

Serine/threonine Kinase Unc-51 Like Kinase-1 (Ulk1) Phosphorylates the Co-chaperone
Cdc37 and Thereby Disrupts the Stability of Cdc37 Client Proteins

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Running title: Regulation of Cell Division Cycle Protein 37 (Cdc37) client proteins by Ulk1

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ABSTRACT

The serine/threonine kinase Unc-51 like kinase-1 (Ulk1) is thought to be essential for induction of autophagy, an intracellular bulk degradation process that is activated by various stresses. Although several proteins have been suggested as Ulk1 substrates during autophagic process, it still remains largely unknown about Ulk1's physiological substrates. Here, by performing *in vitro* and *in vivo* phosphorylation assay, we report that the co-chaperone cell division cycle protein 37 (Cdc37) is an Ulk1 substrate. Ulk1-mediated phosphorylation of Ser339 in Cdc37 compromised the recruitment of client kinases to a complex comprising Cdc37 and heat shock protein 90 (Hsp90), but only modestly affected Cdc37 binding to Hsp90. Since the recruitment of protein kinase clients to the Hsp90 complex is essential for their stability and functions,

Ser339 phosphorylation of Cdc37 disrupts its ability as a co-chaperone to coordinate Hsp90. Hsp90 inhibitors are cancer chemotherapeutic agents by inducing depletion of clients, many of which are oncogenes. Upon treatment with an Hsp90 inhibitor in cancer cells, Ulk1 promoted the degradation of Hsp90-Cdc37 client kinases, resulting in increased cellular sensitivity to Hsp90 inhibitors. Thus, our study provides evidence for an anti-proliferative role of Ulk1 in response to Hsp90 inhibition in cancer cells.

Autophagy is a tightly regulated pathway which can be stimulated by multiple forms of cellular stress (1,2). During autophagic process, cells form double-membraned vesicles, autophagosomes, which sequester organelles, proteins, or portions of the cytoplasm, for delivery to the lysosome

(3,4). The molecular mechanism of autophagosome formation is conserved in evolution and depends on several autophagy-related proteins (ATGs) (5). Atg1 is a serine/threonine protein kinase which plays important roles in autophagy initiation (6,7). In mammalian cells, Atg1's functional homologues are Unc-51-like kinases 1 (Ulk1) and 2 (8-13). During autophagy process, the activated Ulk1/2 phosphorylates Atg13, Fip200 and Beclin-1, leading to autophagy induction (14-16).

Besides autophagy-related protein substrates, several autophagy-unrelated substrates of Ulk1 were also found. For example, during deprivation of amino acid and growth factors, Ulk1/2 directly phosphorylates key glycolytic enzymes to sustain glycolysis (17). Stimulator of interferon genes (STING) is also phosphorylated by Ulk1 to prevent the persistent transcription of innate immune genes (18). Ulk1/2 phosphorylates SEC16A and regulates endoplasmic reticulum (ER) export which is essential for cellular homeostasis (19).

Since Ulk1 has a variety of substrates, it plays important roles in many biological processes, especially in cell death and proliferation. Autophagy facilitates cell survival by maintaining cellular metabolism and eliminating harmful damaged proteins and organelles, therefore Ulk1-autophagy axis is generally considered as a cyto-protective response. However, there are several reports showed that Atg1 induced autophagy inhibits cell growth and induces apoptotic cell death in *Drosophila* (20). Overexpression of wild-type Ulk but not kinase-dead Ulk changed cell morphology and caused cytotoxicity in NIH3T3 cells (12). Recent studies also showed that Ulk1 contributes to cell death in autophagy-dependent or -independent

manner (21-23). For example, upon DNA damage, p53 up-regulated Ulk1 is necessary for the sustained autophagy which results in the subsequent cell death (22). In addition, nuclear Ulk1 can also promote cell death by regulating the activity of the DNA damage repair protein poly (ADP-ribose) polymerase 1 (PARP1) (23). Therefore, the mechanism of Ulk1 induced cell death is very complex and needs further exploration.

In this study, we identified cochaperone Cell Division Cycle Protein 37 (Cdc37) as a new phosphorylation target of Ulk1. Phosphorylation of Cdc37 at Ser339 by Ulk1 decreases its interaction with client kinases, resulting in the instability of the clients. In addition, we also found that Ulk1 kinase affected loss of clients' stability and activity upon Hsp90 inhibition. Finally, silencing Ulk1 decreased cancer cell sensitivity to Hsp90 inhibitors, showing that Ulk1 plays an important role in cellular response to Hsp90 inhibition.

RESULTS

Ulk1 phosphorylates Cdc37

It has been reported that the interaction between Ulk1 and Hsp90-Cdc37 stabilizes and activates Ulk1 (24) (supplemental Fig. 1). Since Ulk1 is a well-known serine-threonine kinase, we tested whether Ulk1 was able to phosphorylate Cdc37. As shown in Fig. 1A-B, mass spectrometry analysis indicated that highly conserved sites S127, T131 and S339 of Cdc37 were phosphorylated by Ulk1. In addition, by performing *in vitro* phosphorylation assay, we found that GST-Cdc37-WT was phosphorylated by wild-type Ulk1, but not the kinase impaired K64R Ulk1 mutant (Fig. 1C). To further confirm these phosphorylation sites, Cdc37(WT), Cdc37(S127A), Cdc37(T131A), Cdc37(S339A) or

Cdc37(S127/T131/S339A)-GST fusion proteins were purified. As shown in Fig. 1D, after incubation with Ulk1(WT), the radiolabeling of Cdc37(WT), Cdc37(S127A) and Cdc37(T131A) was much higher than that of Cdc37(S339A) and Cdc37(S127/T131/S339A), indicating that S339 of Cdc37 is a phosphorylation target of ULK1. Moreover, we also test whether Cdc37 is the target of Ulk2, an Ulk1 close homolog. As shown in supplemental Fig. 2, Cdc37 was phosphorylated by Ulk2; however, the difference of phosphorylation signal intensity between Cdc37-WT and Cdc37-3A was not obviously, indicating that S339 of Cdc37 is a unique substrate site of Ulk1 *in vitro*.

Next, we tried to determine whether Cdc37 is phosphorylated by Ulk1 *in vivo*. Protein extraction of HCT116 cells was immunoprecipitated with an anti-Cdc37 and then probed with anti-phosphorylated serine antibody. A significant increase of phosphorylated Cdc37 was detected in Ulk1-overexpressed HCT116 cells (Fig. 1E-F), suggesting that Ulk1 plays a critical role in regulating Cdc37 phosphorylation.

Phosphorylation of Cdc37 decreases its interaction with clients

We next tested whether Ulk1 mediated phosphorylation site on Cdc37 is necessary for its function. It has been reported that Cdc37 is a general cochaperone for protein kinases, by recruiting client kinases into the Hsp90 system (25). As shown in Fig. 2A, although the interaction between Hsp90 and Cdc37 was not dramatically changed, several protein kinases, for example, Cdk4, AuroraB, Src, Cdk6 and Raf1 cannot be recruited to Cdc37(S339E) which mimics Cdc37 phosphorylation, indicating that S339 dephosphorylation is essential for Cdc37 binding to these client kinases. We

then tried to further confirm the role of Ulk1 in regulation of Cdc37-clients binding. As shown in Fig. 2B-C, expression of wild-type Ulk1 decreased the interaction between Cdc37 and its client kinases Cdk4, AuroraB, Src, Cdk6 and Raf1. Consistently, we also found that Ulk1 suppressed the interaction of clients with chaperone Hsp90 (Fig. 2D-E). It has been reported that AMPK directly phosphorylates and activates Ulk1 (26-28). Therefore we used AMPK activator phenformin to treat HCT116 cells. We found that phenformin also blocked Cdc37(WT) (but not S339A) binding to its client kinases (Fig. 2F). These data suggested that Ulk1 induced Cdc37 phosphorylation at S339 negatively regulates the recruitment of client kinases to Hsp90-Cdc37 complex, but only modestly affects Cdc37's binding to Hsp90.

Ulk1 suppresses the stability of Cdc37's clients

Since Cdc37 is important for maintaining the stability and functions of its client protein kinases (29,30), we next tested the effect of Cdc37 S339 phosphorylation on client kinases' stability. Wild-type Cdc37, Cdc37-S339E(phosphorylation-mimic) or Cdc37-S339A(phosphorylation-incompetent) were stable transfected into Cdc37-knockdown HCT116 cells. As shown in Fig. 3A, compared with that of wild-type Cdc37 or Cdc37-S339A, expression of Cdc37-S339E was not able to rescue the protein level of client kinases. Consistently, we found that p-RB (T826), H3S10, p-MEK1/2 (Ser217-221) and p-Src (Y416) -which are direct downstream phosphorylated products of CDK4/6, AuroraB, RAF-1 and Src, respectively- were rescued by Cdc37-S339A, but not Cdc37-S339E (Fig. 3A), indicating that Cdc37 S339 phosphorylation compromise

the stability and activity of these clients in cancer cells.

We next examined whether Ulk1 reduces the amount of protein kinases. As shown in Fig. 3B, in Ulk1 knock-out cell line which was generated by using CRISPR/Cas9 technology, Cdk4, AuroraB and Raf-1 were diminished after Ulk1 overexpression. In addition, we also tested the half-life of Cdc37's client kinases in the presence of cycloheximide (CHX), a protein synthesis inhibitor. We found that Cdk4 and AuroraB's half-life were decreased dramatically after Ulk1 was expressed (Fig. 3C-F). Consistently, phenformin decreased Cdc37's clients' expression in wild-type HCT116 cells, but not in Ulk1 KO HCT116 cells (Fig. 3G). Since AMPK activator phenformin is known to phosphorylate Ulk1 to induce autophagy (26-28), we then test whether Ulk1 mediated autophagy is related with Cdc37's clients regulation. By using autophagy-deficient Atg3 knockout HCT116 cell line, we found that Ulk1 was still required for phenformin induced Cdc37's clients degradation (Fig. 3H), supporting the autophagy-independent role of Ulk1 in regulating the stability of Cdc37's client kinases.

Next, to determine whether Cdc37 Ser339 phosphorylation is a necessary modification for Ulk1-suppressed kinases stability, Cdc37(WT) or Cdc37(S339A) were transfected into Cdc37-KD HCT116 cells. As shown in Fig. 4A, Ulk1 overexpression reduced the protein level of clients in a proteasome-dependent manner. Furthermore, the reduction was almost abolished when Cdc37(S339A) was overexpressed (Fig. 4B-C), indicating that Ulk1 suppressed the stability of Cdc37's clients by inducing its phosphorylation.

Ulk1 is involved in the 17-AAG-induced

degradation of several kinase clients of Cdc37

As Cdc37 is required for Hsp90-mediated chaperoning of many clients, we next investigated whether Ulk1 affects Hsp90 inhibitor-mediated abrogation of kinase clients. For this purpose, Ulk1-knockout HCT116 (Ulk1 KO) cells were stably transfected with wild-type Ulk1 plasmid or an empty plasmid. As shown in Fig. 5A, compared with Ulk1(WT)-expressing HCT116 cells, 17-AAG induced clients degradation was alleviated in Ulk1 KO cells. In autophagy induction process, the kinase activity of Ulk1 is highly dependent on its binding partner, for example Atg13 (15,31). We therefore knocked down Atg13 in HCT116 cells and then observed similar reduction of 17-AAG effect that caused by depletion of Ulk1 (Fig. 5B), pointing to a crucial role of Ulk1 complex in sustaining 17-AAG effect. Additionally, we also used phenformin to activate AMPK-Ulk1 in HCT116 cells. Consistently, upon 17-AAG treatment, the presence of phenformin induced more obvious degradation of Cdc37's clients in wild-type HCT116 cells, but not in Ulk1 KO HCT116 cells (Fig. 5C). Next, to determine whether Cdc37 Ser339 phosphorylation is a necessary modification for these events, Cdc37(S339A) was stable transfected into Cdc37-KD HCT116 cells. As shown in Fig. 5D-F, Ulk1-dependent clients degradation was almost abolished in Cdc37(S339A)-expressing HCT116 cells, indicating that Ulk1 sustained 17-AAG effect by inducing Cdc37 phosphorylation.

Furthermore, to determine whether Ulk1 regulates 17-AAG mediated kinases degradation via the ubiquitin-proteasome system, HCT116 cells were co-treated with 17-AAG and proteasome inhibitor MG132. As shown in Fig. 5F-H, less ubiquitin was

associated with Cdk4, AuraroB and Raf-1 in Ulk1 KO cells compared with Ulk1(WT) expressing HCT116 cells. Taken together, our data indicated that Ulk1 plays a role in the degradation of several kinase clients following Hsp90 inhibition.

Ulk1 sensitizes cancer cells to Hsp90 inhibitor

It has been reported that silencing the Cdc37 destabilizes kinase clients and sensitizes cancer cells to Hsp90 inhibitors (32). Therefore we next detected whether Cdc37 phosphorylation is involved in cellular sensitivity to Hsp90 inhibitors. Wild-type Cdc37, Cdc37-S339E or Cdc37-S339A were stable transfected into Cdc37-KD HCT116 cells. As shown in Fig. 6A, the cellular sensitivity to Hsp90 inhibitor 17-AAG is much higher in Cdc37-S339E-expressing cells, compared with the wild-type Cdc37 or Cdc37-S339A-expressing cells. Similarity, the increased sensitivity was reproduced with another clinical Hsp90 inhibitors AUY922 (33), indicating that the effect was likely general to Hsp90 inhibitors (Fig. 6B). In addition, the cell death population (sub-G1 population) was then measured by using FACS analysis. As shown in Fig. 6C-D, 17-AAG treatment induced higher sub-G1 cell population in Cdc37-S339E-expressing cells. Collectively, these data suggested that cellular sensitivity to Hsp90 inhibitor is regulated by Cdc37 S339 phosphorylation.

Since Cdc37's cochaperone activity was dampened by Ulk1 induced phosphorylation, we next tested whether Ulk1 is involved in the cancer cell growth inhibitory effects of pharmacological Hsp90 inhibitor. Firstly, we compared the levels of Ulk1 expression in four human colon cancer cell lines including HCT116, DLD1, HT29

and LoVo. Ulk1 was highly expressed in HCT116, DLD1 and HT29 cells, but virtually no expression was detected in LoVo cells (Fig. 6E). As shown in Fig. 6F, Ulk1 levels seemed to be related to the Hsp90 inhibitor sensitivity, in that Hsp90 inhibitor decreased cell viability in HCT116, DLD1 and HT29 cells, but not in LoVo cells. To further clarify the role of Ulk1 in cancer cell's sensitivity to Hsp90 inhibitor, DLD1 and HCT116 cells were treated with Hsp90 inhibitors. We found that knocking-out Ulk1 decreased the cellular sensitivity to 17-AAG or AUY922 in DLD1 and HCT116 cells (Fig. 6G-H and supplemental Fig. 3A-B). To further determine the effect of Ulk1-induced Cdc37 Ser339 phosphorylation, Cdc37-WT-expressing and Cdc37-S339A-expressing cells were used. As shown in Fig. 6G-H, Ulk1 reduced the cellular sensitivity to Hsp90 inhibitors is dependent on Cdc37 S339 phosphorylation, indicating the importance of the Ulk1-Cdc37 axis in Hsp90 inhibitors-decreased cell viability.

Since Ulk1 plays important roles in autophagy, we next tried to determine autophagy's role in regulation of 17-AAG sensitivity. As shown in supplemental Fig. 3C-D, silencing Atg3, another essential gene in autophagy, cannot change the cellular sensitivity to Hsp90 inhibitor, indicating that Ulk1 sensitized cancer cells to 17-AAG independent on autophagy. On the other hand, we found that phenformin, which induces Ulk1 phosphorylation and activation (27), increased the 17-AAG or AUY922 sensitivity in DLD1 and HCT116 cells, but not in Ulk1 KO cells (Fig. 6I-J and supplemental Fig. 3E-F). In addition, the combination of phenformin with 17-AAG increased the sub-G1 cell population, indicative of increased cell death (Fig. 6K-L). Taken together, these data suggested

that Ulk1 promotes the anti-proliferative response caused by Hsp90 inhibitor in cancer cells.

DISCUSSION

Although Ulk1 is mainly understood to mediate autophagy, here we found that Ulk1 also has a function to negatively regulate Hsp90-Cdc37 system. We demonstrated that Ulk1 induces Cdc37 S339 phosphorylation and inhibits the cochaperone activity of Cdc37. As a result, Hsp90-Cdc37's client kinases' stability decreased, leading to the hyper-sensitivity of cancer cells to Hsp90 inhibitors.

Cdc37 is a cochaperone which promotes the association of Hsp90 with the protein kinases to maintain their stability and functions (29,30). Many of these kinases are oncogenes; therefore the molecular mechanism of their activation/inactivation is important for cancer therapy. Our results illustrated an Ulk1-regulated-pathway in Hsp90-Cdc37 complex-dependent kinase activation. The results presented here identified Ulk1-mediated Cdc37 dephosphorylation as a requirement for release of clients from the Hsp90 chaperone (Fig. 2B-D). It has been reported that phosphorylation of N-terminal Ser13 is required for the proper function of Cdc37 (34). This site is phosphorylated by the constitutively active kinase CK2 (35) and dephosphorylated by PP5 (36,37). Although Ser13 are responsible for binding client kinases, Cdc37 also interacts with several client kinases (for example B-Raf) dependent on its C-terminal (38). Previous study also showed that phosphorylation on Y4 and Y298 of Cdc37 disrupts client-Cdc37 association (39). Here in our study, we showed that Ulk1-mediated phosphorylation of Cdc37 is also one of PTMs (post-translational modification) that

controls Hsp90-Cdc37 chaperone function by affecting cochaperone and clients association. We identified Ser339 as a new phosphorylation site of Cdc37, which was also important for its activity. Although it is not essential for the interactions between Cdc37 and each kinase client (supplemental Fig. 4), Ser339 dephosphorylation status is obviously a requirement for several clients recognition, at least for the kinases CDK4, AuroraB, Src, Cdk6 and Raf1 (Fig. 2A). However, among these kinase, only Cdk4, AuroraB and Raf-1 were diminished by Ulk1 overexpression (Fig. 3B), raising the possibility that Ulk1 may regulate Src and Cdk6 via other pathways besides Cdc37 phosphorylation.

Recently, a growing number of studies have shown that ATG proteins also play a role in autophagy-independent functions (40-43). Besides initiation of autophagy, Ulk1's autophagy-independent functions were also expanded by several reports (17-19). Here in our study, we found that Ulk1 affect many kinases stability and activity. Moreover, knocking-down of Atg13, an essential gene for the kinase activity of endogenous Ulk1, also reduced 17-AAG effect on Hsp90-cdc37's client kinase (Fig. 5B). However, no significant change of 17-AAG effect was detected in the Atg3 KD cells (supplemental Fig. 5A-B). In addition, Ulk1-mediated Cdc37's phosphorylation on S339 is not required for phenformin induced autophagy (supplemental Fig. 5C), indicating that Ulk1 regulates Hsp90-cdc37's clients independent on the downstream autophagic events. Moreover, compared with wild-type cell, cellular sensitivity to Hsp90 inhibitor was not changed in autophagy deficient cell line, further supporting Ulk1's autophagy-independent role (supplemental Fig. 3C-D).

Inhibition of mitochondrial Hsp90 activates AMPK and Ulk1/FIP200, which in turn inhibits FAK-directed tumor cell motility and metastasis (44). Here we showed that Hsp90 inhibition induced cyto-toxicity is also related with Ulk1. It has been reported that Ulk1 is a client of the Hsp90-cdc37 complex chaperone (24). We also observed that Ulk1 protein level gradually decreased upon Hsp90 inhibitor treatment, although less prominent than other clients such as CDK4 (supplemental Fig. 6). Therefore we treated colon cancer cells with the combination of Hsp90 inhibitor and Ulk1 activator. The data showed that AMPK-Ulk1 activation promotes the anti-proliferative response caused by Hsp90 inhibitor (Fig. 6I-L). In contrast to our findings, Akt-mTOR-Ulk1 signaling pathway was reported to be involved in Hsp90 inhibitor BIIB021-triggered autophagy which played cytoprotective role in BIIB021-treated CML cells (45). However, our results showed that silencing of Ulk1 increased the cell viability upon Hsp90 inhibitor treatment in colon cancer cell lines HCT116 and DLD1 cells (Fig. 6G-H, supplemental Fig. 3A-B). Moreover, autophagy deficiency did not change the cellular sensitivity to Hsp90 inhibitor (supplemental Fig. 3C-D). We believed the conflicting results uncovered by our group and others could be explained by the different cell lines used in the respective studies: solid tumor cell lines were used in our study, and leukemia cells were used in the other study.

It has been reported that Hsp90-Cdc37-Akt form complex and the degradation of Akt is controlled by Hsp90 (46-48). In our study, we also tested whether Ulk1 can regulate Akt level by phosphorylating Cdc37. As shown in supplemental Fig. 7A, we did not observe that Ulk1 had any

noticeable effect on Akt or p-Akt in HCT116 cells. Moreover, the human colon cancer cell lines HCT116 and DLD1 which we used harbor activating PI3KCA mutation, which results in a constitutively active form of Akt (49). Therefore, in our system, Akt pathway is unlikely to play an important role in 17-AAG's effect regulation. Although the mechanism of how Hsp90 inhibitors affect cell viability is very complex, Ulk1-Cdc37 axis apparently acts as an important component in regulating Hsp90 inhibitors' effect.

EXPERIMENTAL PROCEDURES

Cell culture and plasmid transfection.

Cells were grown in DMEM or McCoy's 5A with 10% (vol/vol) fetal bovine serum and the appropriate amount of penicillin/streptomycin in a37 °C incubator with a humidified, 5% CO₂ atmosphere. Transient and stable transfections were performed using Lipofectamine 2000 (invitrogen) following the manufacturer's protocol.

Plasmids and siRNA. cDNA of Cdc37 was amplified and cloned into p3xFLAG-CMV-10, pEGFP-C1 and pGEX-6p1. Ulk1/2, Raf1, Cdk4 and AuroraB DNA were inserted into p3xFLAG-CMV-10. Cdc37 and Ulk1/2 mutation constructs were generated with a Fast mutagenesis kit (Vazyme). Ulk1 Cas9 cell lines was generated by using Ulk1 sgRNA (5'-CGAAGGCGCCGTGGCCGATC-3').

Cdc37 siRNA sequence: 5'-CGGCAGUUCUUCACUAAGA-3'.

Atg13 siRNA sequence: 5'-CCAUGUGUGUGGAGAUUUCACUUA-3'.

Atg3 siRNA sequence: 5'-CAUUGAGACUGUUGCAGAA-3'.

Antibodies and reagents. The antibodies

used were anti-HA-tag, anti-GFP-tag, anti-Myc-tag, anti- α -tubulin (MBL); anti-Flag-tag (Sigma-Aldrich); anti-CRAF (Cell Signaling, #9422S), anti-phospho-MEK1/2(S217/221) (Cell Signaling, #9154P), anti-Src (Cell Signaling, #2123P), anti-phospho-Src(Tyr416) (Cell Signaling, #6943P), anti-Atg13 (Cell Signaling, #13468S), anti-Atg3 (Cell Signaling, #3415S), anti-phospho-AMPK(T172) (Cell Signaling, #2535S), anti-Ulk1 (Cell Signaling, #8054S); anti-phospho-serine (Abcam, #ab9332), anti-AuroraB (Abcam, #ab45145), anti-phospho-H3S10 (Abcam, #ab32107), anti-MRK (Abcam, #ab166895); anti-MOK(Proteintech, #23926-1-AP), anti-MAK (Proteintech, #13638-1-AP), anti-AKT1 (Proteintech, #10176-2-AP), anti-Hsp90(Proteintech, #13171-1-AP); anti-phospho-pRb(Thr826) (Ruiying, #RLP0556). Phenformin(#S2542), MG132(#S2619), 17-AAG(#S1141) and AUY922(#S1069) were purchased from Selleck.

Co-immunoprecipitation. Cells were harvested and then lysed in lysis buffer [1% NP-40, 150 mM NaCl, 50 mM Tris at pH 7.5, 5 mM EDTA, 0.05% SDS, and 1% EDTA-free protease and phosphatase inhibitor cocktails(Roche)] on ice for 30 min. After centrifugation at 4 °C at 12,000 rpm for 15 min, 2 μ g of the indicated antibody was added into the supernatant and incubated at 4 °C overnight. Then, 30 μ l of protein G or A Sepharose slurry (GE healthcare) was added and incubated for 2 h at 4 °C. The beads were washed by NP-40 buffer three times. The precipitated components were analyzed by Western blotting.

HA-Ub Immunoprecipitation. The cells were harvested and washed with PBS. After centrifugation at 1000 rpm for 5 min, the

cell pellet was resuspended in lysis buffer (50 mM Tris, pH 8.0, 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and 1% EDTA-free protease and phosphatase inhibitor cocktails(Roche)) and incubated on ice for 30 min. After centrifugation at 12000rpm for 10 min, the supernatant was harvested as Whole cell extract (WCE). The pellet was resuspended in buffer S (20 mM Tris, pH 7.4, 150 mM NaCl, and 1% SDS), sonicated for 10s, and then left on a rocking platform at room temperature for 30min. The lysate was passed through a 22-G needle 30 times to break the DNA. After centrifugation at 12000rpm for 10 min, the supernatant was harvested as SDS-extract. The SDS-extract was diluted ten times with lysis buffer and then mixed with WCE for immunoprecipitation with anti-Flag (M2) affinity gel (Sigma-Aldrich). Immunoprecipitations were carried out for 2 h at 4 °C. Immunoprecipitates were washed five times with lysis buffer. The beads were then eluted with 3xFLAG peptide (Sigma-Aldrich) for 8 h at 4 °C.

Western Blotting. Equal amounts of proteins (20–150 μ g) were size fractionated by 6–15% SDS polyacrylamide gel electrophoresis.

Protein purification. GST-fusion proteins were expressed in Escherichia coli induced with isopropyl-D-thio-galactoside and purified by glutathione-Sepharose 4B beads (GE Healthcare, Kings Park, NY, USA) and then washed with TEN buffer [20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, and 100 mM NaCl]. Recombinant His-tagged proteins were expressed in and purified from E. coli by Ni (ii)-Sepharose affinity (GE Healthcare) and the bound protein was eluted with 250mM imidazole in PBS and desalted by buffer exchange with PBS. For GST pull-down assay, GST-fusion proteins were incubated with

His-tagged proteins in TEN buffer. The proteins were incubated at 4°C overnight. The beads were washed 3 times with TEN buffer and boiled with 2xSDS loading buffer, and the proteins were analyzed by Western blot with an anti-His or anti-GST antibody.

***In vitro* kinase assay.** HCT116 cells were grown and each dish transfected with 8μg of Flag-Ulk1/2. After 24 h post-transfection, cells were lysed in MLB (10 mM Tris at pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM Na₃VO₄ and 1% EDTA-free protease and phosphatase inhibitor cocktails(Roche)). Ulk1/2 proteins were immunoprecipitated with anti-Flag-tag (Sigma-Aldrich) antibodies, and then washed with MLB once and RIPA buffer (50 mM Tris at pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.05% SDS, 1% Triton X-100 and 0.5% deoxycholate) twice, followed by washing with kinase assay buffer containing 20 mM HEPES at pH 7.4, 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl₂ and 0.05 mM DTT (dithiothreitol). For Ulk1/2 autophosphorylation assay, the immunoprecipitated Ulk1 bead was

incubated in kinase assay buffer containing 10 μM cold ATP and 2 μCi [γ-³²P]ATP per reaction. For kinase assays with GST–Cdc37 and GST–Cdc37-339A, GST–Cdc37 and GST–Cdc37-339A were bacterially purified. The kinase reaction was performed at 37 °C for 30 min and the reaction was terminated by adding SDS sample buffer and subjected to SDS–PAGE and autoradiography.

Cell proliferation assay. Cellular sensitivity to compounds was measured by MTT cell counting Kit (Vazyme).

Ni-NTA-Agarose Purification. Cells were lysed in buffer A (6 M guanidinium chloride, 0.1 M Na₂HPO₄, NaH₂PO₄, pH 8.0, 10 mM imidazole). The cell lysates were incubated with 50μl of Ni-NTA beads for 3 h at room temperature; this was followed by the intense washing of beads with buffer A and 25 mM Tris-HCl, pH 6.8, 20 mM imidazole. The bound proteins were eluted by 5xSDS sample buffer supplemented with 200 mM imidazole at 100 °C for 5 min, resolved on SDS-PAGE, and detected by the appropriate antibodies.

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Conflict of interest

The authors have no conflict of interest.

Author contributions

R.L. performed most of the experimental work. F.Y. and W.F. performed quantifications and western blots. L.Z. and Y.W. assisted in protein purification. K.M., X.L. and L.W. assisted in autophagy deficient cell line generation. W.G.Z. supervised the project. Y.Z. designed and supervised experiments and wrote the manuscript. All authors critically read the paper.

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Figure Legends

Figure 1. Ulk1 phosphorylates Cdc37. (A) Identification of S127, T131 and S338 phosphorylation on GST-Cdc37 incubated *in vitro* with Ulk1 by using mass spectrometry analysis. (B) Schematic of the phosphorylation sites S127, T131 and S338 in human and other species Cdc37. (C) Flag-Ulk1(WT) or Flag-Ulk1(K46R) (a mutant Ulk1 lacking kinase activity) proteins were immunopurified from transfected HEK293 cells. Then *in vitro* kinase assays were performed in the presence of GST-Cdc37. Phosphorylated proteins were visualized with autoradiography. (D) Flag-Ulk1(WT) protein was immunopurified from transfected HEK293 cells. Then *in vitro* kinase assays were performed in the presence of GST-Cdc37 proteins. Phosphorylated proteins were visualized with autoradiography. (E) An empty plasmid and an expression plasmid for Flag-Ulk1 were cotransfected with or without GFP-Cdc37 into HCT116 cells for 24 h. Proteins were immunoprecipitated with an antibody to GFP followed by immunoblot with an antibody to phosphorylated-serine. (F) An empty plasmid and an expression plasmid for Flag-Ulk1 were cotransfected with or without GFP-Cdc37 into HCT116 cells for 24 h. Proteins were immunoprecipitated with an antibody to HA followed by immunoblot with an antibody to flag or phosphorylated-serine.

Figure 2. Phosphorylation of Cdc37 decreases its interaction with clients. (A) HCT116 cells transfected with Flag-Cdc37(WT), Flag-Cdc37(S127E), Flag-Cdc37(T131E) or Flag-Cdc37(S339E) were subjected to Flag-IP. (B-C) HCT116 cells were cotransfected with Flag-Ulk1 plus GFP-Cdc37 (B) or GFP-Cdc37-S339A (C). At 24h after transfection, cells were subjected to GFP-IP. (D) HCT116 cells transfected with Flag-Ulk1 and myc-Hsp90 were subjected to myc-IP. (E) HCT116 cells transfected with Flag-Ulk1 and myc-Hsp90 were subjected to immunoprecipitated with an antibody to Cdc37. (F) A set of RNAi-resistant rescue forms of Cdc37 plasmids were transfected into stable Cdc37-RNAi HCT116 cells. At 24h after transfection, cells were treated with phenformin and then subjected to flag-IP.

Figure 3. Ulk1 suppresses the stability of Cdc37's clients. (A) A set of RNAi-resistant rescue forms of Cdc37 plasmids were transfected into stable Cdc37-RNAi HCT116 cells. Western blotting was then performed to detect Cdc37's clients and their targets. (B) Different amount HA-Ulk1 plasmid was transfected into Ulk1-KO HCT116 cells. Western blotting was then performed to detect Cdc37's clients and their targets. (C-F) HA-Ulk1 plasmid or an empty plasmid was transfected into Ulk1 KO HCT116 cell. 24 h after transfection, CHX was added at 10μg/ml for different times as indicated. Western blotting was then performed to detectd AuroraB (C) or CDK4 (E). The line graph shows the change in AuroraB(D) or CDK4(F) level at different times. (G) Wild-type and Ulk1-KO HCT116 cells were treated at different concentrations of phenformin for 24 h. (H) Atg3-KO and Atg3-KO/Ulk1-KO HCT116 cells were treated at different concentrations of phenformin for 24 h.

Figure 4. Ulk1 suppressed the stability of Cdc37's clients by inducing its phosphorylation. (A) Ulk1-KO HCT116 cells were transfected with the indicated plasmids in the presence or absence of MG132. 24 h after transfection, proteins were extracted for Western blot. (B) HCT116 cells were transfected with the indicated plasmids. 24 h after transfection, proteins were extracted for Western blot. (C) HCT116 cells were transfected with

the indicated plasmids. 24 h after transfection, cells were treated with phenformin for another 24 h. Proteins were then extracted for Western blot.

Figure 5. Ulk1 is involved in the 17-AAG-induced degradation of several kinase clients of Cdc37.

(A) Wild-type Ulk1 or an empty plasmid was stably transfected into Ulk1-knockout HCT116 (Ulk1 KO) cells. Cells were treated at different concentrations of 17-AAG for 12 h, proteins were then extracted for Western blot. (B) Atg13 RNAi was performed in Ulk1(WT)- expressing HCT116 cells. Cells were treated at different concentrations of 17-AAG for 12 h, proteins were then extracted for Western blot. (C) Wild-type and Ulk1-KO HCT116 cells were treated with the combination of 17-AAG and phenformin for 12 h. Proteins were then extracted for Western blot. (D) RNAi-resistant rescue form of Cdc37(S339) plasmid was transfected into Cdc37-KD or Cdc37-KD/Ulk1-KO HCT116 cells. Cells were treated at different concentrations of 17-AAG for 12 h, proteins were then extracted for Western blot. (E) RNAi-resistant rescue form of Cdc37(S339) plasmid was transfected into Cdc37-KD or Cdc37-KD/Ulk1-KO HCT116 cells. Cells were treated with the combination of 17-AAG and phenformin for 12 h. Proteins were then extracted for Western blot. (F-H) Ulk1 expressing and Ulk1-KO HCT116 cells were transfected with the indicated plasmids. 24 h after transfection, cells were treated for 12 h with MG132 and/or 17-AAG. Then CDK4 (F), AuroraB (G) and Raf-1(H) conjugated to ubiquitin was purified by using Ni-NTA beads under denaturing conditions and then detected by Western blotting.

Figure 6. Ulk1 sensitizes cancer cells to Hsp90 inhibitor.

(A-B) A set of RNAi-resistant rescue forms of Cdc37 plasmids were transfected into stable Cdc37-RNAi HCT116 cells. Then cells were treated with 17-AAG (A) or AUY922 (B) at different concentration for 36 h, and cell proliferation was tested with the MTT assay. (C-D) A set of RNAi-resistant rescue forms of Cdc37 plasmids were transfected into stable Cdc37-RNAi HCT116 cells. Cells were treated with 100 nM 17-AAG for 72h, and the cells were then harvested for flow cytometry analysis. The percentage of sub-G1 was shown in (D). (E) Western blotting was performed to determine Ulk1 expression in human colon cancer cell lines DLD1, LoVo, HT29 and HCT116. (F) DLD1, LoVo, HT29 and HCT116 cells were treated with 17-AAG at different concentration for 36 h, and cell proliferation was tested with the MTT assay. (G-H) A set of RNAi-resistant rescue form of Cdc37 plasmids were transfected into Cdc37-KD or Cdc37-KD/Ulk1-KO HCT116 cells. Cells were then treated with 17-AAG (G) or AUY922 (H) at different concentration for 36 h, and cell proliferation was tested with the MTT assay. (I-J) Wild-type and Ulk1-KO HCT116 cells were treated with 40 nM 17-AAG (I) or 20 nM AUY922 (J) combined with phenformin, and cell proliferation was tested with the MTT assay. (K-L) Wild-type and Ulk1-KO HCT116 cells were treated with 100 nM 17-AAG combined with phenformin for 72h, and the cells were then harvested for flow cytometry analysis. The percentage of sub-G1 was shown in (L).

Figure 1

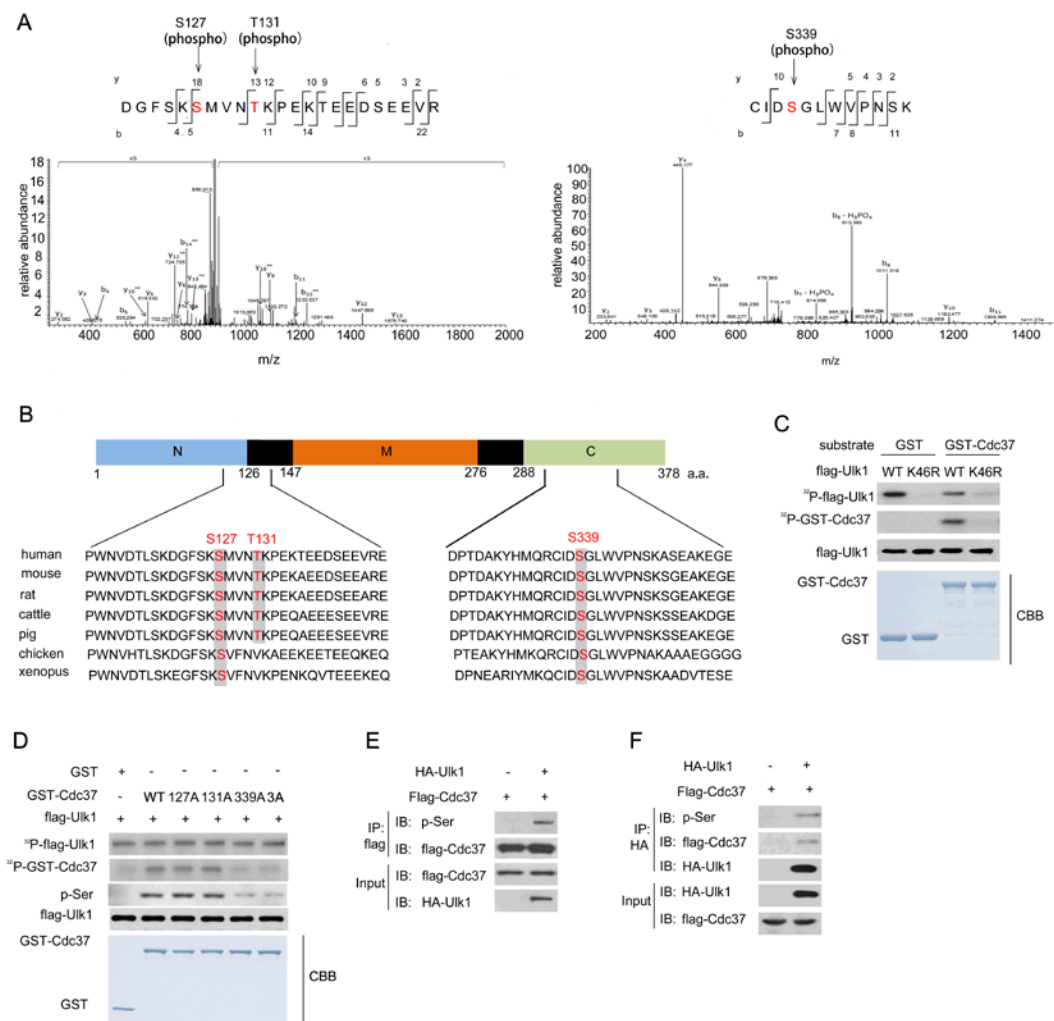


Figure 2

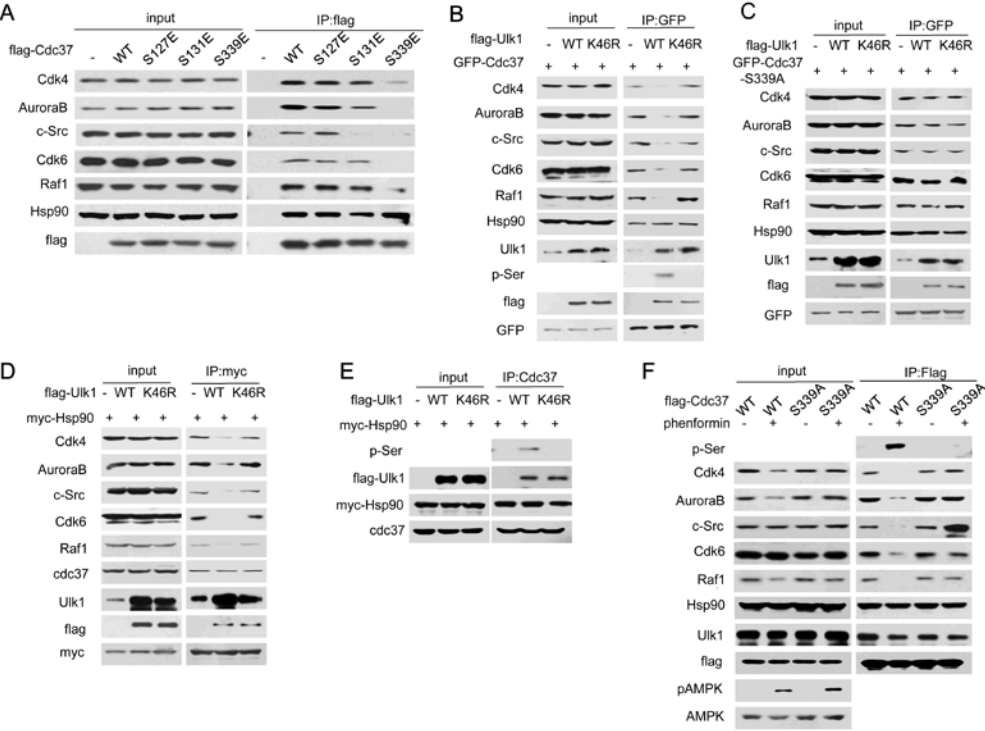


Figure 3

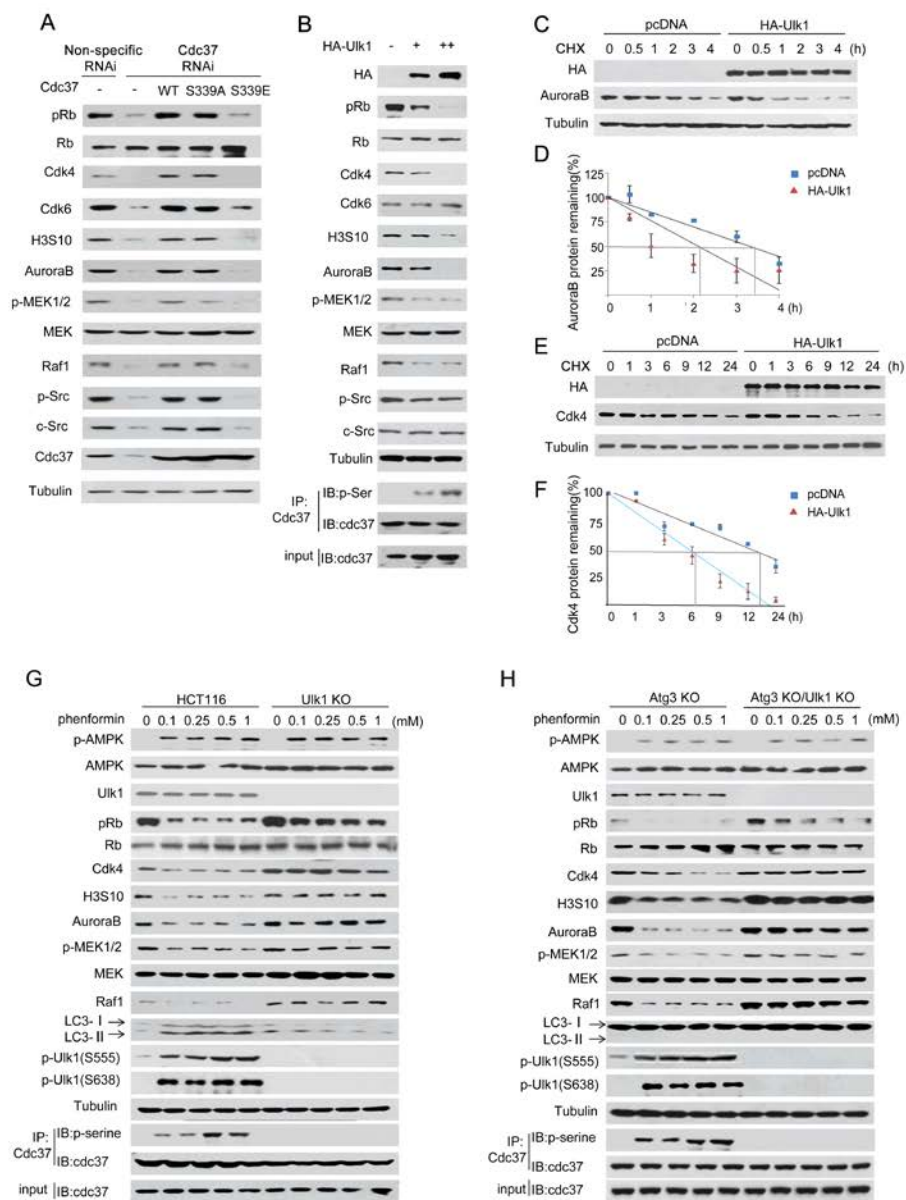


Figure 4

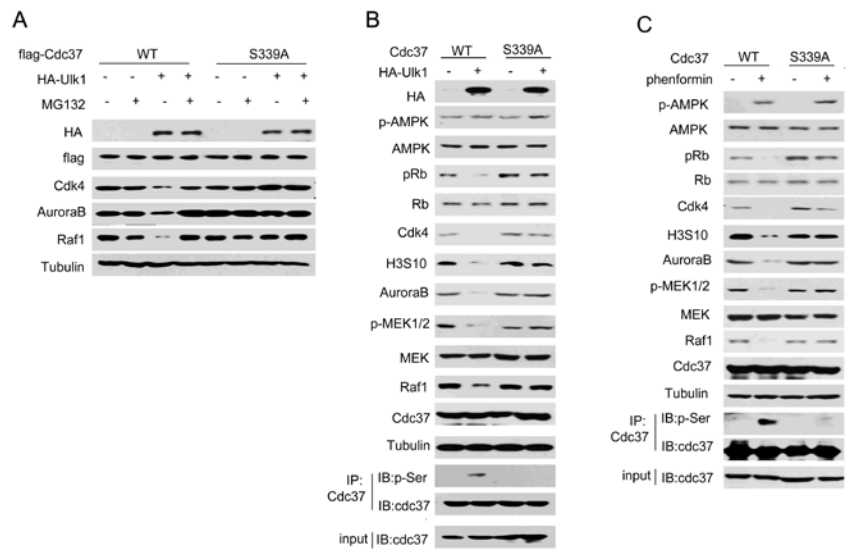


Figure 5

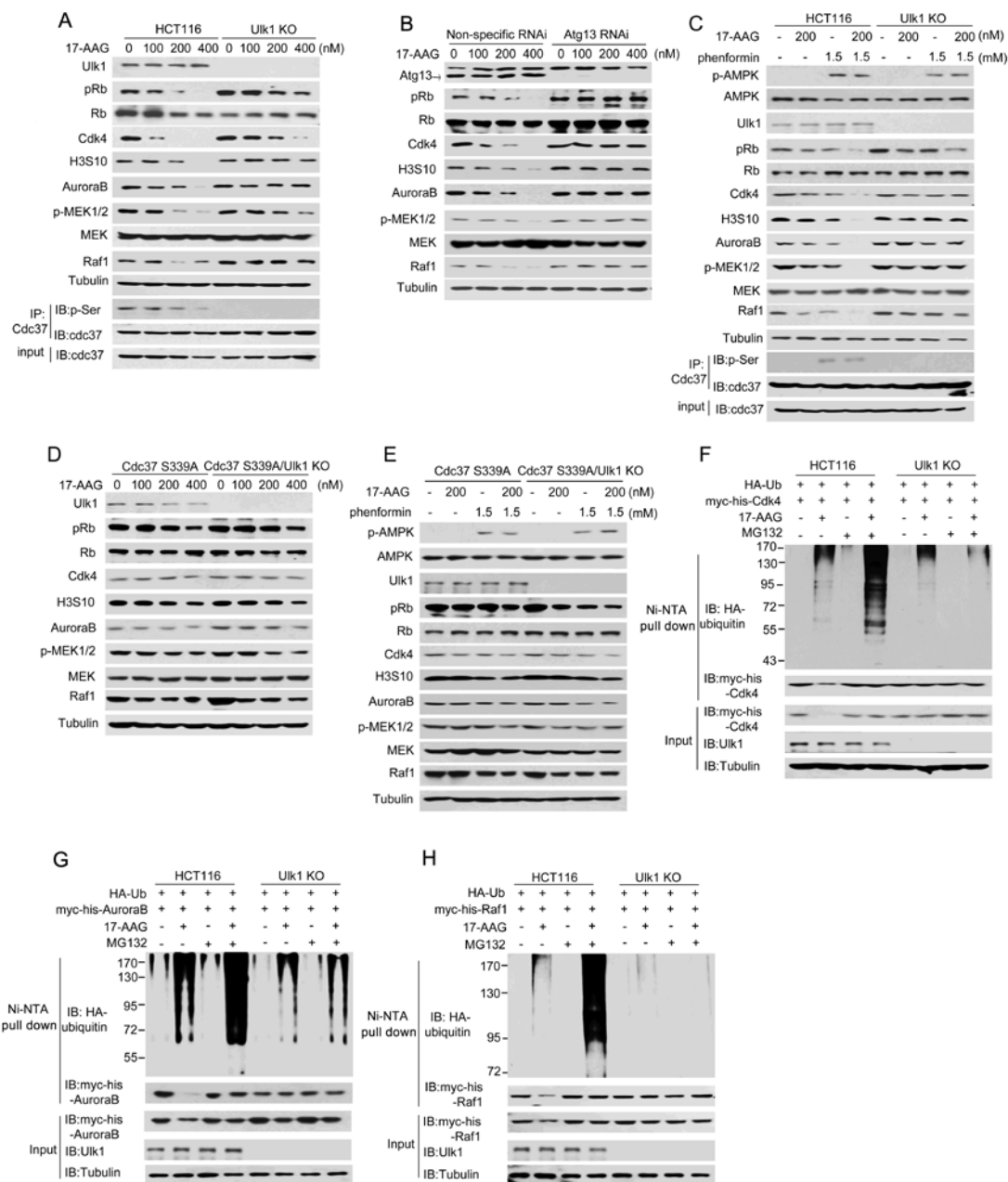
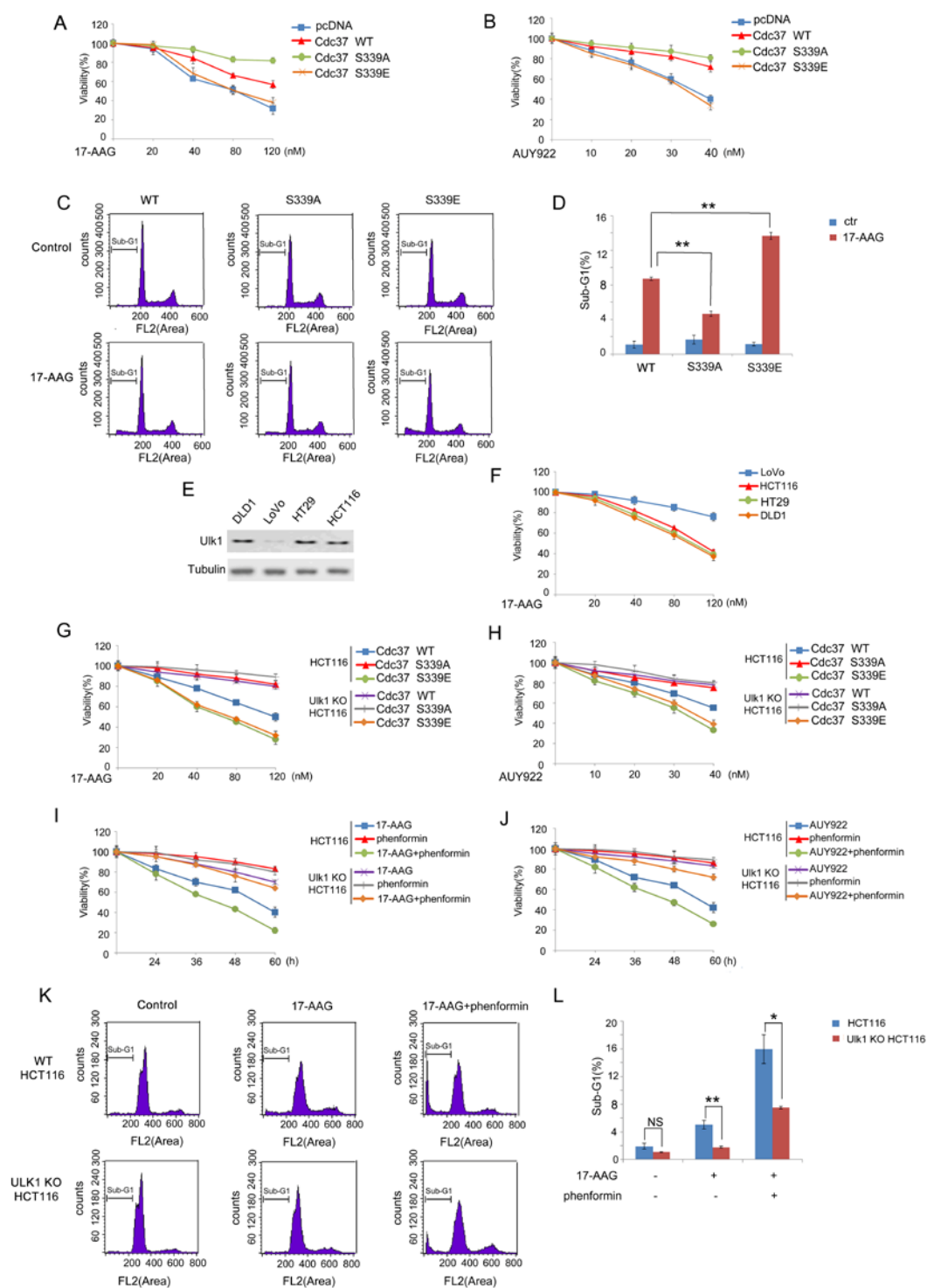


Figure 6



Supplemental Figure Legends

Supplemental Figure 1. GST or GST-Cdc37 fusion protein were expressed in bacteria and purified; these proteins were then incubated with Ulk1 (translated *in vitro*). Western blotting was performed to detect the interaction of Ulk1 with GST-Cdc37.

Supplemental Figure 2. Flag-Ulk2(WT) or Flag-Ulk2(KI) (a mutant Ulk2 lacking kinase activity) proteins were immunopurified from transfected HEK293 cells. Then *in vitro* kinase assays were performed in the presence of GST-Cdc37(WT) or GST-Cdc37(3A). Phosphorylated proteins were visualized with autoradiography.

Supplemental Figure 3. (A-B) Wild-type and Ulk1-KO DLD1 cells were treated with 17-AAG (A) or AUY922 (B) at different concentration for 36 h, and cell proliferation was tested with the MTT assay. (C-D) Wild-type, Ulk1-KO, Atg3-KO Ulk1/Atg3-KO HCT116 cells were treated with 40 nM 17-AAG (C) or 20 nM AUY922 (D), and cell proliferation was tested with the MTT assay at different time point. (E-F) Wild-type and Ulk1-KO DLD1 cells were treated with 40 nM 17-AAG (E) or 20 nM AUY922 (F) combined with phenformin, and cell proliferation was tested with the MTT assay.

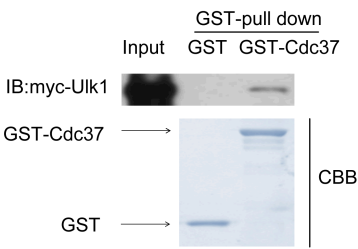
Supplemental Figure 4. HCT116 cells transfected with Flag-Cdc37(WT), Flag-Cdc37(3A) or Flag-Cdc37(3E) were subjected to Flag-IP.

Supplemental Figure 5. (A) Atg3 RNAi was performed in Ulk1(WT)- expressing HCT116 cells. Cells were then treated at different concentrations of 17-AAG for 12 h, proteins were extracted for Western blot. (B) Atg3-KO and Ulk1/Atg3-KO HCT116 cells were treated with the combination of 17-AAG and phenformin for 12 h. Proteins were then extracted for Western blot. (C) A set of RNAi-resistant rescue forms of Cdc37 plasmids were transfected into stable Cdc37-RNAi HCT116 cells. Cells were then treated with phenformin for 12 h.

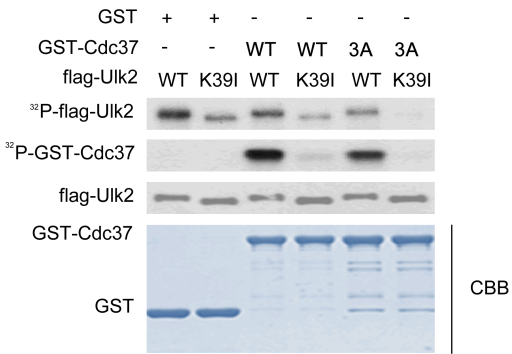
Supplemental Figure 6. (A) HCT116 cells were treated with 17-AAG at different concentration for 36 h. Proteins were then extracted for Western blot. (B) HCT116 cells were treated with 40 nM 17-AAG combined with phenformin. Proteins were then extracted for Western blot.

Supplemental Figure 7. (A) Different amount HA-Ulk1 plasmid was transfected into Ulk1-KO HCT116 cells. Western blotting was then performed. (B) Wild-type and Ulk1-KO HCT116 cells were treated with the combination of 17-AAG and phenformin for 12 h. Proteins were then extracted for Western blot.

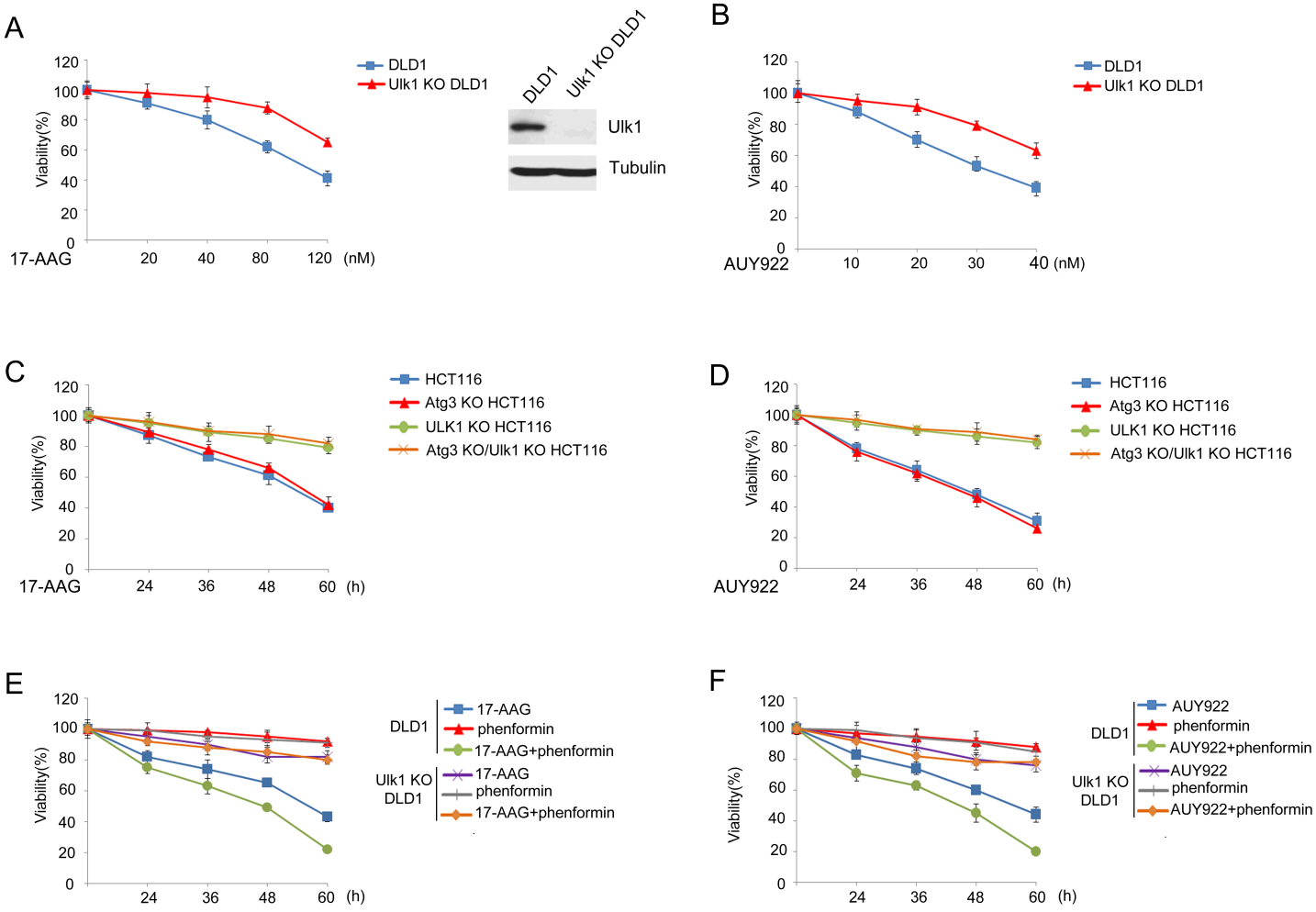
Supplemental Figure 1



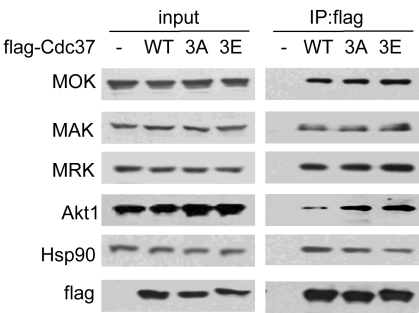
Supplemental Figure 2



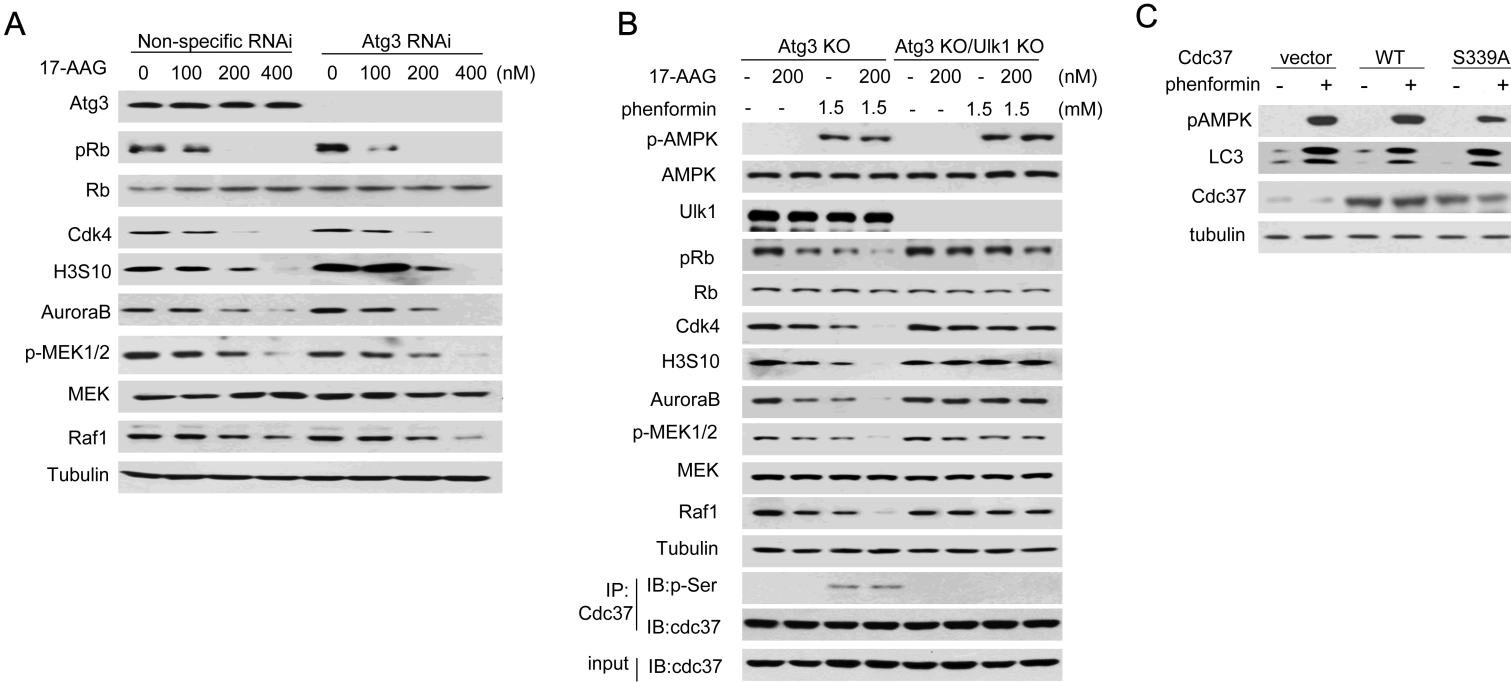
Supplemental Figure 3



Supplemental Figure 4

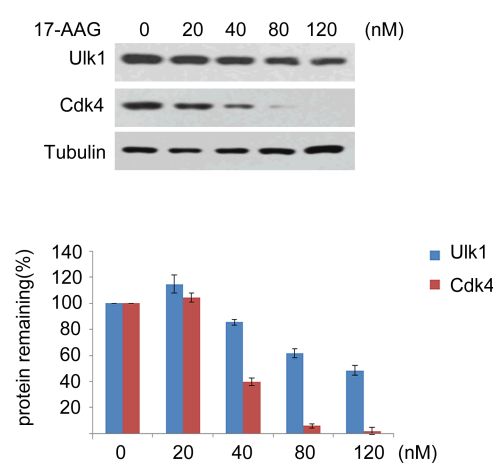


Supplemental Figure 5

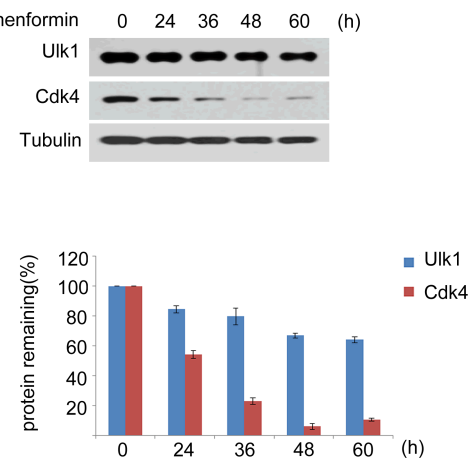


Supplemental Figure 6

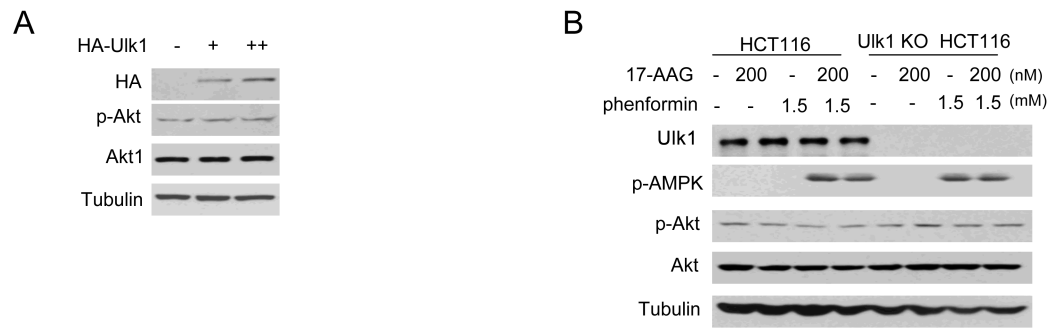
A



B



Supplemental Figure 7



Serine/threonine Kinase Unc-51 Like Kinase-1 (Ulk1) Phosphorylates the Co-chaperone Cdc37 and Thereby Disrupts the Stability of Cdc37 Client Proteins

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