;274:2893-8.
15. Cho YY, Yao K, Kim HG, Kang BS, Zheng D, Bode AM, et al. Ribosomal S6 kinase 2 is a key regulator in tumor promoter induced cell transformation. Cancer Res 2007;67:8104-12.
16. Cho YY. Molecular targeting of ERKs/RSK2 signaling in cancers. Curr Pharm Des 2017;23:4247-58.
17. Prior IA, Hood FE, Hartley JL. The frequency of Ras mutations in cancer. Cancer Res 2020;80:2969-74.
18. Spiegelman VS, Tang W, Chan AM, Igarashi M, Aaronson SA, Sassoon DA, et al. Induction of homologue of Slimb ubiquitin ligase receptor by mitogen signaling. J Biol Chem 2002;277:36624-30.
19. Arsuria M, Cavin LG. Nuclear factor-κB and liver carcinogenesis. Cancer Lett 2005;229:157-69.
20. Shirane M, Hatakeyama S, Hattori K, Nakayama K, Nakayama I. Common pathway for the ubiquitination of IκBα, IκBβ, and IκBε mediated by the F-box protein FWD1. J Biol Chem 1999;274:28169-74.
21. Saitoh A, Katoh M. Expression profiles of IκBα, IκBβ, and IκBε in gastric cancer. APMIS 2001;115:127-33.
22. Lee CJ, Lee MH, Yoo SM, Choi KI, Song JH, Jang JH, et al. Magnolol inhibits cell migration and invasion by targeting the
ERKs/RSK2 signaling pathway. BMC Cancer 2015;15:576.
23. Swaney DL, Beltrao P, Starita L, Guo A, Rush J, Fields S, et al. Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. Nat Methods 2013;10:676-82.
24. Koepp DM, Schaefer LK, Ye X, Keyomarsi K, Chu C, Harper JW, et al. Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. Science 2001;294:173-7.
25. Welcker M, Orian A, Grim JE, Eisenman RN, Clurman BE. A nucleolar isoform of the Fbw7 ubiquitin ligase regulates c-Myc and cell size. Curr Biol 2004;14:1852-7.
26. Wei W, Jin J, Schlissio S, Harper JW, Kaelin WG Jr. The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. Cancer Cell 2005;8:25-33.
27. Ntziachristos P, Lim JS, Sage J, Alfantis I. From fly wings to targeted cancer therapies: a centennial for notch signaling. Cancer Cell 2014;25:318-34.
28. Cho YY. RSK2 and its binding partners in cell proliferation, transformation and cancer development. Arch Pharm Res 2017;40:291-303.
29. Sharrocks AD, Yang SH, Galanis A. Docking domains and substrate-specificity determination for MAP kinases. Trends Biochem Sci 2000;25:448-53.