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Serine 389 phosphorylation of 3-phosphoinositide-dependent kinase 1 by UNC-51-like kinase 1 affects its ability to regulate Akt and p70 S6kinase

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Phosphorylation of the signaling component by protein kinase often leads to a kinase cascade or feedback loop. 3-Phosphoinositide-dependent kinase 1 (PDK1) signaling pathway diverges into various kinases including Akt and p70 S6 kinase (p70S6k). However, the PDK1 feedback mechanism remains elusive. Here, we demonstrated that UNC-51-like kinase (ULK1), an autophagy initiator kinase downstream of mechanistic target of rapamycin (mTOR), directly phosphorylated PDK1 on serine 389 at the linker region. Furthermore, our data showed that this phosphorylation affected the kinase activity of PDK1 toward downstream substrates. These results suggest a possible negative feedback loop between PDK1 and ULK1. [BMB Reports 2020; 53(7): 373-378]

INTRODUCTION

Altered phosphorylation induces conformational changes in proteins and modifies their function. Phosphorylation events can be considered as vehicles of intracellular communication. Protein kinases mediating the phosphorylation are also regulated via post-translational addition of phosphate groups. This phenomenon occurs repeatedly in a kinase cascade or a feedback circuit.

The phosphatidylinositol 3-kinase (PI3K)-dependent signaling pathway has long been recognized as a key regulatory me-

chanism in several important biological functions, such as cell proliferation, survival, and immunity (1). PI3K increases the level of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and phosphatidylinositol 3, 4-bisphosphate (PIP_2), which elicit membrane translocation of PDK1 and Akt via their pleckstrin homology (PH) domain (2), and in turn, the phosphorylation and activation of Akt by PDK1 in a PIP_3- or PIP_2-dependent manner (3, 4). Activation of PDK1 also leads to phosphorylation of p70S6k (5), serum/glucocorticoid regulated kinase (SGK) (6), p90 ribosomal protein S6 kinase (p90RSK) (7) and members of protein kinase C (PKC) family, all of which belong to AGC kinase family named after protein kinase A, protein kinase G, and PKC (8, 9).

Since PDK1 is considered constitutively active, the activity of PDK1 was measured by the degree of substrate protein modification (10). However, several studies revealed that various molecular switches may regulate PDK1 kinase activity. For example, phosphorylation at the activation loop is essential for its activity (11). Additionally, the localization of PDK1 is one of the factors that affect its function (12-14). Homodimerization of PDK1 may represent an inactive state of the kinase (15, 16).

ULK1 is a protein kinase that is well conserved from budding yeast to humans and is one of the components of the initiating complex downstream of the mTOR in autophagy pathway (17-20). The mTOR phosphorylates and inhibits ULK1 complex to suppress autophagy (17, 21), while ULK1 regulates mTOR/p70S6k signaling via phosphorylation of AMPK, mTOR, and raptor (22-25). This feedback loop between mTOR and ULK1 is important to prevent excessive autophagy. Other upstream components of mTOR signaling pathway may be regulated by ULK1 as a negative or positive feedback.

In the present study, we showed that ULK1 phosphorylated Ser389 of PDK1 at the linker region. This phosphorylation did not affect the kinase activity of PDK1. However, the nonphosphorylatable mutant of PDK1 showed weak activity toward the downstream kinases, such as Akt and p70S6k. Our data suggest a possible feedback loop between ULK1 and PDK1.

*Corresponding authors. Byoung Chul Park, Tel: +82-42-860-4260; Fax: +82-42-879-8596; E-mail: parkbc@kribb.re.kr; Jeong-Hoon Kim, Tel: +82-42-860-4264; Fax: +82-42-879-8596; E-mail: jhoonkim@kribb.re.kr; Sunhong Kim, Tel: +82-42-860-4278; Fax: +82-42-879-8596; E-mail: sunhong@kribb.re.kr

RESULTS

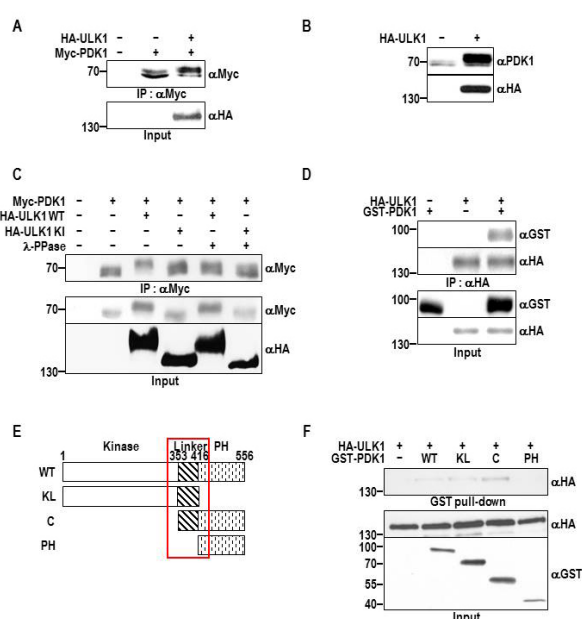### ULK1 interacts with PDK1 and induces phosphorylation

All known substrates of PDK1 are located in the activation loop of AGC kinase family (26). Since the activation loop of ULK1 is very similar to the PDK1 substrate consensus sequence, we thought that PDK1 may regulate ULK1, and therefore, we evaluated the band shift of each protein by western blot analysis. Interestingly, the overexpression of HA-ULK1 increases the upper band of overexpressed and endogenous PDK1 (Fig. 1A and 1B). To confirm that this increase is related to the phosphorylation, we used ULK1 kinase inactive mutant (ULK1 KI) and λ-phosphatase. ULK1 KI failed to induce slower migrating bands of PDK1 and the λ-phosphatase treatment reduced the up-shifted bands of PDK1 by ULK1 WT (Fig. 1C), indicating that ULK1 induced the phosphorylation of PDK1. To examine the direct interaction between PDK1 and ULK1, exogenously expressed GST-PDK1 and HA-ULK1 were analyzed via co-immunoprecipation. We found that GST-PDK1 strongly binds to HA-ULK1 (Fig. 1D). To identify the domains of PDK1 interacting with ULK1, we generated the truncation mutants of PDK1 (Fig. 1E) and performed the GST pull-down assay. HA-ULK1 was bound to GST-PDK1 WT, KL and C but not PH (Fig. 1F), suggesting that the linker region of PDK1 interacts with ULK1.
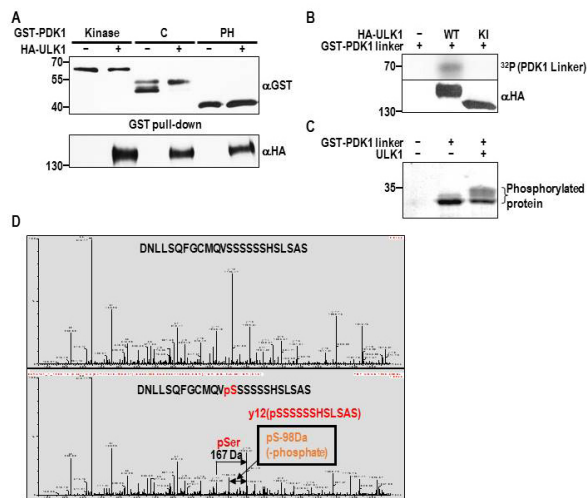
ULK1 phosphorylates Ser389 of PDK1 linker region

To identify the region of PDK1 that is phosphorylated by ULK1, GST-PDK1 kinase domain, C, and PH were overexpressed with HA-ULK1. Only GST-PDK1 C showed the slow-migrating species in SDS-PAGE (Fig. 2A), indicating that ULK1 may phosphorylate the linker region of PDK1. Next, the *in vitro* kinase assay using the bacterially purified GST-PDK1 linker protein as a substrate showed that ULK1 WT directly phosphorylated the substrate protein *in vitro*, while ULK1 KI form was not (Fig. 2B). As ULK1 is an evolutionarily conserved ser/thr kinase from yeast to humans, we speculated that the phosphorylation sites of PDK1 may be conserved. Among evolutionarily conserved serine/threonine residues, several sites were mutated to alanine. However, none of the mutants showed the fast-migrating bands compared with wild-type PDK1 (data not shown). To obtain the phosphorylated PDK1 proteins for mass spectrometry analysis, HEK293T cells were transfected with ULK1 and GST-PDK1 linker. The eluted proteins were subjected to mass spectrometry analysis (Fig. 2C). Finally, Ser389 was identified as the phosphorylation site (Fig. 2D).

To confirm this result, Ser389 residues were mutated to alanine (S389A) and aspartate (S389D), and these mutant proteins were expressed with HA-ULK1. PDK1 WT showed band shift in the presence of ULK1, whereas PDK1 S389A and S389D did not (Fig. 3A). In order to investigate the characteristics of PDK1 phosphorylation at Ser389, we generated phospho-specific antibody that recognizes phosphorylated

Ser389 of PDK1 (pS389 PDK1 antibody). HEK293T cells transfected with PDK1 alone or together with ULK1 were lysed and PDK1 proteins were immunoprecipitated using anti-Myc antibody. In line with the above data, pS389 antibody specifically recognized the PDK1 proteins that are co-expressed with ULK1 (Fig. 3B). Additionally, overexpression of ULK1 WT increased the Ser389 phosphorylation of endogenous PDK1, while ULK1 KI did not (Fig. 3C). Next, we tested whether the activation of ULK1 affected the phosphorylation of PDK1. We used oligomycin A, which is known to induce energy depri-

Fig. 1. ULK1 interacts with PDK1 and induces phosphorylation. (A) HEK293T cells were transfected with HA-ULK1 and Myc-PDK1 constructs. After 48 h of transfection, the cells were lysed, and the lysates were subjected to immunoprecipitation using anti-Myc antibody. The immune complexes were washed and the immunoblots for PDK1 were performed as described in Materials and Methods (upper panel). The same cell lysates were used for anti-HA immunoblot (lower panel). (B) HA-ULK1 was expressed in HEK293T cells. Whole cell lysate was analyzed by immunoblotting for the indicated proteins. (C) HEK293T cells transfected with Myc-PDK1 and HA-ULK1 wild-type (WT) or kinase-inactive (KI) mutant were lysed and subject to immunoprecipitation with anti-Myc antibody. The immune complexes were incubated with λ phosphatase, washed, and subjected to anti-Myc immunoblot as described in Materials and Methods (top panel). Anti-Myc and anti-HA immunoblots were conducted using the same cell lysates (middle and bottom panels, respectively). (D) HA-ULK1 and GST-PDK1 were expressed in the HEK293T cells. The cell lysates were subjected to immunoprecipitation using anti-HA antibody. Immunoblots were obtained with the indicated antibodies. (E) The scheme of PDK1 truncation mutants. (F) HEK293T cells transfected with HA-ULK1 and GST-PDK1 WT, kinase and linker (KL), C-terminal (C) or PH domain (PH) constructs were lysed, and the lysates were subjected to co-immunoprecipitation. The results shown are representative of three independent experiments.
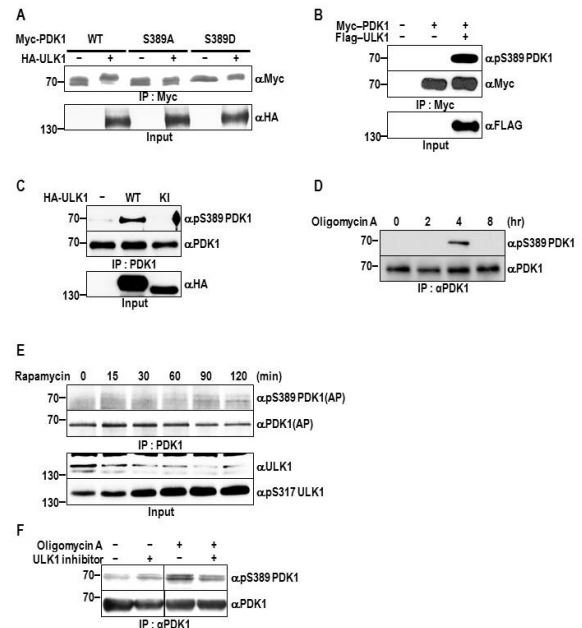
Fig. 2. ULK1 phosphorylates Ser389 of PDK1 linker region. (A) HA-ULK1 proteins were co-expressed with GST-PDK1 Kinase, C or PH proteins in HEK293T cells. The cell lysates were cleared and incubated with glutathione beads as described in Materials and Methods. Western blots for the precipitated proteins were obtained using anti-GST antibody (top panel). The anti-HA immunoblot (bottom panel) was also conducted using the same whole cell lysates. (B) HEK293T cells transfected with HA-ULK1 WT or KI were lysed and ULK1 assays were performed using GST-PDK1 linker protein as a substrate as described in Materials and Methods. The phosphorylated proteins were visualized by autoradiography (top panel). ULK1 expression was detected by the immunoblot obtained from the same whole cell lysates (bottom panel). The above results shown are representative of three independent experiments. (C) HEK293T cells were transfected with GST-PDK1 linker and HA-ULK1 WT. After 48 h of transfection, PDK1 linker proteins were pulled down and eluted as described in Materials and Methods. The fractions of eluted proteins were subjected to SDS-PAGE and the gel was stained by Coomassie R250 solution. (D) Phosphorylated proteins from (C) were analyzed by mass spectrometry as described in Materials and Methods.

vation, and rapamycin, which inhibits mTOR and induces subsequent increase in ULK1 activity. Oligomycin A treatment induced Ser389 phosphorylation of PDK1 at 4 h (Fig. 3D). Rapamycin also elicited PDK1 phosphorylation (Fig. 3E). Furthermore, the addition of ULK1 inhibitor decreased PDK1 phosphorylation induced by oligomycin A (Fig. 3F). Taken together, these results confirmed that ULK1 phosphorylates PDK1 at Ser389.

Phosphorylation at Ser389 may affect downstream signaling
To elucidate the signaling effect of Ser389 phosphorylation of PDK1, we used the PDK1 knockout cell line. Stable cell lines expressing PDK1 wild type or S389A mutant in PDK1 knockout cell line were established (Fig. 4A). First, the *in vitro* kinase assay was carried out to measure the activity of PDK1. It was intriguing that the activity of PDK1 S389A is very similar to wild type *in vitro* (Fig. 4A). When the phosphorylation status

Fig. 3. ULK1 phosphorylates Ser389 of PDK1. (A) HEK293T cells were transfected with the indicated constructs. After 48 h of transfection, the cells were lysed and the lysates were subjected to immunoprecipitation using anti-Myc antibody (top panel). ULK1 immunoblot was performed using the same cell lysates (bottom panel). (B) Myc-PDK1 and Flag-ULK1 were overexpressed in the HEK293T cells. Immunoprecipitated Myc-PDK1 proteins were probed with an anti-pS389 PDK1 antibody (top panel). (C) HA-ULK1 WT and KI were overexpressed in the HEK293T cells and endogenous PDK1 proteins were immunoprecipitated using the anti-PDK1 antibody (top and middle panel). ULK1 expression was detected in the same cell lysates (bottom panel). (D) After the addition of 5 μM Oligomycin A to HEK293T cells at the indicated time points, the cells were lysed for immunoprecipitation of PDK1. The immune complexes were subjected to western blot using anti-pS389 and anti-PDK1 antibodies (top and bottom panel, respectively) (E) HEK293T cells were treated with 1 μM rapamycin at the indicated time points. Immunoprecipitated PDK1 proteins were probed with anti-pS389 and anti-PDK1 antibodies (top and upper middle panels, respectively). AP indicates alkaline phosphatase blotting. (F) HEK293T cells were treated with ULK1 inhibitor (1 μM, SBI-0206965) for 20 h, followed by addition of oligomycin A (5 μM) or DMSO for 4 h. Immunoprecipitated proteins using PDK1 antibody were analyzed via immunoblotting using the indicated antibodies. The above results shown are representative of three independent experiments.

of the downstream targets of PDK1 were investigated, however, S389A mutant showed relatively weak activity toward Akt and p70S6k compared with wild type (Fig. 4B). These data suggested that Ser389 phosphorylation of PDK1 by ULK1 is necessary for the expression of upstream signals.
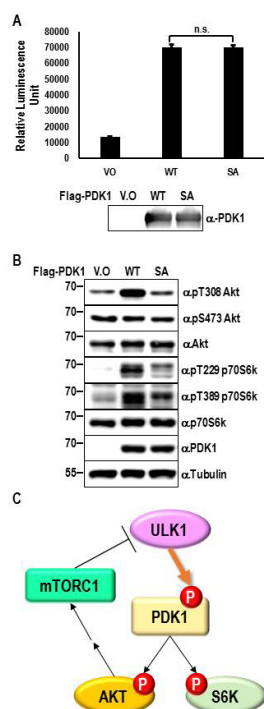
Fig. 4. Phosphorylation of PDK1 Ser389 regulates substrate phosphorylation. (A) Flag-PDK1 WT and S389A proteins were purified from HAP1 stable cells as described in Materials and Methods. PDK1 kinase activity was measured using ADP-Glo™ PDK1 kinase assay kit according to the manufacturer's instructions. The data represent means ± SD of three experiments. Statistical analysis was performed using Student's t-test, and P-value < 0.05 was considered significant; however, the calculated P value was 0.69. n.s.: not significant (B) HAP1 PDK1 knockout cells stably expressing PDK1 WT and S389A mutant were lysed and the whole cell lysates were analyzed by immunoblotting using the indicated antibodies. (C) Negative feedback loop model between PDK1 and ULK1.

DISCUSSION

Since PDK1 is known as a master kinase belonging to the AGC kinase family and is a nearly constitutively active enzyme, its activity depends on the readiness of substrates for phosphorylation by PDK1. For example, the phosphorylation of p70S6k by PDK1 depends on the phosphorylation at a C-terminal Ser/Thr residue located in the hydrophobic motif (27). This phosphorylation facilitates binding of PDK1 to this kinase via a specific substrate-docking site termed the 'PIF pocket' (28), whereas the activation of Akt by PDK1 is independent of phosphorylation at the hydrophobic motif (27, 28).

Other groups investigated the regulation of PDK1 itself. For instance, sphingosine increased PDK1 phosphorylation over 25-fold (29). In addition, PDK1 autophosphorylated Ser241 residue, and this phosphorylation is required for Akt activation

(11). Serine residues in the linker region (Ser393, 396, and 410) were phosphorylated in HEK293 cells. However, this phosphorylation was not required for downstream signaling. Furthermore, insulin promoted the phosphorylation of PDK1 at Tyr9 and 373/376 in the plasma membrane (30). Although Tyr-373/376 residues were located in the linker region, none of these was involved in PDK1 activity.

To our knowledge, this work is the first report demonstrating the phosphorylation of Ser389 in PDK1. Based on previous reports, a few hypotheses can be proposed: First, since Ser389 is located in the linker region between kinase and PH domains of PDK1, phosphorylation at this residue might induce conformational changes in PDK1 protein, leading to altered substrate recognition of the kinase, which is supported by the results suggesting that PDK1 kinase activity was not altered in S389A mutant (Fig. 4A). Further, the phosphorylation by ULK1 may impede inhibitory homodimerization of PDK1 similar to the phosphorylation in the PH domain (15, 16, 31). Finally, there is a possibility that the subcellular localization of PDK1 may be modulated by the phosphorylation at Ser389.

ULK1 protein complex is directly regulated by mTORC1 as mentioned above. In addition, the feedback mechanisms of ULK1 to mTORC1 have been investigated. Two independent groups demonstrated that ULK1 phosphorylated raptor, a component of mTORC1 complex, and subsequently inhibited mTORC1 activity (24, 25), which was consistent with previous data showing that ULK1 blocked p70S6k (22). However, another report suggested that ULK1 phosphorylated all three subunits of AMP-activated kinase (AMPK) resulting in its inhibition (23). However, the role of these phosphorylations in mTORC1 signaling is unknown. It is well known that the inhibition of AMPK activity generally leads to activation of mTORC1 via TSC1/2 complex and raptor (32), in line with our data suggesting that PDK1 phosphorylation by ULK1 might be necessary for the activation of downstream targets of PDK1 (Fig. 4B). Thus, ULK1 might activate or inhibit mTORC1, which might appear inherently contradictory. However, specific conditions might determine the direction of feedback. Further studies are needed to elucidate the mechanism underlying this novel feedback loop.

In conclusion, our study suggests that Ser389 phosphorylation of PDK1 regulates its signaling function and the existence of a novel negative feedback loop between PDK1 and ULK1/autophagy pathway.

MATERIALS AND METHODS

Cell culture, transfection, and establishing stable cell lines

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (WELGENE, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (FBS) (ThermoFisher, Waltham, MA, USA) and antimycotics (ThermoFisher). Human PDK1 knockout HAP1 cells were purchased from Horizon and cultured in Iscove's Modified Dulbecco's Medium (WELGENE)

supplemented with 10% FBS and antimycotics. Cells were transfected using X-tremeGENE™ (Roche, Basel, Switzerland) according to the manufacturer's protocol. PDK1 knockout HAP1 cells were infected with lentivirus particles harboring PDK1 wild-type or S389A constructs and selected by adding puromycin (1 μg/mL) for 7 to 10 days. Western blot confirmed PDK1 protein expression by the selected cells.

Antibodies and reagents

Anti-Flag M2 (F1804), anti-GST (G7781), anti-Tubulin (T6074), Oligomycin A (75351) and Rapamycin (R8781) were obtained from Merck (St. Louis, MO, USA). Anti-Myc (sc-40), anti-HA (sc-805) and anti-GAPDH (sc-47724) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-Myc (2278S), anti-Phospho-ULK1 Ser317 (6887S), anti-ULK1 (8054S), anti-Phospho-Akt Ser473 (4060S), anti-Phospho-Akt Thr308 (4056S), anti-Akt (4691S), anti-Phospho-p70S6k Thr389 (9234S), anti-p70S6k (2708S) and anti-PDK1 (3062S) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-Phospho-p70Sk Thr229 (MAB8964) was purchased from R&D systems (Minneapolis, MN, USA). The phospho-specific antibody recognizing PDK1 phosphorylated at Ser389 was raised in rabbits against the peptide CMQV$\underline{S}$SSSSSHS (corresponding to residues 385-396 of human PDK1) in which the underlined residue is phosphoserine (Peptron, Daejeon, Republic of Korea). The antibodies were affinity-purified on activated-Sepharose (ThermoFisher) covalently coupled to the phosphorylated peptide and then passed through a column of CH-Sepharose coupled to the non-phosphorylated peptide. Antibodies that did not bind to the latter column were selected. ULK1 inhibitor, SBI-0206965 (S7885) were obtained from Selleckchem (Houston, TX, USA). Anti-HA Magnetic Beads (88837), Glutathione Magnetic Agarose Beads (78601), Anti-c-Myc Magnetic Beads (88843), Anti-DYKDDDDK Magnetic Agarose (A36798) and Dynabeads™ Protein G for Immunoprecipitation (10004D) were purchased from ThermoFisher and used for immunoprecipitation.

Preparation of cell lysates, immunoprecipitation, and *in vitro* kinase assay

Stimulation was terminated by washing cells with ice-cold PBS (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137mM NaCl, 2.7 mM KCl). Cell lysates were prepared in Buffer A (20 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1X protease inhibitor cocktail (Roche)).

To perform ULK1 kinase assay, the protein-bead complexes were washed twice with Buffer A, then twice with Buffer A containing 500 mM NaCl. Finally, the complexes of ULK1 were washed with Buffer B containing 20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 0.1 mg/ml BSA, and 3 mM β-mercaptoethanol. ULK1 activities were assayed in a reaction mixture consisting of 1X Buffer B, 1 μg GST-PDK1 Linker protein, 20 μM ATP,

and 10 μCi of [γ-^{32}P]ATP at 30°C for 15 min.

PDK1 KO HAP1 cells stably expressing Flag-PDK1 wild-type and S389A mutant cells were harvested and lysed with Buffer A containing 1X phosphatase inhibitor (Roche). Lysates were immunoprecipitated with Anti-DYKDDDDK magnetic beads. The protein-bead complexes were washed twice with Buffer A containing 500 mM NaCl, twice with Buffer A containing 250 mM NaCl, and finally once with Buffer A, and then eluted using 3X flag peptide. Purified PDK1 enzymes were used for ADP-Glo™ PDK1 kinase assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Mass spectrometry

HEK293T cells were transfected with pEBG-PDK1 linker and after 48 h, the cells were lysed with Buffer A. The overexpressed proteins were pulled down by glutathione Sepharose 4B (GE Healthcare, Chicago, IL, USA). The protein-bead complexes were washed twice with Buffer A, twice with Buffer A containing 500 mM NaCl, and finally once with ST buffer containing 50 mM Tris-HCl pH 7.4 and 150 mM NaCl. The proteins were eluted with Buffer C containing 30 mM glutathione and PBS. Eluted proteins were concentrated by Microcon (Merck) and analyzed using 2D electrophoresis, MALDI-TOF, and Q-TOF by In2Gen.

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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